Ants (Formicidae): Models for Social Complexity

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INTRODUCTION

The family Formicidae (ants) is composed of more than 12,000 described species that vary greatly in size, morphology, behavior, life history, ecology, and social organization. Ants occur in most terrestrial habitats and are the dominant animals in many of them. They have been used as models to address fundamental questions in ecology, evolution, behavior, and development. The literature on ants is extensive, and the natural history of many species is known in detail. Phylogenetic relationships for the family, as well as within many subfamilies, are known, enabling comparative studies. Their ease of sampling and ecological variation makes them attractive for studying populations and questions relating to communities. Their sociality and variation in social organization have contributed greatly to an understanding of complex systems, division of labor, and chemical communication. Ants occur in colonies composed of tens to millions of individuals that vary greatly in morphology, physiology, and behavior; this variation has been used to address proximate and ultimate mechanisms generating phenotypic plasticity. Relatedness asymmetries within colonies have been fundamental to the formulation and empirical testing of kin and group selection theories. Genomic resources have been developed for some species, and a whole-genome sequence for several species is likely to follow in the near future; comparative genomics in ants should provide new insights into the evolution of complexity and sociogenomics. Future studies using ants should help establish a more comprehensive understanding of social life, from molecules to colonies.

BACKGROUND INFORMATION

Despite their small body size, ants are among the most dominant animals in terrestrial ecosystems (Hölldobler and Wilson 1990). There are an estimated 20,000+ ant species, varying greatly in size, social organization, and ecology. This diversity enables comparative research on nearly all aspects of their biology. Records of natural history observations of ants date back millennia. The literature on ants is extremely rich, with journal publications numbering in the thousands. Historically, ants have been used to explore social behavior, ecology, and evolution; with the aid of modern tools and theory, they can be used as models to address fundamental questions across many disciplines in biology.

Much of the ecological success of ants stems from their sociality. Ants are eusocial: The members of their society cooperate in brood care, are multigenerational, and exhibit reproductive division of labor (Hölldobler and Wilson 1990). Ant colonies are composed of one or more queens, dozens to millions of workers, and brood (eggs, larvae, and pupae). Queens are the reproductive female caste. They are typically larger than workers, modified for dispersal and reproduction, and can live decades. Workers are nonreproductive females that perform the supporting tasks of brood care, nest maintenance, defense, and foraging. They are wingless, shorter-lived, and often functionally sterile (although they can possess ovaries). Moreover, some species produce physically distinct worker castes (e.g., majors/soldiers and minors) that tend to specialize in a subset of work within a colony. During the mating season, ant colonies can also contain males, but with few exceptions, these disperse to mate

without contributing to colonial work; all workers are females. Ants, like all Hymenoptera, have a haplodiploid sex determination system; males are produced from unfertilized, haploid eggs, whereas females develop from fertilized, diploid eggs.

Ants are holometabolous insects and go through the following developmental stages: egg, larva, pupa, and adult. The number of larval stages (instars) varies among ant species (typically four to five). Ant larvae are generally incapable of locomotion and rely entirely on nurse workers for transport and feeding. The larvae of many species are critical for the processing of complex foods; workers feed food to the larvae, and, after some processing, the larvae share portions of this food with workers and the queen. Eggs and pupae are nonfeeding developmental stages, but can provide feedback to the queen and the rest of the colony, informing them about the quantity and quality of individuals that have recently been produced.

When larvae have surpassed a critical size, they undergo a molt into a pupa, at which time the imaginal discs that have been developing internally are evaginated and develop into adult tissues (metamorphosis); in some ant subfamilies, the pupae are encased in a cocoon, whereas in others they are "naked" (e.g., Myrmicinae). When an individual ecloses, its cuticle is unhardened and typically lighter in color compared to older individuals; this early adult stage is called "callow." Callow individuals typically do not venture far from where they are born (e.g., the brood pile or brood chamber). As workers age, they begin a centrifugal progression away from the queen and brood chambers, culminating in leaving the confines of the nest and foraging. The major morphological features of adult ants are shown in Figure 1.

Ant species vary widely in colony size, queen number, queen mating frequency, colony growth rate, and timing of reproduction, providing many opportunities for comparative research (see Hölldobler and Wilson 1990, 2008). In broadly simplified terms, mating and colony initiation usually occur in one of two ways. In the first method, virgin queens and males both disperse to engage in nuptial flights, after which, males die and mated queens shed their wings and start new colonies on their own (haplometrosis) or with unrelated cofoundresses (pleometrosis). In the other method, queens mate within or near their natal nest (typically with foreign males, although inbreeding does occur in some species), into which they are readopted. Colonies with multiple queens (i.e., polygynous) typically reproduce by budding or fission, in which the queens and workers divide into multiple new colonies. Colonies founded without the aid of workers typically have high mortality rates early on, but high survivorship thereafter. In contrast, colonies that reproduce by budding tend to have much higher initial survival rates. Colonies that survive the founding stage generally proceed through a period of ergonomic growth during which only workers are produced. On reaching a critical colony size (which can take several years), both workers and reproductive forms are reared thereafter. Following the death of the queen(s), workers persist for a short period before the colony dies.

SOURCES AND HUSBANDRY

Although worker ants can be purchased to stock hobbyist ant farms, a mated queen is needed to maintain a functional research colony. In many countries, it is illegal to sell or transport reproductive ants across state or international borders without a permit. Therefore, laboratory colonies are typically
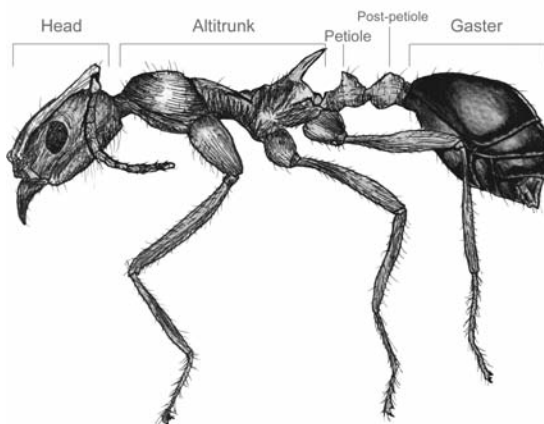

FIGURE 1. The anatomy of a standard ant. The nomenclature of ant body segments differs from that of most insects because the constriction (waist) is within the abdomen. Thus, the abdomen includes the last segment of the alitrunk, the petiole (and post-petiole when present), and gaster. The ant pictured is *Aphaenogaster cockerelli* in the subfamily Myrmicinae. (Drawing courtesy of Clint Penick.)

initiated by collecting an established colony or a founding queen from its natural habitat. Entire colonies including the queen(s) and brood can sometimes be removed from nests in soil, leaf litter, wood, nuts, or rock (see **Collecting Live Ant Specimens (Colony Sampling)** [Smith and Tschinkel 2009a] for a procedure to collect ground-nesting species). A less labor-intensive alternative is to collect newly mated queens immediately following a nuptial flight, the timing of which depends on the species and its environment.

Colonies of many ant species have been cultured successfully in the laboratory, typically housed in plastic containers connected by tubing (Fig. 2). Moisture is provided in test tubes partially filled with water and plugged with cotton or by lining the floor of the nest chamber(s) with plaster kept moist by adding water regularly. To prevent ants from escaping, the nest walls can be coated with Fluon, an aqueous solution of polytetrafluoroethylene (i.e., Teflon) that, once dried, provides a smooth surface most ants cannot climb. Many species acclimate well to indoor light, but nests can be covered with aluminum foil or a colored film to filter light. Food is typically supplied to a foraging arena in a drier container or region of the nest. Common food sources include honey water, insects (fresh or frozen), and/or seeds, depending on the species' diet (Table 1). Speed of brood development and colony growth rate both increase with temperature, which can be regulated using an incubator or heating pad. See Hölldobler and Wilson (1990) for more about culturing ants.

Ants are notoriously difficult to breed in the laboratory. Reproductive forms are usually not reared until colonies reach a critical size, which can require several years of growth. Moreover, in most species, mating is triggered by a unique combination of abiotic variables (e.g., temperature, humidity) and courtship behavior during nuptial flights, and therefore cannot be induced in the laboratory. Some species, however, regularly inbreed within their nests, and thus colonies can be perpetuated and multiplied under artificial conditions. Examples include the Pharaoh ant (*Monomorium pharaonis*), *Cardiocondyla* spp., and the jumping ant (*Harpegnathos saltator*).

RELATED SPECIES

Ants (Formicidae) are a monophyletic clade of Hymenoptera that evolved from wasps between 115 and 135 million years ago. Their large-scale phylogenetic relationships have recently been resolved using molecular markers, which largely confirmed the monophyletic status of subfamilies and tribes previously described using morphological characters (Brady et al. 2006; Moreau et al. 2006). These phylogenies also suggest that two major radiations occurred during the evolution of the Formicidae, splitting ants into two major groups: the poneromorphs and the formicoids, the latter of which contains the majority of described ant species (more than 10,000) (Ward et al. 2005). Members of at least three subfamilies of the formicoid clade can be collected with very little effort on each continent
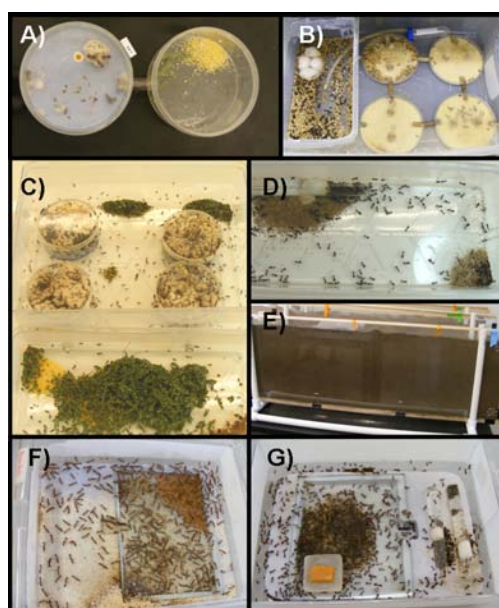

FIGURE 2. Examples of laboratory colony experimental and rearing designs. The species pictured is listed after each nest description. (*A*) For small nests (e.g., for founding queens), two chambers are connected. One (for living) is filled with plaster, with a watering tube to keep the plaster moist. The other chamber (without plaster) is used for foraging (*Acromyrmex versicolor*). (*B*) Similar to *A*, but for a larger colony. Each chamber (Petri dish) can be maintained at a different level of hydration. The dishes are interconnected; only one is connected to the larger foraging area (*Pogonomyrmex badius*). (*C*) As for *B*, but the chambers are taller; this is useful for leafcutter ants that grow large fungus gardens (*A. versicolor*). (*D*) A simple nest with only a test tube as a chamber and an open area where food can be placed (*Pogonomyrmex rugosus*). (*E*) A traditional "ant farm": a dirt-filled space between two glass plates (*Pogonomyrmex californicus*). (*F,G*) Plaster nests covered with a glass plate inside a larger box where food can be provided (*F: Harpegnathos saltator*, *G: Camponotus floridanus*). (Photographed nests courtesy of R. Clark, C.T. Holbrook, J. Liebig, and C.R. Smith.)

Table 1. Brief description of several ant species

Subfamily	Example genus	Example species	Common name (___ ant)	Economic importance?	Maintain well in lab?[a]	Diet[b]	Individual size[c]	Colony size	Genetic/genomic resources[d]	Geographic distribution[e]
Dolichoderinae	Linepithema	humile	Argentine	Yes	5	G, I	S	10^4	E, M	AF, AU, EU, NA, SA
	Tapinoma	sessile	Odorous	Yes	5	G, I	S	10^4		NA
Formicinae	Camponotus	floridanus	Carpenter	Yes	4	G, I	L	10^3	M	NA
	Lasius	niger	Garden	No	3	G, I	S	10^3	E, M	EU
	Myrmecocystus	mexicanus	Honeypot	No	3	G, I	M	10^3	M	NA
Myrmicinae	Acromyrmex	versicolor	Leafcutter	Yes	4	L	M	10^3	L, M	NA
	Monomorium	pharaonis	Pharaoh	Yes	5	G, I	S	10^4	M	AF, NA, SA
	Pheidole	morrisi	Big headed	No	3	G, I	M	10^2		NA
	Pogonomyrmex	badius	Harvester	No	3	G, I, S	M	10^3	E, L, M	NA
	Solenopsis	invicta	Fire	Yes	5	G, I	S-M	10^4	E, M	AF, AS, AU, NA, SA
	Temnothorax	albipennis	Acorn	No	5	G, I	XS	10^2	M	NA
Ponerinae	Dinoponera	australis	Dinosaur	No	2	G, I	XL	10^1		SA
	Harpegnathos	saltator	Jumping	No	4	G, I	L	10^2		AS
	Odontomachus	brunneus	Trap-Jaw	No	3	G, I, S	L	10^2		NA

[a](1) Low; (5) high.
[b](G) Sugar; (I) insect; (L) leaf; (S) seed.
[c](XS) Extra-small: (S) small; (M) medium; (L) large; (XL) extra-large.
[d](E) Expressed genes; (L) linkage map; (M) DNA markers.
[e](AF) Africa; (AS) Asia; (AU) Australia/Oceania; (EU) Europe; (NA) North America; (SA) South America.

excluding Antarctica (Formicinae, Myrmicinae, Dolichoderinae) (Table 1). Members of the ponero-morph clade are usually more difficult to collect because of their rarity, small colony sizes, or cryptic lifestyle. More detailed phylogenies are available for many subfamilies and genera.

USE OF THE ANT MODEL SYSTEM

Amazing diversity in social form and function, coupled with ecological dominance, have made ants a model system for exploring social life, including helping humans better understand our own sociality (Wilson 1975). They are readily studied in the field and the laboratory, enabling researchers to unveil both the proximate mechanisms and ultimate significance of social behavior. The integration of analyses across levels of organization, from genes to physiology to emergent social phenotypes, has begun to yield a more comprehensive understanding of life in complex societies and other systems. Science in the 21st century will continue to build on the detailed observations of ant naturalists in centuries past and will be augmented by next-generation molecular methods to gain insights into how ant societies work and what attributes have made them flourish: "Go to the ant, thou sluggard; consider her ways, and be wise …" (Proverbs 6:6).

Ecology

Ant dominance in terrestrial ecosystems is not achieved by a single, extremely successful species. Instead, there are an estimated 20,000+ ant species, and these occupy most conceivable ecological niches. In many communities, ants function at all trophic levels. For example, both leafcutter ants (*Atta*) and army ants (*Eciton*) exist in colonies with millions of workers and consume massive quantities of resources; the former are primary consumers, and the latter top predators. Ease of observation and collection also make them ideal for assessing community properties including diversity, organization, and competition. Additionally, ants participate in extraordinary symbioses that are models for the study of coevolution. Moreover, because individuals in a colony constantly interact, share resources, and live in dense aggregations, ants are emerging as a model for epidemiology and disease transmission.

The taxonomic and ecological diversity of ants has made them models for the study and assessment of biodiversity. Ants have been used successfully as bioindicators of the overall richness of animal and plant species in some habitats (Alonso 2000). The great advantage of ants lies in their relative ease of sampling and their diversity in ecological function. Thus, surveyors can potentially use ant assemblages to make inferences about the well-being of particular ecosystem processes. For example, many ant species are susceptible to environmental change, and their persistence or local extinction from sites can be used to evaluate the effects of perturbations or the relative success of remediation efforts (Alonso 2000). As poikilotherms, ants are extremely sensitive to temperature fluctuations, and future studies of ant communities are likely to yield insights into the effects of global climate change. Evidence of adaptation by ants to urban heat islands (Angilletta et al. 2007) supports the concept that natural selection for heat tolerance in ants can occur rapidly. Furthermore, global warming is likely to select for species with small body size and large colonies, characteristics shared by many already invasive species (Kaspari 2005). Thus, studies of ant ecology likely will intensify as investigations of the causes and consequences of global climate change and globalization progress.

Some ant species exert very strong effects on their environment, altering both species composition and resource distributions, and these species can act in a keystone capacity in communities. One ant species (*Myrmelachista schumanni*) creates monospecies vegetative plots (i.e., "Devil's Gardens") within the hyperdiverse Amazon rainforests (Frederickson et al. 2005). Leafcutter ant colonies consume as much vegetation as mature cows, cutting leaves in a selective manner, thus affecting the structure and function of whole plant communities (Hölldobler and Wilson 1990). Moreover, the nests of some harvester ant species (e.g., *Pogonomyrmex*) are veritable islands of nutrient abundance: They forage for resources far from their nests and concentrate nutrients in and around their mounds, as well as cycle chemicals in the soil column as they excavate (Wagner and Gordon 1999).

Ants are also notoriously invasive species. In a list of the 100 most harmful invasive species, there were five ants (see Table 1 for some examples; Lowe et al. 2000); many other ant invaders narrowly missed inclusion. These ant invaders have devastating impacts on communities (Holway et al. 2002), and most are facilitated by human-mediated habitat destruction and fragmentation (Holway et al. 2002; Tschinkel 2006). Studies of some invasive species have been informative in understanding the basic processes of invasion and why some species succeed as invaders whereas others fail

(Suarez et al. 2005). Observations of some invasive species have helped unravel fundamental aspects of social organization and communication (Tsutsui et al. 2003).

Because it is possible to observe many interactions among individuals, ants have been models for studying inter- and intraspecific competition. In particular, species with easily visible nests facilitate the mapping of territories, and competition can be observed easily by placing baits near territorial boundaries. Because ants are often abundant members of communities, it is possible to get large sample sizes. Methods have also been developed to both exclude and/or remove species in order to assess directly dominance in competition (LeBrun et al. 2007). Studies of interspecific competition have been very informative in developing an understanding of how communities are structured (Davidson 1977). Also, ants and plants show striking similarities in aspects of their biology (e.g., immobility and modularity), and the rich theoretical literature on plants has been applied successfully to ants (e.g., Kaspari and Byrne 1995).

One of the most spectacular qualities of ants is their involvement in intricate and obligate symbioses. The most intensively studied mutualism involves leafcutter ants, which farm an obligate mutualist fungus for food. The complexity of this system is enhanced by a parasitic fungus and an antibiotic bacterium harbored by the ants (Currie et al. 2003). Investigation into the coevolution of the three mutualists and their "arms race" against their parasite has yielded important insights into the evolution and maintenance of symbioses. This system also represents an evolution of agriculture independent from humans, providing insights into how agricultural systems evolve and are maintained. Similarly, several ant species have evolved herding, such that the ants tend and manipulate sap-feeding bugs in much the same way as humans do with cattle (Hölldobler and Wilson 1990).

In addition to mutualistic symbioses, ants often host an incredible number of commensal and parasitic organisms in their colonies. Thomas et al. (2005) estimated that more than 10,000 insects are social parasites of ants; others are commensal and use ant nests for shelter (Hölldobler and Wilson 1990). Social parasites have broken the chemical code of the ant colony (Lenoir et al. 2001) and not only trick the ants into letting them in, but often successfully solicit food from the ants, sometimes many times in excess of what would normally be fed to a developing ant larva. Some ant species, too, invade other species of ant and trick the residents into rearing unrelated offspring; some of these social parasites no longer produce workers and are obligate parasites (Brandt et al. 2005). Additionally, some ant species raid colonies of the same or different species and steal worker brood that they rear as their own, duping these "slaves" into rearing the host colony's larvae as though they were their own.

Because ants live in very dense societies and are exposed to various parasites and pathogens, some studies have tested human epidemiological models in ant colonies (Fefferman and Traniello 2009). Many parameters that are difficult to measure in human societies (e.g., contact rates, exposure time, etc.) are easily measured in laboratory ant colonies.

Social Evolution

Ants have inspired the major developments in theories on social evolution. To Darwin, the sterile castes of ants posed "one special difficulty, which at first appeared to me insuperable, and actually fatal to my whole theory" (Darwin 1859). His idea that natural selection favors families (or colonies) of altruists anticipated Hamilton's (1964) elegant solution to the problem: "inclusive fitness theory." Also known as "kin selection" (Maynard Smith 1964), inclusive fitness theory explains that altruism and other social traits can evolve by benefiting individuals who share genes with the altruistic actor. Over the past several decades, ants have played a leading role in testing inclusive fitness theory, especially quantitative predictions of sex allocation based on genetic relatedness asymmetries (Trivers and Hare 1976; for review, see Bourke and Franks 1995).

Colony-level traits in ants and other social insects can usefully be interpreted through the lens of modern multilevel selection, conceptualized (although initially dismissed) by Williams (1966), formulated by Price (1972), embraced by Hamilton (1975), and vigorously championed by D.S. Wilson, E.O. Wilson, and others (Wilson and Wilson 2007). The basic logic of multilevel selection is consistent with alternative perspectives of social evolution (e.g., inclusive fitness theory), but emphasizes the underlying processes of within-group versus between-group (i.e., colony) selection.

Following the multilevel selection framework, when intracolonial conflict is suppressed by intense competition among colonies, the colony becomes the primary target of selection and can be referred to as a "superorganism" (Hölldobler and Wilson 2008; see "Social Organization" below). Although all ants are eusocial, species vary in their degree of colonial integration; the smaller societies of many ponerine ants are characterized by internal competition over reproduction, whereas the largest, most

advanced colonies, such as those of leafcutter ants, display a unity of purpose that rivals some unitary organisms. In this context, social evolution in ants can shed light on the other major transitions in evolution (e.g., prokaryotes to eukaryotes, unicellularity to multicellularity), each of which involved the integration of lower-level units into higher-level units of biological organization (Maynard Smith and Szathmáry 1995).

Social Organization

The superorganism concept has gained traction recently with the support of multilevel selection, but it can be traced back a century to the pioneering myrmecologist W.M. Wheeler (1911), who noted several deep similarities between ant colonies and unitary organisms. These include differentiation into "germ plasm" (male and female reproductives) and "soma" (workers), and the properties of individuality, homeostasis, nutrition, reproduction, and protection. Moreover, many ant colonies undergo a stereotyped sequence of changes in composition and behavior during development (termed "socio-genesis"), which can be compared to the process of morphogenesis at the level of cells and tissues (Wilson 1985). Thus, ants can serve as models not only for studying the organization and evolution of animal societies, but also for probing the organization of complex systems and the evolution of development ("evo-devo").

In the definitive volume of myrmecology, Hölldobler and Wilson (1990) outlined two major aims of research on ants: "first to identify more fully the mechanisms by which colony members differentiate into castes and divide labor, and second to understand why certain combinations of these mechanisms have generated more successful products than others." These fundamental questions are best answered through integrative approaches, from developmental genetics to complexity theory, which also hold the potential to reveal general principles of biological organization and evolution. Ants offer great advantages over unitary organisms in this pursuit. Live colonies can be experimentally decomposed, manipulated, and reassembled, exposing the nature of a complex system from its most basic components to its emergent properties. Moreover, extensive population-level and macroevolutionary variation in attributes such as colony size, polyphenism, division of labor, and communication can be used to relate social organization and development to questions of ecology, life history, and adaptation, something that is not possible with existing embryonic evo-devo models (Yang 2007).

Division of Labor and Colony Integration

Division of labor, a pattern whereby different individuals within a group (also organelles within a cell, cells or tissues within an organism, etc.) perform different functions, is essential to both the evolution of complexity (Maynard Smith and Szathmáry 1995) and the spectacular success of ants (Wilson 1971; Oster and Wilson 1978). Reproductive division of labor is a hallmark of ant societies that enhances queen fecundity and allows for further labor specialization among workers. Most ant species studied to date exhibit temporal polyethism: Workers change roles as they age, typically progressing from in-nest tasks such as brood care, to foraging outside the nest. Furthermore, some derived species have evolved physical worker castes that differ in body size and/or shape, often to the extent that nestmates look like members of different species. In an extreme example, major and minor workers of the Asian marauder ant *Pheidologeton diversus* differ 10-fold in head width and 500-fold in mass. In these polymorphic ants, worker behavior is caste-specific, with majors specializing in defense (e.g., *Cephalotes*, *Pheidole*), milling seeds (e.g., *Pheidole*, *Solenopsis*), or food storage (e.g., *Melophorus*, *Myrmecocystus*), whereas minors perform brood care, housekeeping, and foraging.

Unlike solitary or primitively social organisms, where demography is an epiphenomenon of individual life histories, the age and size distributions of workers in an ant colony can be shaped by natural selection (i.e., adaptive demography) (Oster and Wilson 1978). Yang et al. (2004) showed that in *Pheidole morrisi*, major-to-minor worker ratios vary in a manner that is consistent with local adaptation: Colonies from populations in more competitive environments produce higher proportions of majors, potentially conferring a defensive advantage. Moreover, physical caste systems can be regulated homeostatically. When colonies of *Pheidole dentata* were altered to contain a uniform percentage of major workers and then allowed to grow freely, they quickly returned to their original caste ratios (Johnston and Wilson 1985). In *Pheidole bicarinata*, an excess of major workers inhibits further production of majors, evidently because of a pheromone produced by majors (Wheeler and Nijhout 1984).

An ant colony, however, is more than the sum of its parts. It must be able to monitor changes in its internal and external environment, allocate its members among tasks to meet the most urgent

colony needs, and coordinate the actions of many individuals (up to millions) into a coherent whole. These functions, and the underlying networks of communication that make them possible, represent the social physiology of a superorganism (Seeley 1995). A distinctive feature of insect societies is that order is not derived from a central command structure as in most human institutions, but instead emerges via self-organization, a process by which interactions among individuals based solely on local information give rise to group-level patterns (Camazine et al. 2001). Self-organized systems are flexible and robust to perturbations. For example, when minor workers are removed from a *Pheidole* colony, the majors respond by spontaneously expanding their behavioral repertoire to cover the tasks normally performed by minors, sustaining the colony until a workforce of minors can be restored (Wilson 1984). Even in monomorphic ant species, nonreproductive division of labor can be generated by amplification of initially slight differences in task preference, which can be associated with genotype and/or physiology (Beshers and Fewell 2001). Simple rules and positive feedback can also lead to fast and accurate collective decision-making, whether during recruitment to alternative food sources using a trail pheromone (Camazine et al. 2001) or selection of a new nest site (Pratt and Sumpter 2006). Beyond their basic scientific value, algorithms based on the self-organized behavior of ants can be applied to new designs in computing, telecommunications, and artificial intelligence, and provide insights into the networking of neurons in the human brain (Bonabeau et al. 1999).

Caste Determination

Investigations of caste determination in ants demonstrate how developmental and behavioral variation is affected by a dynamic interplay between nature and nurture (Smith et al. 2008a). Both environmental and genetic factors influence queen/worker and worker/worker caste differentiation. Additionally, the social environment (e.g., interactions among individuals in the nest, such as nurse workers and larvae) is critically important to caste determination. Generally, queen/worker differentiation involves nutrition, with queen-destined larvae receiving a larger quantity or better quality of food, which is likely controlled by the workers (Smith et al. 2008b). Nutrition is believed to affect caste by acting on the endocrine system, specifically juvenile hormone (JH, a hormone governing development across insect taxa) (Nijhout 1998). For example, JH levels increase with increased assimilated nutrition, and developmental pathways diverge based on JH levels relative to a threshold. Typically, if the threshold is surpassed, larvae begin queen-specific development; otherwise, worker development ensues. In some species, queens influence the fate of their larvae via pheromonal signals, which can inhibit sexual development during key periods (Keller and Nonacs 1993). Some queens also control hormone levels in their eggs, which gives them partial control over the caste fate of their offspring (Schwander et al. 2008). Queens might also be able to manipulate the nutritional starting point of their eggs: Laying eggs with decreased nutritional content will likely result in worker development (Wheeler 1986).

Genetic effects are also important in caste differentiation, although they have historically been thought to be rare and maladaptive. When larval genotype is a strong predictor of caste (i.e., strong genetic caste determination) (Anderson et al. 2008), the ability of colonies to manipulate caste ratios based on cues from the external environment, and thus adaptively change caste allocation, is reduced. For example, when colonies have few resources, they typically produce workers rather than expensive queens, but genetic caste determination constrains a colony's ability to manipulate which castes are produced. How strong genetic caste determination evolves and is maintained is not well understood (Anderson et al. 2008; Smith et al. 2008a). Genetic differences among larvae might affect the nutritional threshold needed to trigger queen development, such that some genotypes are more likely to develop into queens than others. Genetic caste determination appears to be widespread in ants, although typically the correlation between caste and genotype is weak (Anderson et al. 2008; Smith et al. 2008b). Moreover, interactions between an individual's own genes (direct genetic effects) and genes expressed in social partners (indirect genetic effects), such as between nurses and larvae, can influence caste determination (Linksvayer 2006). The importance of genetic and social facets of caste determination also makes ants an exemplary system to study the interactions between genetics and the environment.

Studies of species with polymorphic workers have yielded insights into the development and evolution of body size variation and allometry. The incredible variation sometimes seen among the worker castes of species can be explained by variation in two basic developmental processes: critical size and growth rules. Critical size is the size at which a developing larva metamorphoses into the next phase of development. Reaction norms for critical size can differ among larvae, resulting in continuous

variation in adult size, or with the addition of a threshold, discrete size classes of adult. Changes in growth rules correspond to differential allocation of nutrients and energy to body parts, which thus differ in relative growth rate. Critical size and growth rules can be influenced jointly at reprogramming events (i.e., sensitive windows during larval development) and thus give rise to individuals varying greatly in size and shape (Wheeler 1986). Both critical size and growth rules are likely affected by JH, which, in turn, is likely affected by nutrition.

The Comparative Method

Given the range of ant social structures and the availability of phylogenies across taxonomic levels (family, subfamily, genus, and species-group), ants are an ideal system for comparative tests of both proximate and ultimate questions. One important trait that varies among ant species is intracolonial genetic diversity. Despite the potential for high nestmate relatedness to favor the evolution of altruism (see "Social Evolution" above), it has long been known that colonies can be composed of individuals that are not highly related. For example, many ant colonies contain a queen that mated with multiple males (polyandry) and/or multiple queens within the nest (polygyny). Such genetic diversity can be selectively advantageous for division of labor, disease resistance, or other reasons (Oldroyd and Fewell 2007). A recent study of 241 species, controlling for phylogeny, found a significant negative relationship between polyandry and polygyny, indicating that they are alternate routes to greater intracolony genetic diversity (Hughes et al. 2008).

The diversity of female castes has drawn much attention for its variability across the ant phylogeny. Large degrees of worker polymorphism in ants appear to have evolved in excess of 18 times (Oster and Wilson 1978; Brady et al. 2006). Worker size variation was found to be more common in colonies with higher genetic diversity and greater queen/worker dimorphism (Fjerdingstad and Crozier 2006). Studies of size variation within genera have also been fruitful for assessing proximate and ultimate causation of worker size variation (e.g., Schöning et al. 2005). Furthermore, the worker caste has been secondarily lost in many species of socially parasitic ants. Social parasites occur across most ant subfamilies and have been used to address questions on conflict, coevolution, and chemical communication (Lenoir et al. 2001; Brandt et al. 2005).

Chemical Communication

Communication is the glue that holds an ant society together. It allows ants to discern friend from foe, to spread information regarding the location of food or the presence of an enemy, and to maintain reproductive division of labor, all of which are important in the maintenance of eusociality. Living in a predominantly dark environment, ants rely mostly on chemical signals for communication. A typical worker body contains more than 10 different semiochemical-producing glands. The surface of the cuticle is covered with lipids that serve, in addition to their traditional role in water conservation, as recognition signals (Howard and Blomquist 2005).

Trail and alarm pheromones are the most widely studied and chemically characterized of all ant semiochemicals. The same chemicals can be used by different genera, although they sometimes serve different functions (Keeling et al. 2004). The chemical composition and structure of a pheromone correlates with its function. Alarm pheromones, for example, can be single, small volatile chemicals, whereas trail pheromones, which must provide more information than can be conveyed by a yes-or-no answer, are usually mixtures of chemicals varying in volatility (Hölldobler and Wilson 1990). Male ants usually produce aggregation pheromones to attract conspecific alates of both sexes to mating areas, although they have not been chemically identified to date. Similarly, few queen pheromones are chemically known. The slave-making *Polyergus rufescens* queen produces a pheromone, decyl butanoate, which repels host workers (Keeling et al. 2004). In the red imported fire ant, *Solenopsis invicta*, a queen pheromone of unknown structure induces workers to kill sexual larvae (Klobuchar and Deslippe 2002). Two other queen pheromones in the same species are responsible for retinue formation and for inhibiting reproductive development in virgin queens (Vargo 1998).

Cuticular hydrocarbons play a major role both in nestmate and caste recognition. These hydrocarbons are produced in specialized cells, oenocytes, and transported to the surface of the cuticle and other body parts via the lipid carrier protein lipophorin (Fan et al. 2003). In small colonies, ants can learn individual hydrocarbon profiles of their nestmates, but in large colonies it is assumed that a shared, average colony profile is learned by the ants (Howard and Blomquist 2005). In addition to the genetic influence on the hydrocarbon profile, explained by the relatedness of colony members,

environmental effects such as microclimate and food can distinguish ant colonies from one another (Buczkowski et al. 2005). The mechanisms underlying nestmate recognition might help to explain the success of some invasive ant species in their new environment, as well as the significance of recognizing one's self from other individuals (Payne et al. 2004).

Ants can also distinguish each other within a colony because of the nature of the different tasks they perform. Ants that spend more time outside (e.g., foragers) will have different hydrocarbon profiles than ants that stay inside the nest; the differences are triggered by environmental conditions encountered by the ants (Wagner et al. 2001; Howard and Blomquist 2005). The information provided by such task-related differences in cuticular hydrocarbon compositions can help ants in making decisions. For instance, in the seed-harvester ant *Pogonomyrmex barbatus*, the rate at which ants encounter patrollers at the nest entrance helps them decide whether to initiate foraging (Greene and Gordon 2007).

Finally, fertility correlates with the presence of specific hydrocarbons, probably as a result of ovarian activity. Specific hydrocarbons have been implicated in determining dominance hierarchies in queenless species or following the death of the queen in some ponerine ants, as well as maintaining reproductive monopoly by the queen in the carpenter ant, *Camponotus floridanus* (Howard and Blomquist 2005; Endler et al. 2006). Thus, ants can serve as a model system for understanding the development of chemicals as honest signals (Peeters and Liebig 2009).

Sociogenomics

The increasing development of molecular toolkits for use in ants is helping to establish them as an important source of information on sociogenomics, that is, understanding social life in molecular terms (Robinson et al. 2005). Because of the behavioral diversity seen within colonies, among colonies, and between species of ants, the investigation of molecular mechanisms underlying social organization in ants offers exciting possibilities. This approach has provided insights into task allocation in the seed-harvester ant *P. barbatus*. In both honey bees and these ants, the *foraging* gene has significant expression differences between task groups: In honey bees, high levels of expression correlate with high foraging activity, whereas the opposite is true in *P. barbatus* (Ingram et al. 2005). Thus, a conserved gene accomplishes similar organization effects, but differently in independent eusocial lineages.

Another example of the role of genes in social behavior is the discovery of *Gp-9* (General protein 9) in *S. invicta* fire ants. Differences in genotype correspond with the presence of one or more queens (monogyny vs. polygyny). The gene codes for a putative pheromone-binding protein believed to be involved in recognition (Gotzek and Ross 2007). Not only is this difference in queen number associated with relatively few genes, but gene expression also is strongly affected by the genotypic makeup of the colony, independent of the genotype of an individual worker (Wang et al. 2008). This, and other examples from other ant species (e.g., Linksvayer 2006), provide opportunities for exploring indirect genetic effects and their role in organizing social life (Linksvayer and Wade 2005).

Reproductive caste determination (i.e., whether a larva becomes a queen or worker) is most often dependent on environmental factors and not the genetic makeup of the individual. Therefore, any phenotypic differences between queens and workers are attributable to differences in gene regulation and ontogeny rather than differences in their genes. Research on *Lasius niger*, which shows significant queen/worker dimorphism, showed that queens overexpress genes linked to reproductive division of labor as well as some linked to somatic and immune system maintenance. This highlights the usefulness of ants as a model system for exploring the effects of gene expression on division of labor and aging (Gräff et al. 2007).

Ants have also been used as a model for evolutionary development. Abouheif and Wray (2002) found that the loss of wings in workers of different species is not governed by a single, conserved disruption in the gene network of wing development. Instead, different genes in the regulatory network are disrupted in different species to give the convergent phenotype of wingless workers. Furthermore, in a species producing two discrete worker sizes (*P. morrisi*), the wing development pathway is disrupted at different genes in the forewings and hindwings of soldiers. Thus, one of the most characteristic phenotypes of ants is evolutionarily labile. Future studies using the diversity found in ants should indicate how developmental patterns evolve.

Although these are just a few examples of sociogenomic research using ants, it is obvious that ants are a practical and advantageous model system for studying social organization from a molecular, as well as an evolutionary development, perspective.

GENETICS AND GENOMICS

Chromosome numbers and karyotypes are known for a large number of ants, and the described karyotypes (1N) range from 1 to 47 (Imai and Taylor 1989; Imai et al. 2001). Physical genome size is also known for representatives of most subfamilies and ranges from 210.7 Mb (0.22 pg) in *Cerapachys edentatus* to 690.4 Mb (0.71 pg) in *Ectatomma tuberculatum* (Tsutsui et al. 2008).

One genetic map for an ant species (*Acromyrmex echinatior*) has been published (Sirviö et al. 2006), and the mean recombination frequency is the second highest so far reported for any higher eukaryote species, next to that of another eusocial hymenopteran, the honey bee *Apis mellifera* (Wilfert et al. 2007).

Because of the importance of the intracolonial relatedness structure for sociobiological research, ants are a group for which every major class of molecular marker has been used in multiple studies (e.g., allozymes, DNA fingerprinting, random amplification of polymorphic DNA, microsatellites, amplified fragment length polymorphism, etc.), and protocols for the application of any of these markers can be found in the literature. With the advent of efficient sequencing and array techniques, expressed sequence tags have become available for a couple of ant species (Gräff et al. 2007; Wang et al. 2008), and expression studies using a candidate gene approach have been conducted (Ingram et al. 2005). We are hopeful that the first ant genomes will be sequenced soon. Sequenced genomes of other Hymenoptera, the honey bee (Honey Bee Genome Sequencing Consortium 2006), and the solitary parasitic wasp *Nasonia* will facilitate genome annotation in any upcoming ant genome project. A comparison between the genomes of solitary insects and multiple convergently evolved social insects is likely to provide insights into how sociality has changed the genome of an organism and will help us to understand how sociality is maintained and becomes elaborated to generate an ever higher degree of social complexity.

TECHNICAL APPROACHES

Ants can be examined in their natural habitats to study ecological interactions and assess biodiversity (see **Ecological Sampling of Ants: Competition and Biodiversity** [Smith 2009]). Furthermore, biochemical studies such as assessing stored body fat (see **Ant Fat Extraction with a Soxhlet Extractor** [Smith and Tschinkel 2009b]) can help assess the nutritional status of individual ants and colonies, whereas **Stable Isotope and Elemental Analysis in Ants** (Smith and Tillberg 2009) can provide insights into dietary ecology and the role of ants in food webs.

For laboratory-based experiments, procedures are available for **Collecting Live Ant Specimens (Colony Sampling)** (Smith and Tschinkel 2009a) and for **Marking Individual Ants for Sampling Behavior in a Laboratory Colony** (Holbrook 2009).

Juvenile Hormone Extraction, Purification, and Quantification in Ants (Brent and Dolezal 2009a); **Radiochemical Assay of Juvenile Hormone Biosynthesis Rate in Ants** (Brent and Dolezal 2009b); and **Ant Ecdysteroid Extraction and Radioimmunoassay** (Brent and Dolezal 2009c) present methods for measuring two developmentally crucial classes of insect hormones. **GC-MS for Characterization and Identification of Ant Semiochemicals** (Eliyahu 2009) discusses techniques for the study of the chemical signals used by ants for communication.

DNA Isolation from Ants (Gadau 2009a) presents two methods for isolating DNA from a wide range of ant species suitable for a use in a variety of molecular biological applications, such as **In Situ Hybridization on Ant Ovaries and Embryos** (Khila and Abouheif 2009). **General Dissection of Female Ant Reproductive System and Brain** (Dolezal and Brent 2009) provides protocols to obtain tissues for such studies. Finally, **Phase-Unknown Linkage Mapping in Ants** (Gadau 2009b) discusses the procedures necessary to generate linkage maps when the phase of the marker to be used is not known, as is often the case with ants.

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Collecting Live Ant Specimens (Colony Sampling)

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INTRODUCTION

Because of the great diversity of ants, it is difficult to give a single protocol for the collection of live specimens. Ant body size can be very small or extremely large; the ants can be hard or soft, sting or spray toxic chemicals, live in the open or in hard-to-reach places; and colony size can range from tens of individuals to millions. Thus, collection techniques must be tailored to each particular species. In particular, caution must always be taken when dealing with stinging species, and symptoms and basic first-aid measures, especially for the treatment of anaphylactic shock, should be reviewed before beginning fieldwork. Nonetheless, many species are collectable as whole colonies. This protocol reviews some basic techniques for collecting ground-nesting species and describes how to collect whole live colonies (with queens), which are necessary for long-term laboratory studies and addressing questions of social organization and ecology.

RELATED INFORMATION

The protocol presented here has proven very successful for the excavation of several species of harvester ant with very different nesting ecologies, in soils ranging from very sandy to complete clay. If the focal species has nests deeper than one-half meter and lives in very rocky soils, the use of mechanical assistance (e.g., a small backhoe) is recommended. Colony-collection techniques specific to other particular species are described in the literature (Tschinkel 1987, 1993, 1998, 2005; Seal and Tschinkel 2006).

For a more extensive discussion of the background, husbandry, and potential uses of ants as model organisms, see **Ants (Formicidae): Models for Social Complexity** (Smith et al. 2009).

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <!>, and recipes for reagents marked with <R>.

Reagents

Fluon

Fluon is an aqueous solution of poly(tetrafluoroethylene) that can coat vertical surfaces and prevent climbing and hence escape of ants.

Aspirator

The collecting tube of the aspirator should be appropriate for the size of the ants to be collected. Some ants spray toxic chemicals (e.g., formic acid) that should not be inhaled. An aspiration bulb can be used in lieu of mouth suction, as can a modified vacuum cleaner.

Cotton balls

Forceps

Use soft (floppy) forceps to collect softer-bodied species.

Hand trowel

Knife or small cement spreader

Shovel

Choosing an appropriate shovel is important. If digging >30 cm, use a shorter-handled shovel with a wide blade. A sharpened blade, beveled on only the top side, increases digging accuracy (e.g., exposing chambers/tunnels).

Tarp

Test tubes

Vials or other collection containers (e.g., plastic boxes), equipped with lids

Wooden planks (optional; see Step 11)

METHOD

Preparation

1. Before going into the field, prepare containers to place the ants in once they have been collected:

   i. Cover the sides of the containers with Fluon to prevent escapes.

   ii. Place damp cotton balls in the containers.

   This prevents desiccation of the ants after collection but before arrival at the laboratory.

2. Locate the nest entrance(s).

   These will serve as a starting point for excavation (e.g., Fig. 1A,B*).*

3. Clear debris and obstacles (including plants) from near the nest entrance gently in order to maintain the integrity of upper nest chambers.

4. (Optional) Depending on the species, water the nests days or hours before collection.

   This can decrease the collection of loose dirt when ants are aspirated and also draws ants toward the ground surface, including larvae/pupae.

Excavating the Top of the Nest

(See Fig. 1A.1-1A.6.*)*

5. Collect foragers leaving and entering the nest.

   Often 10% of the worker population will be foraging. If a complete collection of the population is desired, it is necessary to patiently collect those individuals not physically in the nest. Do not collect foragers away from the nest; these could be from neighboring colonies.

6. Gently excavate the area centered on the nest entrance using a flat-bladed shovel to "peel" away layers of dirt.

   In many species, numerous chambers are located just below the surface and can be distributed quite widely around the nest entrance. It is important to explore the surface around the nest fully, collecting ants from the top chambers as each chamber is exposed.

7. Carefully expose the vertical shafts that extend downward from the exposed chambers (Fig 1C,E).

   Use a knife (or small cement spreader) so as not to destroy or plug the holes, as can occur when using a shovel or trowel. Blow loose dirt out of the way using the aspirator hose to help expose chamber edges and tunnels.
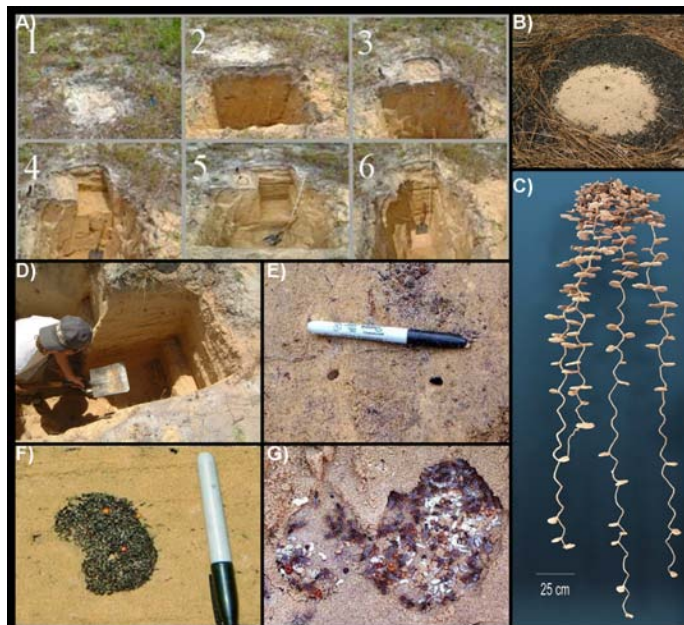
FIGURE 1. An illustrated guide to nest excavation and colony structure for the Florida harvester ant, *Pogonomyrmex badius*. (*A*, panels *1-6*) Sequential stages of the excavation. (*B*) The nest mound. (*C*) Plaster cast of the nest's underground architecture. (*D*) An excavator in the pit works the horizontal surface of the excavation. (*E*) The openings of two vertical shafts extend downward from the horizontal working surface. (*F*) A seed chamber. Note the vertical shaft to the *left* of the chamber. (*G*) A brood chamber. White objects are pupae and larvae.

8. Lay a tarp down next to a proposed pit location adjacent to the colony.

   Piling excavated dirt on the tarp facilitates cleanup and minimizes the footprint of excavation.

9. Dig a pit adjacent to the colony located such that it won't intersect with the nest, but near enough so that all of the exposed chambers can be reached (Fig. 1A,D).The pit should be broad enough to accommodate both you and your equipment.

   Generally, the breadth of the nest decreases as it gets deeper. If you are excavating a species known to have deep nests, make the initial pit large enough so that you will be able to dig deeper (i.e., it must be wider than your shovel).

10. Using the shovel, follow the vertical shafts downward:

    i. Dig from the side, removing horizontal layers of dirt to continue exposing the vertical shafts (Fig. 1A,D). Prevent loose dirt and debris from cluttering the horizontal surface.

    ii. Continue collecting ants as you expose more chambers (Fig. 1F,G).

    Many ants will fall into the pit if care is not exercised; be diligent about collecting all ants as chambers are exposed.

Excavating the Bottom of the Nest

*The queen(s), brood, and younger workers tend to occur at the bottom of the nest (*Fig. 1G*). Take extra care not to damage the soft-bodied larvae and queen.*

11. If digging very deep, reinforce the walls of the pit using wood planks.

12. Widen and deepen the pit as needed to follow chambers and tunnels. Always keep the pit deeper than the working surface (i.e., the exposed chambers), and always keep the working surface horizontal.

    This makes it easier to follow the nest downward. Exposing the nest below the bottom of the pit can be dangerous and makes it more difficult to collect the ants.

13. Continue digging beyond the point where chambers and tunnels are no longer found to be certain the complete colony has been uncovered.
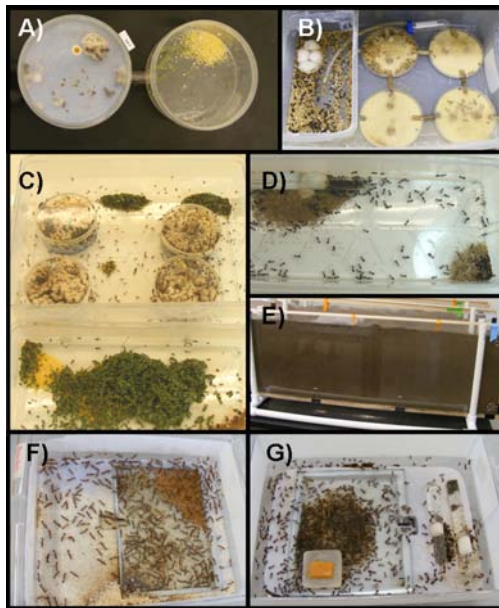
FIGURE 2. Examples of laboratory colony experimental and rearing designs. The species pictured is listed after each nest description. (*A*) For small nests (e.g., for founding queens), two chambers are connected. One (for living) is filled with plaster, with a watering tube to keep the plaster moist. The other chamber (without plaster) is used for foraging (*Acromyrmex versicolor*). (*B*) Similar to *A*, but for a larger colony. Each chamber (Petri dish) can be maintained at a different level of hydration. The dishes are interconnected; only one is connected to the larger foraging area (*Pogonomyrmex badius*). (*C*) As for *B*, but the chambers are taller; this is useful for leafcutter ants that grow large fungus gardens (*A. versicolor*). (*D*) A simple nest with only a test tube as a chamber and an open area where food can be placed (*Pogonomyrmex rugosus*). (*E*) A traditional "ant farm": a dirt-filled space between two glass plates (*Pogonomyrmex californicus*). (*F,G*) Plaster nests covered with a glass plate inside a larger box where food can be provided (*F: Harpegnathos saltator, G: Camponotus floridanus*). [(*A,C*) Photos courtesy of Rebecca Clark; (*D,E*) photos courtesy of C.T. Holbrook.]

Post-excavation

14. Make sure the collected ants are stored properly and remain cool during transport.

15. Fill in the hole and tamp down the dirt.

    Attempt to leave as little of an ecological footprint as possible.

16. Set up the lab colony following a method appropriate for the species.

    For details, see Fig. 2 *and the "Sources and Husbandry" section of* **Ants (Formicidae): Models for Social Complexity** *(Smith et al. 2009).*

TROUBLESHOOTING

Problem: Tunnels cannot be found.
Solution: Carefully using a trowel, scrape the dirt away. Use the aspirator to blow vigorously on the newly exposed dirt. If this does not work, the tunnels might be outside the excavation area. In this case, expand both the horizontal work surface and the pit outward.

Problem: The hole collapses.
Solution: The excavation can usually be salvaged by removing the fallen dirt, but great care must be taken when removing dirt from the horizontal surface of the exposed nest.

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Marking Individual Ants for Behavioral Sampling in a Laboratory Colony

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INTRODUCTION

Ant societies are tractable and malleable, two features that make them ideal models for probing the organization of complex biological systems. The ability to identify specific individuals while they function as part of a colony permits an integrative analysis of social complexity, including self-organizational processes (i.e., how individual-level properties and social interactions give rise to emergent, colony-level attributes such as division of labor and collective decision making). Effects of genotype, nutrition, and physiology on individual behavior and the organization of work also can be investigated in this manner, through correlative and manipulative approaches. Moreover, aspects of colony demography (e.g., colony size, and age and size distributions of workers) can be altered experimentally to examine colony development and regulatory mechanisms underlying colony homeostasis and resiliency. This protocol describes how to sample the behavior of ants living in a colony under laboratory conditions. Specifically, it outlines how to identify and observe individuals within a colony, an approach that can be used to quantify individual- and colony-level patterns of behavior. When a lower-resolution measure of overall group behavior is desired, individual identities might not be required. Given the diversity of ants and their study, this protocol provides a very general methodology; the details can be modified according to the body size, colony size, and ecology of the focal species, as well as to specific research aims. These basic techniques can also be extended to more advanced experimental designs such as manipulation of colony demography and hormone treatment.

RELATED INFORMATION

For a more extensive discussion on the background and potential uses of ants as model organisms, as well as information for maintaining colonies in the laboratory, see **Ants (Formicidae): Models for Social Complexity** (Smith et al. 2009).

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <!>, and recipes for reagents marked with <R>.

Reagents

<!>CO_2 (carbon dioxide) (optional; see Step 1)
Paint
Solvent-based hobby coating or Sharpie oil-based paint markers are recommended.

METHOD

Marking Individual Ants

1. Restrain an individual ant.

   Active ants can be handled more easily following a period of cooling or sedation with CO_2. Depending on body size and aggression, an ant can either be grasped gently between the thumb and index finger or immobilized on a piece of soft foam with insect pins or a wire or thread harness.

2. Apply a drop of paint to dorsal body surface(s) using a size-appropriate paint applicator.

   Use different color combinations on the head, alitrunk, and gaster (see Fig. 1_) to increase the number of unique individual identities. Take care not to paint over eyes or inhibit movement of antennae and legs. A dissecting microscope can be helpful when painting small ants._

3. Once the paint has dried, return the ant to the colony.

Observing and Analyzing Behavior

4. Wait for ants to resume normal activity (e.g., hours or days).

5. Observe and record behavior of marked individuals.

   A variety of sampling methods (e.g., focal-animal continuous sampling or instantaneous scan sampling) (Altmann 1974) can be used, depending on the research objectives. Data can be recorded in real time by a human observer. Alternatively, if individual identities and behaviors are detectable from a fixed position, the entire colony can be videotaped and reviewed later.

   See Troubleshooting.

6. Analyze behavior (see Discussion).

TROUBLESHOOTING

Problem: Paint marks do not persist once ants are returned to the colony.
[Step 5]
Solution: Depending on the cuticle texture and grooming behavior of a particular species, some types of paint (e.g., enamel, lacquer, fuel-proof dope, nontoxic) might work better than others. Also, in
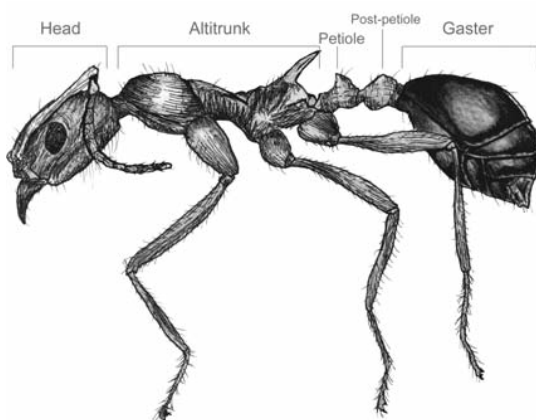

FIGURE 1. The anatomy of a standard ant. The nomenclature of ant body segments differs from most insects because the constriction (waist) is within the abdomen. Thus, the abdomen includes the last segment of the alitrunk, the petiole (and post-petiole when present), and gaster. The ant pictured is _Aphaenogaster cockerelli_ in the subfamily Myrmicinae. (Drawing courtesy of Clint Penick.)

some species, workers will groom more frequently if only a subset of the colony is painted; painting the entire colony, if feasible, can resolve the problem. Alternative marking methods include tying fine-gauge colored wires around the petiole (and post-petiole, if present) (Fig. 1), or gluing colored, numbered bee tags to the alitrunk of large ants.

DISCUSSION

A standard method for analyzing worker ant behavior is to compile a list of distinct behavioral acts that can be grouped into tasks, that is, a particular sequence of acts that accomplishes a specific purpose (e.g., brood care, nest maintenance, foraging). This list can then be used to construct an ethogram that incorporates behavioral repertoires and transition probabilities connecting individual acts (Hölldobler and Wilson 1990). These techniques provide quantitative measures of behavior that facilitate comparisons within and between species. However, they have been criticized for their subjective assessment of acts and tasks and the loss of contextual information, including social interactions (Bourke and Franks 1995). Gorelick et al. (2004) developed a framework for quantifying division of labor that can be applied across social systems. Researchers are increasingly coupling behavioral observations with agent-based modeling to explore algorithms underlying collective behavior (e.g., Pratt and Sumpter 2006).

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Ecological Sampling of Ants: Competition and Biodiversity

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INTRODUCTION

Ants are among the most dominant taxa in terrestrial ecosystems, despite their small individual size. Furthermore, they are a hyperdiverse family with an estimated 20,000 species. Together, these two properties make ants a model for ecological interactions (specifically competition) and biodiversity estimation. Although there are many means of measuring diversity, the two most common among myrmecologists are baiting and pitfall trapping. Pitfall traps provide an almost unbiased estimate of the ground foraging community, whereas baiting allows the estimation of ecological dominance and the competitive outcomes between species. This protocol describes an approach to assay both abundance (pitfall traps) and ecological interactions (baits) in the same community.

RELATED INFORMATION

This protocol presents only some of many sampling methods available. There is an extensive body of literature on both baiting and pitfall trapping, as well as differences in opinion as to the "best" method. Agosti et al. (2000) discuss the advantages and disadvantages of using different bait types, as well as different sizes of pitfall traps, killing agents, etc. They also discuss many other methods for sampling ant diversity. Other studies have assessed the relative effectiveness of different monitoring methods across habitats (e.g., Schlick-Steiner at al. 2006). Before beginning a project to sample ant communities, reviewing the merits of different approaches is recommended.

For a more extensive discussion on the background, ecology, and potential uses of ants as model organisms, see **Ants (Formicidae): Models for Social Complexity** (Smith et al. 2009).

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <!>, and recipes for reagents marked with <R>.

Reagents

Propylene glycol (animal-friendly antifreeze)

Propylene glycol is a good preservative, and samples can be stored in it until they are washed with water and stored permanently in 95% ethanol.

Tuna fish (canned)

Equipment

Card stock, laminated, >10-cm diameter

METHOD

Baiting

1. Define the site to be sampled. Randomly or pseudorandomly (i.e., stratified random) define points in transects or grids, depending on the question being addressed.

2. Flag all points. Map them for future reference.

   Each point will be a sampling locale for both baiting and pitfall trapping.

3. Prepare a datasheet for each sampling locale with the following column headings: "Sample Time," "Species 1," "Ant Numbers," "Species 2," "Ant Numbers," and "Comments."

   Each row will represent an observation of the activity at the bait.

4. In the early morning (or when ant activity is perceived as high), place laminated cards at each of the sampling locales. Place a dollop of tuna 20 mm in diameter in the center of each card.

5. At regular intervals (e.g., every 5-10 min or more frequently), check the baits for the presence of ants.

   The first ant of a species observed at a bait "discovers" the bait. Any calculations based on recruitment and foraging are calculated as "Time Since Discovery."

   i. Note the species, morphological type, and number for each species present.

   Often, "recruitment" is defined as the presence of more than 10 individuals of a species at a bait. Because many ant species recruit foragers en masse to baits, it might be necessary to estimate ant numbers once they go beyond 20 individuals (e.g., 20-50, 50-100, etc.).

   ii. Record the displacement of one species by another at a bait.

   iii. Use the metrics of discovery and recruitment to compare species.

Pitfall Trapping

Pitfall trapping should not precede baiting at sites because ants will avoid the area where trapping was conducted.

6. At the same points where baits were collected, but at least 24 h afterward, begin pitfall trapping.

   The time lag is necessary to ensure that ant trails are not biased to that particular site because of recruitment to the bait; 24 h is the minimum suggested time between baiting and pitfall trapping.

7. Using the pipe and mallet or the trowel, make a hole in the soil for the 50-mL centrifuge tube.

   In forgiving soils, hammering the pipe into the ground and removing it to create a hole will disturb the ground less than digging a hole with a trowel.

8. Insert the centrifuge tube into the newly created hole so that the top of the tube is flush with the ground surface; no lip should be visible.

9. Cover the tube with the cap inverted. Do not leave tubes open or fill them with propylene glycol for at least 24 h.

   The soil disturbance can affect ant activity and bias results (a so-called "digging-in effect").

10. After 24 h, open the tubes. Fill the tubes one-third full with propylene glycol.

    This is most easily accomplished by measuring out the desired quantity with an extra centrifuge tube and pouring it from the measuring tube into the tube in the ground.

11. Leave the traps open for 24-48 h.

12. After the collection period, remove the tubes from the ground and cap them.
    See Troubleshooting.

13. Extract the samples from the propylene glycol:

    i. Open the tube. Cover the top with fine-knit fabric.

    ii. Pour out the propylene glycol.

    iii. Wash the samples with water repeatedly until all of the propylene glycol is removed.

TROUBLESHOOTING

Problem: It rained and the pitfall traps filled with water.
[Step 12]
Solution: The data are likely unreliable, because samples might have been washed out of the tubes. Do not use these data.

DISCUSSION

The metrics collected with the baiting sample collection method can be used to compare the general foraging ecology of species (e.g., solitary foragers, group foragers, mass recruiters) and dominance hierarchies of species within communities (based on displacements of species at baits). Furthermore, these data can also provide evidence of trade-offs between the ability of ant species to discover versus dominate food resources. However, because the probability of discovery of a bait is proportional to the relative abundance of that species, a measure of abundance unbiased by recruitment should also be collected (e.g., pitfall trapping). In contrast, pitfall trap samples will yield data on the relative abundances of ants. However, when sampling for biodiversity, it is important not to confound the individual ant with the colony. Thus, for conservative analysis, presence/absence occurrence data are often preferred.

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Stable Isotope and Elemental Analysis in Ants

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INTRODUCTION

Over the past 20 years, the use of stable isotopes to infer feeding ecology and the examination of how energetic and elemental exchanges are affected by and affect life (ecological stoichiometry) have gained momentum. The ecological diversity of ants makes them interesting models to explore dietary ecology and their role in food webs. Moreover, their ecological dominance in most habitats facilitates sampling. The protocol described here will produce samples adequate for submission to most labs that specialize in high-throughput analysis of stable isotopes; one should check with any particular lab for specific submission instructions. Note, however, that this protocol is designed specifically for the quantification of the natural abundance of stable isotopes; it does not cover the preparation of trace samples.

RELATED INFORMATION

For a more extensive discussion on the background and potential uses of ants as model organisms, see **Ants (Formicidae): Models for Social Complexity** (Smith et al. 2009).

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with **<!>**, and recipes for reagents marked with **<R>**.

Reagents

Ants of the species of interest, live, fresh
Desiccant (e.g., Drierite)
> *Place it in a pouch made of tissue for Step 12.*

Ethanol

Equipment

Capsules, tin, sterile (e.g., Costech)
Cell culture plates, 96-well (e.g., Fisher)
Drying oven, preset to 50°C
> *In addition, an oven set to low temperature is optional for Step 10.*

Forceps, hard and soft
Freezer

Laboratory tissues
Microbalance, accurate to <1 mg
Plastic bags, sealable (e.g., ziplock)
Tape
Vials, sterile

METHOD

Sample Collection and Preservation

Samples stored in ethanol cannot be used for hydrogen or carbon analysis; storage in ethanol does not affect nitrogen analysis.

1. Collect live, fresh ants of the species of interest. Separate them from soil and other debris before placing them in a sterile collection vial.

2. Kill the samples by freezing or by other nonchemical means.

   Minimize the time the samples spend in the freezer; 12 h or less is usually sufficient. Do not store samples in the freezer.

3. Dry dead samples in an oven for 48 h at 50°C.

   Longer drying times will not hurt the samples.

4. Store samples in the oven in partially open vials with color-changing desiccant (to indicate if samples become exposed to moisture).

   If stored outside of an oven, maintain with desiccant in a sealed container.

   See Troubleshooting.

Sample Processing

5. Prepare a datasheet on which to record "Cell Coordinates" (row and column from the 96-well plate, i.e., A1, A2, etc.), "Sample Identity," and "Sample Mass."

6. Using a precision microbalance, weigh and tare a sterile tin capsule.

7. Using forceps sterilized in 100% ethanol, remove the gaster (i.e., the portion of the abdomen after the constricted "waist," Fig. 1)

   The gaster should not be included in any sample because this portion will likely be contaminated by the recent diet of the ant.

8. Weigh the ant or ant tissue to be analyzed:

   For nitrogen and carbon quantification, a sample range of 800-1200 g is suggested. Thus, depending on the focal ant species, portions of, entire, or multiple individuals might be needed to meet this target. For example, in large Pogonomyrmex *harvester ants, only a portion of the ant is necessary. For Argentine ants* (Linepithema humile)*, 10-15 individuals are necessary. For the smallest of ants, even more are needed.*

   i. If the entire ant is desired but too heavy, pulverize the ant using forceps. Weigh the appropriate mass.

   ii. Record the mass on the datasheet.

   Sterilize the forceps after each use.

9. Close the tin capsule to seal the contents inside. Compact the closed capsule into a square or ball to facilitate sampling with an autosampler.

   Stray pieces of foil should be avoided.

   See Troubleshooting.

10. Place the closed capsule in the appropriate well in the 96-well plate.

    If all samples cannot be processed in one session, secure the lid of the 96-well plate (e.g., with tape). Store the plate in a low-temperature oven or a sealed bag with desiccant.
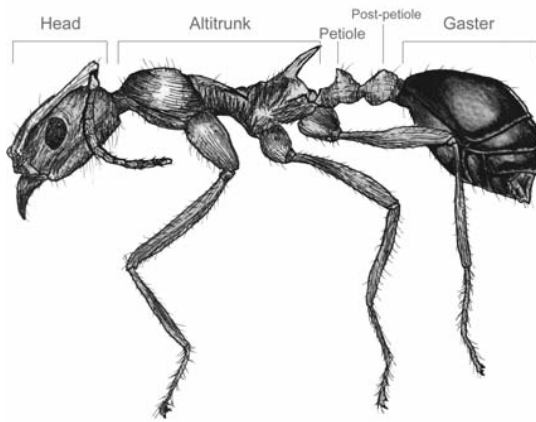
FIGURE 1. The anatomy of a standard ant. The nomenclature of ant body segments differs from most insects because the constriction (waist) is within the abdomen. Thus, the abdomen includes the last segment of the alitrunk, the petiole (and post-petiole when present), and gaster. The ant pictured is *Aphaenogaster cockerelli* in the subfamily Myrmicinae. (Drawing courtesy of Clint Penick.)

11. When all samples have been collected, label the plate clearly; be sure the datasheet is labeled similarly. Tape the lid of the plate closed securely so that samples cannot leave their well.

12. Enclose the plate in a sealable plastic bag with desiccant.

    Place the desiccant in a pouch made of tissue to keep dust from reaching the samples.

TROUBLESHOOTING

Problem: The sample appears/feels tacky/sticky after taking it out of the oven.
[Step 4]
Solution: Return it to the oven; it probably is not completely dry.

Problem: The tin capsule breaks.
[Step 9]
Solution: Enclose the broken capsule within a second capsule and refold.

DISCUSSION

Because natural selection generally operates at the level of the colony in ants, sampling ants for stable isotope analysis requires a different sampling regimen than for solitary species. Studies have shown that populations of the same species can differ dramatically in feeding ecology (Tillberg et al. 2007), as can colonies within a population (LeBrun et al. 2007) and individuals within a colony (Smith et al. 2008). Thus, a robust sampling approach is recommended for most ecological questions. For example, sampling a minimum of five individuals per colony and five colonies per species is necessary to understand basic components of variation in community feeding ecology. Furthermore, because habitats and populations differ, it is recommended that insects of known trophic position, as well as plant matter, be collected to calibrate the trophic position of the focal species and to serve as references for interpretation. Note, however, that the preparation of plant matter differs slightly from that described above; a specific protocol for handling plant material should be obtained from the laboratory that will process the samples.

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Ant Fat Extraction with a Soxhlet Extractor

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INTRODUCTION

Stored fat can be informative about the relative age of an ant, its nutritional status, and the nutritional status of the colony. Several methods are available for the quantification of stored fat. Before starting a project involving fat extraction, investigators should weigh the advantages and disadvantages of different methods in order to choose the one that is best suited to the question being addressed. This protocol, although not as accurate as some alternatives, facilitates the rapid quantification of many individuals.

RELATED INFORMATION

For a more extensive discussion on the background and potential uses of ants as model organisms, see **Ants (Formicidae): Models for Social Complexity** (Smith et al. 2009).

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <!>, and recipes for reagents marked with <R>.

Reagents

Ants of the species of interest (see Step 1)
<!>Diethyl ether (ether)

Equipment

Capsules, gelatin (www.Capsuline.com)
> Different sizes are available; use a size that best fits the species of interest.

Condenser
Drying oven preset to 60°C
Flask, flat-bottom
Freezer
Fume hood
Hotplate preset to 37°C
Microbalance, accurate to 50-100 µg
Needle
Pen, fine-point, permanent (e.g., Pigma Pen)

www.cshprotocols.org

Ring stand
Rubber tubing
Soxhlet extractor
Wire and wire cutter

METHOD

Specimen Collection

1. Collect ant specimens live. Keep fresh.

2. Kill specimens by nonchemical means such as in a freezer for 12 h.
   Do not use ethanol; it leaches hydrocarbons out of the body.

Sample Preparation

3. Dry specimens completely; at least 48 h in an oven at 60°C.
   A longer time will not hurt and is preferable to ensure that all water is out of specimens: Excess water will bias measurement of fat content.

4. Make a datasheet with columns for "Sample ID," "Dry Mass," and "Lean Mass."

5. Using a needle, puncture small holes at both the top and bottom of gelatin capsules.
   These holes will allow the capsule to fill and drain later.

6. Weigh each dried ant on a microbalance and then enclose in a punctured capsule. Using a fine-point permanent pen, label each capsule with the identity of that individual. Record the ant's mass under "Dry Mass."

Fat Extraction

7. Cut segments of wire that are slightly shorter in length than the distance between the topmost elbow in the drainage tube and the bottom of the reservoir in the Soxhlet extractor. Bend the bottom of the wire so that it is shaped like a fish hook (Fig. 1A-C).

8. Thread ant-filled capsules onto the wires (the hooked end being the bottom of the wire; Fig. 1C).
   For a typical-sized Soxhlet extractor, you can stack the capsules four high. Depending on the size of the extractor available, many more could be stacked.
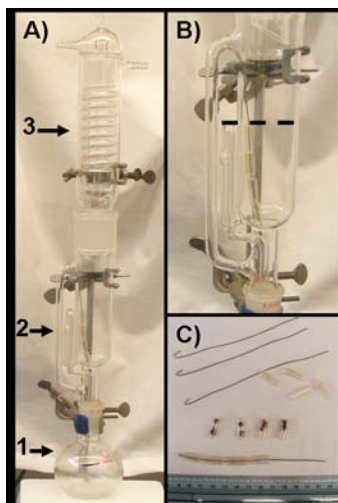

FIGURE 1. Equipment and set-up for ant fat extraction with a Soxhlet extractor. (*A*) Glassware arranged for extraction on top of a hotplate. (1) Flat-bottomed flask (the dark line on the flask indicates the fill point as described in the protocol); (2) Soxhlet extractor with four gelatin capsules arranged on a wire; (3) condenser (in/out water hoses not attached). (*B*) Close-up view of the Soxhlet extractor. (Dashed line) Maximum height at which objects to be extracted can be stacked. (*C*) Cut and bent wires, empty size "1" gelatin capsules, capsules filled with ants, and capsules strung on a wire.

9. Under a hood, construct the apparatus (Fig. 1A):

   i. Place the hotplate at the base of the ring stand.

   ii. Place the flat-bottomed flask on top of the hotplate.

   iii. Place the Soxhlet extractor on top of the flask.

   iv. Place the condenser on top of the Soxhlet extractor.

   v. Connect rubber tubing to the in and out pieces of the condenser.

   vi. Hook the "in" tube to a cold water outlet. Place the "out" tube down a drain. Turn on the water and make sure it is cycling through the condenser and draining into an unclogged drain.

   vii. Fill the flask about two-thirds full with ether.

   When the extractor is running, ether will evaporate, and thus the total volume in the flask will decrease; this quantity should never be allowed to decrease below the volume necessary to completely fill the Soxhlet extractor. If ether dips below this level, the reservoir in the extractor will not drain. The amount you fill the flask with is therefore important, and only practice and observation will inform you whether the amount is correct.

   viii. Place the capsules threaded on their wires into the Soxhlet extractor.

   Make sure that no capsule is above the top elbow of the drainage tube (dashed line in Fig. 1B*).*

10. Heat the hotplate to just above 37°C (the boiling point of ether).

    Time the duration of one cycle and adjust the temperature of the hotplate such that one full cycle lasts ~30 min. One cycle constitutes the filling and draining of ether from the Soxhlet extractor. Ether will escape from the system if the hotplate is greatly in excess of 37°C, joints are leaky, and/or the water running through the condenser is not very cool.

11. Let the system run for ~24 h.

    Most of the fat will be extracted in the first few hours; the remaining time ensures that fat has been extracted to completion.

12. Remove the wires and capsules from the Soxhlet extractor. Place them in a tray under the hood.

    Let all excess ether evaporate under the hood before removing them.

    See Troubleshooting.

13. After several hours of drying under the hood, transfer the samples to a 60°C oven for additional drying (in case humidity from the hood was absorbed) or storage before proceeding.

Measurement

14. Remove each ant from its capsule. Reweigh on the same precision microbalance.

    The resulting mass is the ant's "Lean Mass."

    See Troubleshooting.

15. Calculate the fat mass as the difference between dry and lean masses.

16. Calculate the proportion of ant that was fat as (dry mass – lean mass)/(dry mass).

    Proportional fat content is typically of most interest to biologists, because this accounts for changes in fat content with growth.

TROUBLESHOOTING

Problem: Too much ether evaporates when the extraction runs overnight.
[Step 12]
Solution: Refill the flask with enough ether so that the extractor can drain, but not too much so that the flask is not over-full when the ether drains from the Soxhlet extractor.

Problem: After the fat extraction, the capsules won't open.
[Step 14]
Solution: This is a common problem. Break the capsule open at one end, taking care not to damage the ant within.

DISCUSSION

The amount of fat an individual ant stores in its body can be informative in a variety of contexts. The fat stores of individual ants are often a way for the colony to store excess resources, and these can be shared with colony members when resources are low (sharing likely occurs through trophic egg production). Thus, the fat stores of workers can be informative about the nutritional status of a colony (Tschinkel 1999; Smith 2007). As in many social insects, workers change tasks as they age (age-related polyethism), beginning with nursing and culminating in foraging. As they age, they tend to deplete their fat stores. Thus, the fat content of behaviorally different individuals often correlates with task and age, and fat depletion is causal in the transition between hive and foraging behavior in honey bees (Toth et al. 2005). Analyzing fat content across castes/behavioral groups, however, is only valid within colonies because colonies can vary in nutritional status, which in turn affects the fat content of individuals with the colony (Smith 2007). Finally, before leaving their natal nest for mating and dispersal, queens often accumulate massive fat stores (up to 50% of their body mass). Fat stores in queens have been shown to vary with social organization (single queen colonies vs. multi-queen colonies) and founding strategy (whether or not queens foraged when they found a nest), which both have great consequences for the ecology and evolution of a species (e.g., Keller and Passera 1989).

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General Dissection of Female Ant Reproductive System and Brain

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INTRODUCTION

Dissection of the reproductive system of ant workers and queens can be useful for answering many questions. Observations of ovarian status in both female castes can be used to identify relationships between other factors and the ovaries, determine whether an individual has laid eggs, and, with more advanced molecular techniques, identify the end product of the oocyte in question (e.g., trophic egg, viable egg, nonviable egg). In addition, dissection of queens allows for observation of the spermatheca (the sperm storage organ), and thus identification of whether an individual has mated. Sperm can also be sampled for genetic analyses. In this protocol, we describe the dissection of the female ant reproductive system. We also discuss dissection of the corpora allata glands, where juvenile hormone is produced.

RELATED INFORMATION

Protocols for **In Situ Hybridization on Ant Ovaries and Embryos** (Khila and Abouheif 2009) and **Radiochemical Assay of Juvenile Hormone Biosynthesis Rate in Ants** (Brent and Dolezal 2009) are also available. For a more extensive discussion on the background and potential uses of ants as model organisms, see **Ants (Formicidae): Models for Social Complexity** (Smith et al. 2009).

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <!>, and recipes for reagents marked with <R>.

Reagents

Ants of the species of interest

For best results, start with live ants, or samples stored frozen without a solvent. Dissection of solvent-stored individuals is possible but more difficult, and might not be appropriate for some purposes.

Liquid medium

The choice of medium will depend on the final use of the tissue. For general observation, deionized water is acceptable.

Equipment

Blade, cutting, steel or sapphire/diamond (recommended for thicker cuticles)

Clay (for corpora allata dissection)
Dish, culture (11.25-cm or other, as appropriate for species)

For best results, dishes with a silicon or wash wax dissection surface are preferred.

Forceps, Dumont (#4 and #5)

For especially delicate work, mirror-tipped forceps are preferred.

Insect pins (sizes 00 and 2)
Microscope, dissection, equipped with an appropriate light source

METHOD

Ovary and Spermatheca Dissection

In most ant species, workers do not have a spermatheca, and hence cannot mate.

1. Fill a dish with liquid medium. Place the ant in the dish, dorsal side up. Place the dish under the dissection microscope at low magnification.

2. With blunt #4 forceps, grasp the petiole (Fig. 1).

3. Remove the reproductive organs by grasping the last 1-2 posterior segments (tergites) of the abdomen with forceps and pulling gently.

    The tissue connecting the segments will tear, and the ovaries, oviducts, spermatheca (if present), and often even the crop and poison gland (if present) will pull free of the abdomen proper.

4. If the organs do not pull cleanly away with the cuticle, remove the segments individually:

    i. Grasp the petiole with forceps.

    ii. Slide the other forceps between segments of cuticle and pull them away.

    iii. Once a few segments are removed, try pulling the organs free again (as in Step 3).

5. Using one pair of forceps, grasp the common oviduct as close to the cuticle as possible. With another pair of forceps, pull the remaining abdominal segments away from the organs.

6. If the crop (a food storage organ) is still attached, remove it.

    Usually, the crop is dark, but its color depends on the food that is stored within.

7. Hold the common oviduct with one set of forceps. Using forceps or a fine 00 insect pin, drag fat cells, Malpighian tubules, and tracheoles away from the ovaries.

    At this point, only the ovaries, connected by the common oviduct, and the spermatheca (if present) are connected.
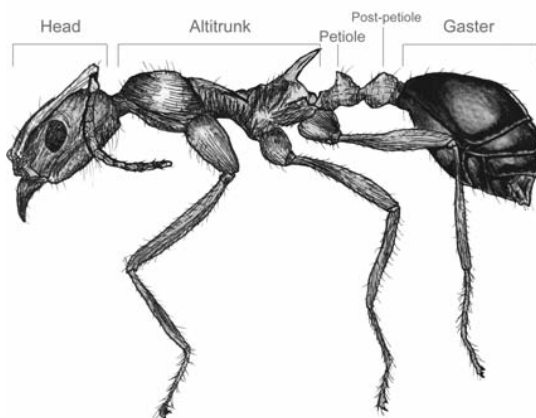

FIGURE 1. The anatomy of a standard ant. The nomenclature of ant body segments differs from most insects because the constriction (waist) is within the abdomen. Thus, the abdomen includes the last segment of the alitrunk, the petiole (and post-petiole when present) and gaster. The ant pictured is *Aphaenogaster cockerelli* in the subfamily Myrmicinae. (Drawing courtesy of Clint Penick.)

8. Observe the ovarian status (Fig. 2):

   i. Note the number of oocytes in each ovariole, and whether any are vitellogenic (distinguished by whitish opacity).

   ii. Look for yellow bodies.

   These collapsed areas, where mature oocytes were recently stored, will appear as yellow dots and indicate that the individual has recently oviposited.

9. If necessary (e.g., for certain types of staining, in situ hybridization), remove the peritoneal sheet surrounding each ovariole (for details, see **In Situ Hybridization on Ant Ovaries and Embryos** (Khila and Abouheif 2009).

   This is analogous to removing a sock from a foot by pulling at the toe of the sock. This will leave an ovariole, with the sheet removed, separated from the ovaries. Repeat on as many ovarioles as necessary.

10. Remove the spermatheca (if present):

    i. Grasp the common oviduct (or the cuticular segments if still attached) with forceps.

    ii. Gently pull off the tissue connecting the spermatheca to the ovaries.

11. Observe the status of the spermatheca.

    If the spermatheca is transparent, the individual has not mated. If it is opaque or translucent with a bluish-white color, she has mated. If this is the case, the spermatheca can be saved and preserved for use in genetic analyses of the sperm stored within.

Corpora Allata Dissection

12. Place some clay in a dissection dish.

13. Hold the head of the subject animal down by crossing two size 2 insect pins behind the head, with the pins driven into the wax or silicon of the dish, and pressing the head firmly into the clay.

14. Using forceps, clip off the antennae of the ant to prevent them from getting in the way.

15. Using an appropriate blade, cut away the section of the cuticle near the posterior (occipital region) of the head, leaving a large opening.

    See Troubleshooting.

16. Push mandibular head muscles aside, removing them if necessary.

    This can be risky; removing the muscles can also pull away desired tissue.

17. Remove the tracheal elements from the area. This should reveal the corpora allata/corpora cardiaca complex, connected anteriorly and posteriorly by filaments.

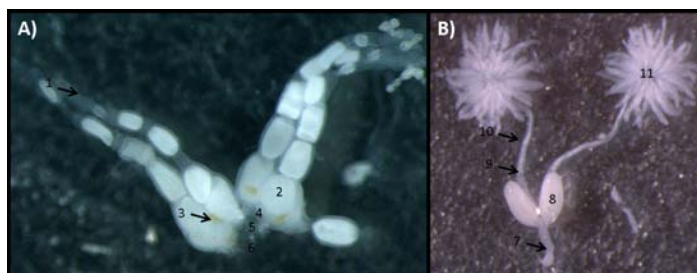18. Grasping the filaments on both sides of the corpora allata, gently remove the glands.

FIGURE 2. Reproductive systems in ants. (*A*) Female worker reproductive system. (1) Terminal filament; (2) vitellogenic oocyte (note its white opacity); (3) yellow bodies (denoting recently laid oocytes); (4) lateral oviducts connect each ovary to the common oviduct; (5) common oviduct; (6) location of spermatheca, if present. Many worker ants, such as the one shown here, lack spermathecae. (*B*) Male reproductive system. (7) Ejaculatory duct; (8) accessory glands; (9) seminal vesicles; (10) vas deferens; (11) testis. [(*A*) Photo courtesy of Adrian A. Smith; (*B*) photo courtesy of R. Overson.)]

19. Minimize the amount of brain tissue attached to the glands. Allow attached tracheal elements to stay if tissues will be used for radiochemical assays; this will provide buoyancy promoting oxidative reactions necessary for juvenile hormone production.

*The corpora allata can now be used for **Radiochemical Assay of Juvenile Hormone Biosynthesis Rate in Ants** (Brent and Dolezal 2009) to quantify juvenile hormone production.*

TROUBLESHOOTING

Problem: The blade will not cut through the cuticle of the head.
[Step 15]
Solution: Invest in a sharper steel blade, or, if necessary, use a sapphire or diamond blade.

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DNA Isolation from Ants

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INTRODUCTION

Many different DNA isolation methods have been employed successfully in ants. Parameters such as the size and developmental stage of the specimen (egg, larvae, or adult) and the subsequent use of the DNA will mostly determine which method should be used. Ant body sizes range from minute (1-2 mm in length) to large (30 mm), and the volume of the initial digestion should be adjusted accordingly. Whereas workers usually have low concentrations of storage proteins and fat, queens and larvae can contain considerable amounts of these substances that can interfere with the subsequent use of the isolated DNA. Ants also have many glands in the head and abdomen, and the contents of these glands can also interfere with the successful application of polymerase chain reaction (PCR) or restriction digests of the isolated DNA. This protocol presents two DNA isolation methods that have worked reliably for a wide range of ant species: a "quick and dirty" technique using Chelex isolation, and a more elaborate, classical phenol:chloroform procedure.

RELATED INFORMATION

For a more extensive discussion on the background and potential uses of ants as model organisms, see **Ants (Formicidae): Models for Social Complexity** (Smith et al. 2009).

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with **<!>**, and recipes for reagents marked with **<R>**.

Reagents

<R>Ant homogenization buffer (for phenol:chloroform extraction only)
<R>Ant solubilization buffer (for phenol:chloroform extraction only)
Ants of the species of interest
<R>Chelex solution (5%) (for Chelex extraction only)
<!>Chloroform:isoamyl alcohol mixture (25:1 [v/v]) (for phenol:chloroform extraction only)
Ethanol (for phenol:chloroform extraction only)
<R>Low TE (optional; see Step 27)
<!>Phenol (pH 8.0) (for phenol:chloroform extraction only)
<!>Proteinase K (20 mg/mL for Chelex extraction; 10 mg/mL for phenol:chloroform extraction)
Sodium acetate (3 M, pH 5.2) (for phenol:chloroform extraction only)

METHOD

Chelex Isolation for a Small- to Medium-Size Ant Species

1. Stir the 5% Chelex solution to generate a good emulsion of the resin before pipetting.

2. Pipette 50 μL of 5% Chelex solution into a 1.5-mL reaction tube.
   Check that each tube contains a good proportion of Chelex resin at the bottom.

3. Add the specimen/tissue to the solution.
   If the specimen was stored in alcohol, let it dry for ~10 min to evaporate the alcohol before adding it to the solution.
   See Troubleshooting.

4. Crush each ant with a pestle. Try to remove all ant bits from the pestle.
   It is helpful to leave the pestle in the tube as a place marker. Do not reuse pestles.

5. Once all samples have been crushed, remove and discard all the pestles.
   Be careful not to cross-contaminate samples.

6. Add 1 μL of proteinase K (20 mg/mL) to the lid of each tube. Close the tubes.

7. Centrifuge on a "quick spin" in a microcentrifuge to combine the solutions and pellet any tissues that stick to the wall of the tubes.

8. Incubate the samples in a water bath for 1 h at 57°C.

9. Boil the samples in a heat block for 5 min at 95°C.
   Close the lid of the reaction tube securely; otherwise the lid will pop during heating, possibly contaminating the sample.

10. Centrifuge samples in a microcentrifuge at maximum speed (e.g., 14,000 rpm) for 15 min.

11. While centrifuging, label a new set of sterile tubes.

12. Transfer supernatants (~20-40 μL) from each sample into appropriately labeled new tubes.
    This DNA solution can be stored for at least 5 yr at -80°C.
    See Troubleshooting.

Phenol:Chloroform Isolation for a Small- to Medium-Size Ant Species

13. Label three sets of 1.5-mL reaction tubes in parallel.
    This facilitates transfers between tubes and the tracking of sample identities.

14. Crush each sample in 100 μL of ant homogenization buffer with a pestle.
    Do not reuse pestles.

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15. Add 100 µL of ant solubilization buffer and 5 µL of proteinase K (10 mg/mL).

16. Incubate for 1 h at 37°C (or overnight at 57°C).

17. Working under a hood, add 250 µL phenol. Shake gently for 10 min.

18. Centrifuge in a microcentrifuge at 8000 rpm for 10 min.

19. Transfer the aqueous, DNA-containing supernatant to a new tube.

20. Add 250 µL of chloroform:isoamyl alcohol. Shake until the solution is milky.

21. Incubate for 5 min at -20°C.

22. Remove the sample from the freezer. Shake for 5 sec. Centrifuge at 8000 rpm for 10 min.

23. Transfer the supernatant into a new tube.

    Do not transfer any of the milky substance at the interface of the phenol or phenol:chloroform and aqueous solution. Be careful not to destroy the separation of the phases when transferring samples.

24. Add one-tenth of the sample volume of 3 M sodium acetate. Add two volumes of 100% ethanol. Precipitate the DNA for 30 min.

25. Centrifuge in a microcentrifuge at maximum speed (e.g., 14,000 rpm).

    A DNA pellet should be visible.

26. Remove as much of the supernatant as possible. Let the ethanol evaporate under a hood for 30 min.

    If the time for evaporation is too short, the ethanol will remain in the DNA solution, which will have a negative impact on any further use of the DNA solution.

27. Dissolve the DNA pellet in 100 µL of low TE or water.

TROUBLESHOOTING

Problem: The ant is large.
[Step 3]
Solution: Increase the Chelex volume by 25 µL. If very large, use 50 µL. Alternatively, extract DNA from only a portion of the ant tissue.

Problem: When pipetting the supernatant, a portion of the ant or Chelex resin was also aspirated.
[Step 12]
Solution: These contaminants must be removed; they can interact negatively with applications involving PCR. Transfer the supernatant to its new tube, then pipette or otherwise extract the ant part and discard it. The Chelex will settle to the bottom of the new vial and the supernatant can be transferred to a new labeled tube.

DISCUSSION

For large-scale population genetic studies using microsatellite markers, the Chelex isolation method is very efficient, because many samples can be processed in parallel and no hazardous chemicals are used. Chelex isolation can be used to isolate DNA from minuscule samples (e.g., sperm collected from a queen's spermatheca, tarsi of an individual ant worker, embryos, small larvae) and is one of the few ways to perform nondestructive sampling in ants. However, DNA generated by the Chelex isolation method is sheared and often contains a huge amount of contaminating compounds, which make this DNA unsuitable for applications susceptible to sheared or contaminated DNA (e.g., amplified fragment length polymorphism analysis or cloning).

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Juvenile Hormone Extraction, Purification, and Quantification in Ants

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INTRODUCTION

Juvenile hormone (JH) is an important insect hormone known to have many effects on development, reproduction, and behavior in both solitary and social insects. A number of questions using ants as a model involve JH. This procedure allows for quantification of circulating levels of JH III, which can be an important factor in many questions relating to insect research. The JH III is extracted from a subject, purified, and converted to a d_3-methoxyhydrin derivative that can be quantified by gas chromatography-mass spectrometry (GC-MS). The major advantages of this protocol are its high resolution, and its ability to quantify significant differences between relatively small quantities of the hormone. Its major limitations are the time necessary to process samples, its relatively high cost, and maintaining the sensitivity of the equipment.

RELATED INFORMATION

For a general review of the role of JH in development, reproduction, and behavior, see Nijhout (1994). For additional details on this protocol, see Bergot et al. (1981), Shu et al. (1997), and Brent and Vargo (2003). A protocol for **Ant Ecdysteroid Extraction and Radioimmunoassay** (Brent and Dolezal 2009) is also available. A more extensive discussion on the background and potential uses of ants as model organisms is available in **Ants (Formicidae): Models for Social Complexity** (Smith et al. 2009).

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <!>, and recipes for reagents marked with <R>.

Reagents

<!>Acetonitrile (HPLC grade; 50% in nanopure water)
Aluminum oxide (Al_2O_3; Sigma-Aldrich)
Ants of the species of interest
<!>Diethyl ether (ethyl ether)
<!>Ethyl acetate
<!>Hexane (HPLC grade)
<!>JH III (HPLC grade) (200 pg/µL in acetone)

> Using increasing quantities of the JH III standard, prepare five to 10 dilutions for a standard curve (e.g., 1, 2, 5, 10, 20, 50, etc. µL of JH standard). Store at -20°C. Use the standards to identify the time point associated with the most robust JH III derivative peak.

<!>Methanol (HPLC grade; 90%) (optional; see Step 4)

<!>Methanol-d$_4$ (1000-µL ampoules)

> *Store at -20°C.*

<!>Trifluoroacetic acid (spectrophotometric grade)

> *Store at -20°C.*

Equipment

Centrifuge
Column holder (e.g., a board with small holes drilled through the entire width)
Drip tray
Drying oven preset to 60°C
GC-MS, including the following:
> Autoinjector or injection syringe (approved for the GC-MS in use) if autoinjector is
> unavailable or its use is not desirable
> Gas chromatograph
> Gas chromatography capillary column (EC-Wax, 30 m × 0.25 mm × 0.25 µm; ECONO-
> CAP)
> <!>Helium gas (UHP grade only)
> *Helium is used as the carrier gas for GC.*

Glass wool
Microcapillary tube (optional; see Step 3)
Micropipettor and tips
<!>Nitrogen gas (UHP grade only)
Pasteur pipettes (flint glass)
Pipettes (glass, Pyrex, equipped with suction bulb)
Stirring rod (glass) or a glass tube that has been melted shut
Syringe (10-µL)
Tubes (centrifuge, glass, 12-mL)
Vacuum concentrator (e.g., SpeedVac)
Vial inserts for autoinjector vials (polymer feet, 100-µL)
Vials (approved for GC autoinjector use)
Vials (glass, Teflon-capped, 2- and 12-mL)
Vortex mixer

METHOD

Sample Homogenization and Purification

1. Prepare ethyl ether:hexane solutions (10:90 [v/v], 30:70 [v/v], and 50:50 [v/v]). Store at -20°C
   until use.

2. Turn on the vacuum concentrator refluxer. Allow the system to cool. If applicable, set the temper-
   ature to 30°C.

3. Place the sample animal in a 12-mL glass centrifuge tube.

   *Alternatively, if there are sufficient quantities, collect hemolymph in a microcapillary tube and expel it into 1.5
   mL of 50% aqueous acetonitrile.*

4. Homogenize the bodies in 0.5 mL of 50% acetonitrile with a glass stirring rod.

   If the stirring rods are too large, use larger vials.

   If ecdysteroid quantification from the same sample is desired, replace the acetonitrile solution with methanol.

5. Centrifuge the homogenate at 5500 rpm for 8 min.

   If larger vials are used, centrifuge the homogenate in the vacuum concentrator with the vacuum disconnected.

6. Collect the supernatant in a 12-mL glass vial using a glass pipette.

7. Repeat Steps 4-6 three times.

8. Using a glass pipette, add 2.5 mL of hexane to the sample. Mix thoroughly.

   The JH should partition into the hexane layer.

9. Pipette the upper hexane layer into a fresh 12-mL vial. Repeat Steps 8-9 twice (i.e., three times total). Discard the bottom layer after the third wash.

   *For ecdysteroid measurement, save the bottom layer. Extract ecdysteroid from this layer and measure it using a radioimmunoassay (see **Ant Ecdysteroid Extraction and Radioimmunoassay** [Brent and Dolezal 2009]).*

10. Prepare a negative control by adding hexane to fresh 50% aqueous acetonitrile.

11. Prepare a standard curve of positive controls by adding 10 µL of the diluted JH stocks to 0.5 mL of 50% aqueous acetonitrile.

    At this point, samples can be stored in the freezer temporarily.

12. Centrifuge the hexane extracts (from Step 9) and controls (from Steps 10 and 11) in the vacuum concentrator until dry (~25 min).

    Alternatively, blow the samples dry with nitrogen.

13. While the samples are drying, prepare glass columns under a laboratory hood:

    i. Place Pasteur pipette glass columns in the holes of the column holder. Place a drip tray underneath.

    ii. Insert a small plug of glass wool at the narrow end of each column.

       Use enough glass wool to plug the column, but note that excess glass wool slows the flow rate.

    iii. Activate Al_2O_3 powder by adding water (6% [v/v]). Mix until dry.

    iv. Add 2 mL of the newly activated Al_2O_3 to the columns.

    v. Add 750 µL of hexane to the columns.

       If the columns drip, add Al_2O_3 until they hold the volume.

    vi. Wash the columns with 1.5 mL of hexane. Allow the hexane to drip into the tray.

       This cleans the Al_2O_3.

14. Add 300 µL of hexane to each vial of dried extract (from Step 12). Mix thoroughly.

15. Using glass pipettes, transfer each sample to a column. Repeat Steps 14 and 15 twice (i.e., three times total).

16. Add 900 µL of hexane to the columns.

    This ensures removal of nonpolar compounds.

17. Wash the column twice with 900 µL of ethyl ether:hexane (10:90), allowing the flow-through to drip into the tray.

18. Wash the column with 750 µL of ethyl ether:hexane (30:70), allowing the flow-through to drip into the tray.

19. Wash the column with 900 µL of ethyl ether:hexane (30:70). Collect the flow-through in vials. Repeat once, pooling both eluates in the same vial (i.e., 1.8 mL). Discard the columns.

20. Vacuum-concentrate the samples to dryness (~15 min).

21. While drying the samples, equilibrate the methanol-d$_4$ ampoules to room temperature.

    Methanol-d$_4$ absorbs water from air, which can quench the reaction.

22. Using a micropipettor, add 53 µL of trifluoroacetic acid to a 1000-µL methanol-d$_4$ ampoule. Mix.

    This makes enough 5% solution for 12 samples. Evacuate air from the trifluoroacetic acid container with nitrogen before storage. Steps 22-24 are the only steps where it is acceptable to use micropipettors.

23. Slowly add 75 µL of methanol-d$_4$ to each sample vial with a micropipettor.

24. Slowly add 75 µL of 5% trifluoroacetic acid:methanol-d_4 to each sample. Mix with the micropipet-tor as the sample is added.

    Mixing plus heating converts the samples to produce the d_3-methoxyhydrin derivative of JH III.

25. Cap the vials tightly. Incubate for 20 min at 60°C.

26. Prepare new columns (as in Step 13).

27. Remove the samples from the oven. Add 500 µL of hexane to each. Mix well. Vacuum-concentrate to dryness.

28. Add 300 µL of hexane to each vial of dried extract (from Step 27). Mix thoroughly. Transfer each sample to a column with glass pipettes. Repeat three times.

29. Wash the column twice with 900 µL of ethyl ether:hexane (30:70), allowing the flow-through to drip into the tray.

30. Wash the column twice with 750 µL of ethyl ether:hexane (50:50), allowing the flow-through to drip into the tray.

31. Wash the column with 900 µL of ethyl acetate:hexane (50:50). Collect the flow-through in vials. Repeat once, pooling both eluates in the same vial (i.e., 1.8 mL). Discard the columns.

32. Vacuum-concentrate the samples to dryness.

33. Add 300 µL of hexane to each sample. Mix well.

    The samples are now ready for analysis in GC-MS. Store in a freezer until the time of analysis. If stored at -20°C, analyze within 7 d. Samples stored at -80°C will last somewhat longer.

34. Evaporate the samples under nitrogen gas to ~25 µL of liquid.

35. Use a glass pipette to transfer the fluid into a vial insert.

36. Wash the sample vial with 200 µL of hexane. Vortex. Repeat Steps 34-35.

37. Using nitrogen, dry the liquid in the vial insert down to ~10 µL. Quantify with a 10-µL syringe.

38. Using the syringe to monitor, dry the hexane under nitrogen to 3 µL. Cap the vial.

GC-MS Setup and Analysis

The specific GC-MS parameters should be tailored for individual applications. For more information and another version of GC-MS settings, see Shu et al (1997).

39. Set temperature protocol:

    i. Set initial temperature to hold for 1 min at 60°C.

    ii. Ramp the temperature up at 20°C/min to a final temperature of 240°C. Hold for 20 min (i.e., a total run time of 30 min).

40. Set inlet protocol:

    i. Set the protocol to pulsed splitless injection at 250°C.

    ii. Set the pressure at 9.98 psi with a 23.8-mL/min flow rate.

    iii. Set the injection quantity to 1 µL.

41. Create a 5-min solvent delay.

    This prevents the recording of highly volatile solvents, which evaporate early on.

42. Load the vials into a GC-MS autoinjector. Run the program.

    If an autoinjector is unavailable (or its use is not desirable), use an injection syringe (approved for the GC-MS in use) to inject 1 µL of the sample manually.

43. Monitor at *m/x* 76 and 225.

    This ensures specificity for the JH-III derivative.

    See Troubleshooting.

TROUBLESHOOTING

Problem: The standard is sensed correctly, but samples have very low or no JH.
[Step 43]
Solution: Depending on the species used, it might be necessary to pool samples (e.g., multiple individuals per sample) to get readable results. This is highly dependent on the size of the ants used, as well as the experimental design.

Problem: Samples appear contaminated, with large, non-JH chromatogram peaks.
[Step 43]
Solution: Consider the following:

1. Hexane, which is used heavily in the protocol, dissolves many plastics, possibly contaminating the samples. Minimize sample contact with plastics of any kind, especially when using hexane or a hexane-containing solvent.

2. It is possible that not all of the fat content of the sample has been removed. In most ants, this is not a problem, but it could occur in larger, sexual caste individuals. To remedy, remove the fat using Sep-Pac cartridges; see Brent and Vargo (2003) for this procedure.

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Ant Ecdysteroid Extraction and Radioimmunoassay

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INTRODUCTION

Ecdysteroids are a group of steroid compounds present in many plant and invertebrate species. In arthropods, they function primarily as hormones involved in the regulation of molting. This protocol describes how to extract ecdysteroid hormones from ant specimens and subsequently quantify circulating levels of the hormone. The hormone can be extracted from hemolymph or from whole-body homogenates of insects and quantified by radioimmunoassay.

RELATED INFORMATION

For a more extensive discussion on the background and potential uses of ants as model organisms, see **Ants (Formicidae): Models for Social Complexity** (Smith et al. 2009). A protocol for **Juvenile Hormone Extraction, Purification, and Quantification in Ants** (Brent and Dolezal 2009) is also available.

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <!>, and recipes for reagents marked with <R>.

Reagents

<!>[^{3}H]-ecdysone stock (1 mg/mL)
20-Hydroxyecdysone stock (1 mg/mL)
<R>Borate buffer
Ethanol
H-22 antibody

> *At present, there is no commercial source for this polyclonal ecdysteroid antiserum; it must be produced in-house or acquired privately.*

<!>Hexane (HPLC grade) (for whole-body extraction only)
Insect saline
<!>Methanol (HPLC grade; 100%, 90%) (chilled for Step 4.i)
Pansorbin (fixed, Protein A-bearing *Staphylococcus aureus* cells; Calbiochem)
Universal scintillation fluid

Equipment

Capillary tubes (for hemolymph extraction only)

Centrifuge
Ice
<!>Nitrogen gas (UHP grade)
Orbital shaker
pH meter
Pipette tips (microcapillary)
Pipettes (glass)
Plastic wrap (or Parafilm)
Radioactive waste container
Refrigerator or cold room preset to 4°C
Scintillation counter
Stirring rod (glass) (for whole-body extraction only)

Alternatively, use a glass tube that has been melted shut.

Tubes (glass, centrifuge, 12-mL) (for whole-body extraction only)
Tubes (culture, 6 × 50 mm)
Tubes (microcentrifuge, 1.5-mL)
Vacuum concentrator (e.g., SpeedVac)
Vials (glass, 12-mL)
Vials (scintillation)
Vortex mixer

METHOD

Be aware of radioactive contamination. Always dispose of radioactive materials in an approved manner.

Preparation for Radioimmunoassay

1. Prepare a working stock of [³H]-ecdysone:

    i. Dissolve 1 µL of the [³H]-ecdysone stock in 3 mL of universal scintillation fluid. Measure the dpm in a scintillation counter.

    The dpm should be ~2.5-4 × 10^5; if it is too much lower, it is most likely degraded.

    ii. Dilute the stock solution in borate buffer such that 100 µL of diluted solution dissolved in 3 mL of universal scintillation fluid measures 2-2.5 × 10^4 dpm.

    Adjusting the concentration to achieve this can take several attempts.

    iii. Dilute enough of the [³H]-ecdysone stock in borate buffer such that 100 µL of the working solution are available for each sample to be tested.

    Prepare a small amount of additional working solution to allow for pipetting errors.

2. Clean the Pansorbin solution:

    i. Vortex the Pansorbin well to resuspend.

    ii. Aliquot sufficient Pansorbin for 20 µL per sample into a microcentrifuge tube.

    Be sure to aliquot a small amount of additional Pansorbin to allow for pipetting errors.

    iii. Centrifuge at 5000 rpm for 5 min. Remove the supernatant. Resuspend the pellet in a comparable volume of borate buffer.

    iv. Centrifuge at 5000 rpm for 5 min. Discard the supernatant. Repeat the washing once.

    v. Resuspend in enough borate buffer to bring the total volume back to its original amount. Vortex well to fully resuspend.

3. Prepare a working stock of antibody:

    i. Add 1 mL of H$_2$O to lyophilized H-22 antibody. Dissolve at room temperature to produce a "neat" serum.

ii. Dilute the serum in borate buffer to a range of concentrations (e.g., 1/100, 1/200, 1/300, 1/400, 1/500, 1/600, 1/700, 1/800).

iii. Aliquot 100 µL of each dilution into microcentrifuge tubes.

iv. Add 100 µL [^{3}H]-ecdysone to each tube. Vortex gently. Cover tubes with plastic wrap or Parafilm.

Assay the samples in duplicate.

v. Incubate overnight (~18 h) at 4°C on orbital shaker.

vi. Add 20 µL of cleaned Pansorbin (from Step 2.v) to each tube. Cover the tubes again. Incubate on an orbital shaker for 1 h at room temperature.

vii. Centrifuge at 5000g (~8000 rpm) for 10 min.

viii. Remove and discard supernatant using microcapillary pipette tips.

ix. Wash the pellet twice with 100 µL borate buffer. Resuspend the final pellet in 50 µL of water.

x. Transfer the contents of each tube to a labeled scintillation vial. Wash each tube with another 50 µL water and add that to vial.

xi. Add 3 mL of universal scintillation fluid to each vial. Determine dpm using a scintillation counter for 3 min.

The radioactivity should decrease at a steady rate with decreasing dilution. The optimal dilution (i.e., working concentration) of the antibody should bind ~25%-33% of the total dpm of the [^{3}H]-ecdysone added.

xii. Dilute the neat serum (from Step 3.i) to the working antibody dilution (as determined in Step 3.xi).

xiii. Divide the diluted antibody into aliquots sufficient for 6 mo worth of experiments; this will vary depending on the volume of samples run per day in a given laboratory.

Store antibody to be used immediately at 4°C until use. Aliquots to be used within the next 6 mo can be stored at -20°C; -80°C is recommended for longer storage.

Ecdysteroid Extraction

4. Extract ecdysteroid by one of the following methods:

For hemolymph collection:

i. If possible, collect hemolymph from the insect with a 10-µL capillary tube. Expel into 500 µL of chilled methanol in a 1.5-mL tube. Vortex.

ii. Centrifuge at maximum speed for 10 min. Collect the supernatant into a fresh 1.5-mL tube.

iii. Add 500 µL of methanol to the original tube. Vortex well to resuspend.

This reextracts the ecdysone.

iv. Centrifuge at maximum speed for 10 min. Pool this supernatant with the supernatant from Step 4.ii.

v. Dry the samples using either a nitrogen stream or the vacuum concentrator.

This can take ~60 min.

vi. Resuspend the pellet in 200 µL of methanol. Freeze at -80°C until assayed.

For whole-body extraction:

vii. Turn on the vacuum concentrator refluxer. Allow the system to cool. If applicable, set the temperature to 30°C.

viii. Place the sample animal(s) in a 12-mL glass centrifuge tube. Homogenize the bodies in 0.5 mL of methanol with a glass stirring rod.

If the stirring rods are too large, use larger vials.

ix. Centrifuge the homogenate at 5500 rpm for 8 min.

If larger vials are used, centrifuge the homogenate in the vacuum concentrator with the vacuum disconnected.

x. Collect the supernatant in a 12-mL glass vial using a glass pipette.

xi. Repeat Steps 4.viii-4.x three times.

xii. Using a glass pipette, add 2.5 mL of hexane to the sample. Mix thoroughly.

The ecdysteroid should partition into the methane layer.

xiii. Remove and discard the upper hexane layer. Repeat Steps 4.xii-4.xiii twice (i.e., three times total). Reserve the bottom layer after the third wash.

*Alternatively, the upper hexane layers can be reserved and pooled for assaying circulating levels of juvenile hormone, as described in **Juvenile Hormone Extraction, Purification, and Quantification in Ants** (Brent and Dolezal 2009).*

xiv. Vacuum-concentrate the vial of methanol down to remove hexane contaminants.

xv. Wash the vial three times with 0.5 mL of 90% methanol. Pool the washes into a 1.5-mL tube.

xvi. Dry the sample down again. Resuspend in 250 µL of 90% methanol.

Radioimmunoassay

5. Prepare serial dilutions of the standard:

i. Dilute 20 µL of 20-hydroxyecdysone stock solution with 80 µL of ethanol (i.e., 2 µg/10 µL).

ii. Dilute 10 µL of the above solution with 90 µL of ethanol (to 0.2 µg/10 µL).

iii. Dilute 10 µL of the above solution with 990 µL of insect saline (to 2000 pg/10 µL).

iv. Dilute the solution from Step 5.iii serially 1:1 with insect saline to concentrations ranging from 15.6 to 1000 pg/10 µL.

Keep standard solutions on ice when not in use.

6. Label and prepare duplicates of glass culture tubes for experimental samples, standards, negative controls (B_0, i.e., no added antibody), and maximum-binding control (B_{max}, i.e., no exogenously added ecdysone).

7. Dispense 10 µL of samples in methanol (from Step 4.vi or 4.xvi) or 10 µL of standards (from Step 5.iv) into appropriate tubes.

When first using this protocol, test a range of volumes (e.g., 1-50 µL) from the experimental samples to determine the quantity that gives the best results while allowing the most possible iterations of the assay per sample.

8. Add 100 µL of [^{3}H]-ecdysone in borate buffer (from Step 1.iii) to all tubes except B_{max}.

9. Add 100 µL of H-22 antibody (from Step 3.xiii) to all tubes except B_0. Vortex gently.

10. Cover the tubes with plastic wrap or Parafilm. Incubate on an orbital shaker overnight (~18 h) at 4°C.

11. The following day, prepare a fresh batch of Pansorbin (as described in Step 2).

12. Add 20 µL of the cleaned Pansorbin (from Step 11) to each tube to precipitate the complexes.

13. Cover the tubes again. Incubate on an orbital shaker for 1 h at room temperature.

14. Centrifuge at 5000*g* (~8000 rpm) in a vacuum concentrator with the condenser disconnected for 10 min.

Because of the length of the tubes, using a vacuum concentrator without the drying apparatus works best. If the concentrator does not have variable speeds or does not spin at 5000g, simply increase the spin time.

15. Remove and discard the supernatants using microcapillary pipette tips.

16. Wash samples twice by centrifugation in 100 µL of borate buffer. Discard the supernatants.

17. Resuspend the samples in 50 µL of water. Transfer the contents of each tube to a labeled scintillation vial. Wash each tube with another 50 µL of water and add that to the vial.

18. Add 3 mL of universal scintillation fluid to each vial.

19. Determine dpm using a scintillation counter for 3 min.

20. Estimate ecdysteroid concentrations by nonlinear regression.
    See Troubleshooting.

TROUBLESHOOTING

Problem: Ecdysteroid levels are lower than expected from samples.
[Step 20]
Solution: Run iterations of possible sample dilutions to find the quantity best suited to the purposes at hand.

DISCUSSION

Tritiated ecdysone binds to the H-22 antibody more strongly than any of the other forms of ecdysteroid with which it is competing in this assay. Therefore, to get a true estimate of the amount of hormone in hemolymph samples, one must correct for this difference. Cross-reactivity is expressed as the ratio of the mass of a particular ecdysteroid needed to displace 50% of the mass of the labeled ecdysone. The cross-reactivity factors for H-22 are: makisterone A (2.88); 20-hydroxyecdysone (3.84); makisterone C (1.60) (see Feldlaufer and Hartfelder 1997).

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Radiochemical Assay of Juvenile Hormone Biosynthesis Rate in Ants

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INTRODUCTION

Juvenile hormone (JH) is an important insect hormone known to have many effects on development, reproduction, and behavior in both solitary and social insects. This protocol describes how to quantify in vitro biosynthesis rates from excised corpora allata (CA), the glands responsible for JH production. Excised glands are incubated with radiolabeled methionine, resulting in the production of radiolabeled JH III, which can then be quantified. This protocol is most useful when quantification of the activity of the glands, rather than circulating JH levels, is desired. Its difficulties lie primarily in the ability to remove and handle the glands correctly.

RELATED INFORMATION

A protocol for **Juvenile Hormone Extraction, Purification, and Quantification in Ants** (Brent and Dolezal 2009) is also available. Like that protocol, the method presented here allows for the quantification of JH. The advantage of this procedure over the other method is that it tests for JH production rather than measuring the circulating titer. The method used will depend on the experimental system to be examined. For more information, see Brent and Vargo (2003). Detailed instructions for dissecting the CA can be found in **General Dissection of Female Ant Reproductive System and Brain** (Dolezal and Brent 2009). For a more extensive discussion on the background, husbandry, and potential uses of ants as model organisms, see **Ants (Formicidae): Models for Social Complexity** (Smith et al. 2009).

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <!>, and recipes for reagents marked with <R>.

Reagents

<!>2,2,4-trimethylpentane, anhydrous (isooctane; 99.8%; Sigma-Aldrich 360066) (prechilled for Step 18)

Corpora allata, ant

Obtain from the species of interest as described in General Dissection of Female Ant Reproductive System and Brain (Dolezal and Brent 2009). Minimize the amount of brain attached, but allow any attached tracheal elements to stay; these provide added buoyancy, allowing the glands to stay near the surface of the medium during incubation.

Corpora allata, cockroach

<!>L-[methyl-³H]-methionine (>97%, 1 mCi/mL, 70-85 Ci/mmol; PerkinElmer NET061X250UC)
<R>M-199 incubation medium
Scintillation fluid (ScintiSafe Econo F)
Stir to mix thoroughly before use.

Equipment

Centrifuge
Magnetic stirrer
Mixer, rotating, variable
<!>Nitrogen, UPC grade
Pipettor
Plastic wrap
Radioactive waste container
Scintillation counter
Stirring plate
Tubes, glass, 6 × 50 mm
Tubes, microcentrifuge
Vacuum concentrator (e.g., SpeedVac) (optional; see Step 21)
Vials, glass, 15-mL
Vials, scintillation
Vortexer
Wire hoop, copper

METHOD

Methionine Wash

1. The day of the experiment, dilute 100 µCi of [³H]-methionine to a final volume of 50 µL in a micro-centrifuge tube, as described on the packaging.

2. Add 500 µL of isooctane to the methionine. Mix well by vortexing for 1 full min.

3. Centrifuge at 10,000 rpm for 10 min.

4. Using a pipettor, remove the isooctane (i.e., upper) layer. Avoid the methionine/saline layer.
   Dispose of the contaminated isooctane in a vial and the pipettor tip in radioactive waste.

5. Repeat Steps 2-4 twice (i.e., three washes total).

6. After the third wash, transfer 100 µL of isooctane to a scintillation vial.

7. Evaporate the isooctane in the vial to near-dryness under nitrogen.

8. Add 3 mL of scintillation fluid to the vial. Cap. Vortex for 1 min.

9. Count the radioactivity of the isooctane in a scintillation counter using the "Autocount" function.

   i. If the background count is sufficiently low (i.e., <1000 dpm), the methionine is ready for use.

   ii. If the background count is >1000 dpm, repeat Steps 2-4.

   iii. If the cleaning process does not reduce the background dpm under 2000 within the first several washes, incubate the methionine in 500 µL of isooctane overnight (or longer) to remove more of the degraded isotope.

10. Evaporate any of the isooctane remaining in the vial (from Step 6) with nitrogen gas.

11. Dilute 50 µL of the methionine solution with 950 µL of M-199 incubation medium in a 15-mL vial.

This should provide a final (recommended) concentration of 100 µM methionine per incubation, with a specific activity of 5 µCi/100 µL. Higher concentrations will ensure that radioactive methionine will be incorporated into any JH synthesized, but will also increase the background count. Adjust the concentration as needed for the particular experimental conditions.

Establishing a Species-Specific Time Course for JH Production

CAs from different insect species produce JH at linear rates for different periods of time. The amount released each hour should be similar until the CA function begins to degrade.

12. Preincubate five to seven dissected corpora allata/corpora cardiaca (CA/CC) complexes in 100 µL of nonradiolabeled M-199 medium for 30 min in labeled glass culture tubes.

13. Aliquot 100 µL of radiolabeled medium (from Step 11) into each of eight sets of labeled glass culture tubes.
    Each set will be used for a separate time point.

14. Using a copper wire hoop, transfer the preincubated CA/CC from the nonradiolabeled medium into the corresponding tube containing the radiolabeled medium. Try to ensure that the tissue stays on the surface of the medium; the CA needs oxygen to produce JH.
    Use radiolabeled medium with no added tissue as a negative control.

15. Loosely cover the tubes with plastic wrap to prevent desiccation, but ensure an adequate supply of oxygen.

16. Incubate the samples on a variable-plane mixer set at a 15°-17° angle at 90 rpm at 27°C.

17. Once each hour for 8 h, transfer the CAs to a new tube containing fresh radiolabeled medium.

18. After the CA is removed from a tube, add 250 µL of chilled isooctane to that tube. Incubate for 15-30 min without mixing to complete the extraction process.

19. Vortex the samples for 1 min. Centrifuge at 10,000 rpm for 10 min.

20. Transfer 100 µL of the upper isooctane layer to a labeled scintillation vial. Repeat with another vial (i.e., assay each extract in duplicate).
    Make sure that both the caps and the bottles of the scintillation vials are labeled.

21. Reduce the volume of the isooctane to near-dryness under nitrogen or in a vacuum concentrator.

22. Add 3 mL of scintillation fluid to the vial. Cap. Vortex for 1 min.

23. Count the radioactivity of the isooctane in a scintillation counter using the "Autocount" function.
    See Troubleshooting.

24. Subtract the average background count (from the negative control samples) from each reading.

25. Determine the JH concentration produced at each time point by converting the dpm counts (2.22×10^9 dpm = 1 mCi).

Measuring JH Production

26. Preincubate dissected CA/CC complexes from the species of interest in 100 µL of nonradiolabeled M-199 medium for 30 min in labeled glass culture tubes.

27. Aliquot 100 µL of radiolabeled medium (from Step 11) into another set of labeled glass culture tubes.

28. Using a copper wire hoop, transfer the preincubated CA/CC from the nonradiolabeled medium into the corresponding tube containing the radiolabeled medium. Try to ensure that the tissue stays on the surface of the media; the CA needs oxygen to produce JH.
    Use a cockroach CA as a positive control and radiolabeled media with no added tissue as a negative control.

29. Loosely cover the tubes with plastic wrap to prevent desiccation, but ensure an adequate supply of oxygen.

30. Incubate the samples on a variable-plane mixer set at a 15°-17° angle at 90 rpm at 27°C for a period of time within the linear range of JH production for that species (as determined in Step 25).

    This allows for an accurate assessment of the rate of release and maximizes the amount of JH available for detection purposes.

31. After the selected interval, add 250 µL of chilled isooctane to each tube. Incubate for 15-30 min without mixing to complete the extraction process.

32. Process and count the samples as described in Steps 19-23.

    See Troubleshooting.

33. Subtract the average background count (from the negative control samples) from each reading.

34. Determine the JH concentration by converting the dpm counts (1 mCi = 2.22×10^9 dpm).

35. Determine the JH release rate by dividing the concentration by the incubation duration.

TROUBLESHOOTING

Problem: No JH production is noted.
[Steps 23 and 32]
Solution: Make sure that the dissections are done correctly and that the CA are present at every step. It is very easy to lose the glands or to accidentally incubate nonglandular tissue.

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GC-MS for Characterization and Identification of Ant Semiochemicals

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INTRODUCTION

Living in a predominantly dark environment, ants rely mostly on chemical signals for communication. Trail and alarm pheromones are the most widely studied and best characterized of all ant semiochemicals, but other such compounds can influence a variety of other behaviors, including reproductive activities, sexual development, nest mate and caste recognition, and defense. A typical worker body contains more than 10 different semiochemical-producing glands, and the surface of the cuticle is covered with lipids that serve as recognition signals. The methods of choice for collection and identification of ant semiochemicals should be determined based on results of behavioral analyses. These can indicate the source (e.g., glandular, cuticular) and the nature (volatile vs. nonvolatile) of the chemical. This protocol presents a number of different methods for collecting lipid semiochemicals. These can be followed by gas chromatography (GC) coupled with mass spectrometry (MS) to better characterize, and possibly identify, the semiochemical in question.

RELATED INFORMATION

For a more extensive discussion on the background and potential uses of ants as model organisms, see **Ants (Formicidae): Models for Social Complexity** (Smith et al. 2009).

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <!>, and recipes for reagents marked with <R>.

Reagents

The choice of solvent is a combination of good judgment and trial and error. Generally, use a solvent with polarity compatible with the semiochemical of interest. Because the identity of a given semiochemical is often unknown, it is a good idea to start with hexane or pentane and, if it does not work, to move on to more polar solvents. Some recommended solvents are listed below.

Ants of the species of interest

Solvents (GC grade) of varying polarity

Possibilities include the following:

<!> *Dichloromethane*

<!> *Diethyl ether (ethyl ether)*

<!>Ethyl acetate

<!>Hexane

<!>Methanol

<!>Pentane

Equipment

<!>CO_2 (carbon dioxide) (optional; see Step 1.ii)
Dissection instruments (optional; see Step 1.i)
GC columns

> The choice of column will depend on the chemicals to be analyzed. DB1 columns should be suitable for most hydrocarbons, whereas a DB5 is preferred for more volatile compounds and hydrocarbons.

GC-MS unit
MS detectors, chemical and/or electron-impact ionization
<!>Nitrogen gas, high purity
Pasteur pipettes, glass
Petri dish, glass, filled with ice (optional; see Step 11)
Pipette tips, polytetrafluoroethylene (optional; see Step 3)
Solid phase microextraction (SPME) needles and fibers (for microextraction only)
Support stand and clamps (optional; see Step 8)
Tubing, Teflon
Vials, glass, 1.5- and/or 4-mL, equipped with Teflon-lined caps

> The size of the vial will be determined by the size of the gland/ant to be extracted. It is generally recommended to use the smallest vial needed. Conical inserts may be needed (see Step 4.ii).

Vials, headspace (i.e., glass vial with airtight seals and Teflon septa)

METHOD

Solvent Extraction

> This method can be used for both volatile and nonvolatile semiochemicals. It does not require any special equipment and is very simple to perform. The yield is usually high, and further actions, such as microchemical derivatizations, can be performed. However, often a large number of chemicals are extracted, and recognizing the compounds responsible for the behavior might require further investigation. Also, this method is destructive and can only be performed once on each individual.

1. Identify the source of the semiochemical:

    i. If the semiochemical is produced by a gland, dissect the gland(s) out.

    This minimizes the presence of compounds from other body parts.

    ii. If the semiochemical is found on the cuticle surface, kill the ant(s) by freezing or CO_2 anesthesia.

2. Transfer the glands (from Step 1.i) or intact ants (from Step 1.ii) to glass vials.

    If individual characterization is not an issue, ants and glands can be extracted in groups.

3. Using glass Pasteur pipettes or polytetrafluoroethylene pipette tips, add just enough of the solvent of choice to cover the ant or the gland. Soak the tissues in solvent for 1-5 min.

4. Using glass Pasteur pipettes, transfer the solvent to a new vial:

    Store in tightly capped vials in the freezer until needed. If the sample contains volatiles, make sure there is enough solvent. If the behaviorally active component is unstable, proceed as soon as possible.

    i. If the semiochemical is highly abundant, and the final volume of solvent is >200 µL, use a regular vial.

ii. If the semiochemical needs to be highly concentrated for GC analysis (i.e., the final volume of the sample must be <200 µL), transfer to a vial containing a conical insert.

If the volume exceeds that of the conical insert, transfer the extract first to a new vial. Evaporate the solvent under a stream of high-purity nitrogen gas (see Step 5) to approximately the volume of the conical insert and then transfer the extract to a fresh vial containing the insert.

5. Evaporate the samples to near-dryness by blowing a gentle stream of high-purity nitrogen gas through a glass Pasteur pipette connected to the gas tank by a Teflon tube.

Take care not to blow the semiochemical out with the solvent. If the semiochemical is highly volatile, do not blow the solvent to complete dryness.

6. Add fresh solvent to the conical insert to achieve the desired concentration (e.g., 1 ant-equivalent per µL).

Headspace Collection with SPME

This method collects only volatiles, but is nondestructive. The amount of semiochemicals collected should be just enough for GC analysis.

7. Place the ant in a small headspace vial. If the ant feels threatened, it can release defense compounds. Unless these are the semiochemicals of interest, let the ant acclimate for a while before sealing the vial.

The vial should be small enough so the concentration of the volatiles of interest will be high enough for detection. Depending on the type of semiochemical, a single ant or multiple ants can be placed in the same vial.

8. Insert the SPME needle into the vial. Expose the fiber.

If the SPME apparatus is too large to balance on top of a small vial, use a support stand and clamps to stabilize the apparatus.

9. Leave the SPME fiber inside the vial for the ant to release its volatiles.

i. If the semiochemical release is associated with a specific behavior, watch the ant to make sure it performs that behavior.

If the fiber is left in too long, volatiles from excretions can be adsorbed as well.

ii. Prevent the ant from climbing on the fiber.

10. Retract the fiber into the needle. Remove the SPME apparatus.

Solid Phase Microextraction

The SPME apparatus can also be used for collecting surface nonvolatiles. The method is largely nondestructive, especially if using sclerotized adult ants. This is a good method for studying differences in cuticular lipid profiles between individuals.

11. Immobilize the ant (e.g., by anesthesia or placing on top of an ice-filled glass Petri dish).

12. Expose the SPME fiber. Rub it gently against the cuticle of the ant for a predetermined amount of time (e.g., 100 strokes, 2 min, etc.). Make sure most of the exposed fiber contacts the ant's surface.

The amount of time should be consistent across all samples collected.

GC-MS Program Setup

For volatile collections, the oven temperature does not need to get too high. A final temperature of 260°C should be enough to remove all chemicals from the GC column. For solvent extracts (e.g., from Step 6) and SPME of cuticular lipids (from Step 12), the oven temperature should range from 60°C to 300°C, because long-chain hydrocarbons might be present.

13. Program the GC-MS oven as appropriate for the semiochemical under investigation:

For volatiles:

i. Use a temperature gradient that is not too steep (e.g., 10°C/min) for the first few minutes and that increases quickly thereafter (e.g., 30°C/min).

This should remove nonvolatiles that are not of interest from the column.

For nonvolatiles:

    ii. Use a temperature gradient that increases quickly in the first few minutes (e.g., 30°C/min) and then levels off to a gentler slope (e.g., 10°C/min).

    This insures that the separation in the range where the chemicals elute from the column is greatest and that the peaks of interest can be more easily discriminated, while keeping the run as short as possible.

Chromatogram Analysis

14. Make sure the peaks in the total ion chromatogram separate distinctly. Otherwise, change the GC oven program accordingly and reanalyze the sample.

See Troubleshooting.

15. Use a chemical ionization MS detector to confirm the molecular weight of a compound.

16. Double-click on a given peak on the total ion chromatogram.

This opens the mass spectrum (MS) window of the peak.

17. Double-click anywhere in the MS window to access the database library.

The mass spectrum pattern could match closely that of a known chemical structure that can be found either in the database library or somewhere in the literature.

18. To interpret the pattern of an unknown chemical structure, refer to Silverstein et al. (2005) or Martin Smith (2004).

TROUBLESHOOTING

Problem: There are not enough, or hardly any, peaks in the chromatogram.
[Step 14]
Solution: Although it is rare, some ants indeed have very little hydrocarbons on their cuticle. Consider the following:

  1. If using SPME, try extracting longer (e.g., more strokes, longer exposure in the headspace) or even try a different SPME fiber.

  2. It might be necessary to resort to solvent extraction. If using solvent extraction, extract more ants and concentrate the sample more. Also, try different solvents and longer solvent extractions.

Problem: There are contamination peaks in the chromatogram.
[Step 14]
Solution: Ignore contaminants that do not interfere with the peak(s) of interest. If they do interfere, determine the source of contamination; common contaminants are identifiable through the database library. Reanalyze the sample, taking care in the extraction process to avoid contamination. Always wear gloves and only use glass and Teflon-lined caps. Do not let the samples come in contact with Parafilm; it contains hydrocarbons.

Problem: There are too many peaks in the chromatogram.
[Step 14]
Solution: Some of these peaks could be contamination; see above. If they are from the sample, reduce the number of peaks by separating the chemicals based on their physical properties before the GC-MS analysis, using flash column chromatography or high performance liquid chromatography. These separation methods produce collectible fractions that can be tested for behavioral activity, and this might reduce significantly the number of peaks.

DISCUSSION

GC-MS analysis is a simple way of identifying chemical structure. It does not require high purity because the GC will separate compounds based on their physical attributes. The MS breaks the molecule in a predictable way to give distinct ions in distinct proportions to allow for identification.

Nonetheless, often GC-MS analysis is not enough, especially if the chemical has never been described before, as is common in insect systems. In some of these cases, the problem can be solved easily with simple microchemical reactions coupled with additional GC-MS analyses. In other cases, however, other types of analyses (such as nuclear magnetic resonance spectroscopy) might be required.

REFERENCES

Silverstein RM, Webster FX, Kiemle D. 2005. *The spectrometric identification of organic compounds*, 7th ed. Wiley, Hoboken, NJ.

Smith CR, Dolezal A, Eliyahu D, Holbrook CT, Gadau J. 2009. Ants (Formicidae): Models for social complexity. *Cold Spring Harb Protoc* (this issue). doi: 10.1101/pdb.emo125.

Smith R Martin. 2004. *Understanding mass spectra: A basic approach*, 2nd ed. Wiley, Hoboken, NJ.

In Situ Hybridization on Ant Ovaries and Embryos

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INTRODUCTION

The detection of transcript distribution throughout a fixed tissue is a major step in studying the transcriptional activity of target genes and their function. In situ hybridization specifically detects the spatial distribution of RNA transcripts using an antisense RNA probe. This protocol describes the preparation of digoxigenin-labeled antisense RNA probes and their hybridization to complementary mRNA sequences; expression can then be localized using an antibody against digoxigenin conjugated to a chromogenic enzyme. In ants, this method can be applied to visualize cell populations of interest among other populations in a tissue, such as insect ovaries, or in the whole organism, such as insect embryos. Specific markers (e.g., genes known to be expressed in particular clusters of cells) can be cloned and used as probes to study the distribution and development of germline cells (e.g., *nanos*, *vasa*), neurons (*repo*), or limb structures (e.g., *distal-less*). Various markers might also allow the study of oogenesis (*nanos*, *par-1*, *oskar*), segmentation in the embryo (e.g., *engrailed*, *wingless*), or other developmental processes.

RELATED INFORMATION

Specific examples of the application of this technique in ant tissues are presented in Figure 1. For a more extensive discussion on the background and potential uses of ants as model organisms, see **Ants (Formicidae): Models for Social Complexity** (Smith et al. 2009). A protocol for **General Dissection of Female Ant Reproductive System and Brain** (Dolezal and Brent 2009) is also available.

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <!>, and recipes for reagents marked with <R>.

Reagents

Agarose gel (1%)
<R>Alkaline phosphatase buffer (APS)
Anti-digoxigenin (DIG), Fab fragments, AP-conjugated (Roche Applied Science 11093274910)
Ants of the species of interest
<R><!>BCIP (50 mg/mL in DMF)
cDNA of the gene of interest
<R>Diethyl pyrocarbonate (DEPC)-treated H_2O
DIG RNA Labeling Kit (SP6/T7) (Roche Applied Science 11175025910)

> *This kit includes NTP labeling mixture (10X), DNase I (RNase-free; 10 U/µL), T7 and SP6 RNA polymerases (20 U/µL), RNase inhibitor (20 U/µL), and Transcription buffer (10X).*

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<!>DMSO (dimethyl sulfoxide)
Glycerol
<!>Heptane (*n*-Heptane)
<R>In situ hybridization solution (ISHS)
M13 RNA probes, antisense

> For the forward primer, use 5'-CGCCAGGGTTTTCCCAGTCACGAC-3'. For the reverse primer, use 5'-GAGCG
> GATAACAATTTCACACAGG-3'.

<!>Methanol (freezer-cold for Step 13)
<R><!>NBT stock solution (75 mg/mL)
<R><!>Paraformaldehyde (4%)
<R>PAT
<R>PBS(P) (pH 7.4)
<R><!>PBS(P) containing 1% Triton X-100 (PBSTx)
<R>PBS(P) containing 0.1% Tween 20 (PBSTw)
Plasmid, T7/SP6 (e.g., pGEM-T or pGEM-T Easy system; Promega)
QIAquick PCR purification kit (QIAGEN 28104)
RNeasy Mini kit (QIAGEN 74104)
Tween 20

Equipment

Boiling water bath
Centrifuge
Dishes, glass, transparent, three-well (VWR)
Electrophoresis unit for agarose gels
Forceps, very fine
Heating block preset to 37°C
Ice
Microscope
Oven or waterbath preset to 58°C-60°C
PCR unit
Slides and coverslips
Tubes, microcentrifuge, 1.5-mL

METHOD

Probe Preparation

1. Clone a cDNA fragment (0.5-kb or larger) from the gene of interest into a T7/SP6 plasmid.

   The fragment must be sequenced and the 5' → 3' orientation established to determine which polymerase will result in an antisense in vitro transcription.
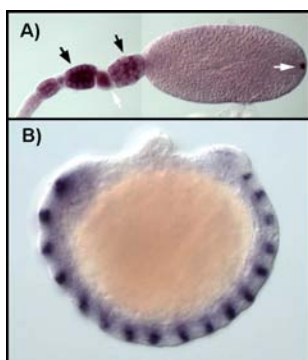

FIGURE 1. In situ hybridizations. (*A*) *Myrmica americana* ovariole stained with a probe specific for the germline marker *nanos* (purple). *nanos* mRNA is produced in the nurse cells (black arrows), then transported to the developing oocytes where it is localized to the posterior pole (white arrows). (*B*) Segmented embryo of *Messor pergandei* stained with a probe specific to the segment polarity gene *wingless* (*wg*). Note the segmental distribution of *wg* mRNA, which suggests that *wg* function in defining segment polarity is conserved in ants.

2. Perform PCR using the M13 forward and reverse primers.

   The resulting PCR fragment is flanked with the T7 promoter from one side and the SP6 promoter from the other. This fragment can be used as a transcription template with the appropriate polymerase.

3. Purify the PCR fragment using the QIAquick PCR purification kit according to the manufacturer's instructions.

4. Verify 1 µL of the purified template on a 1% agarose gel.

In Vitro Transcription Reaction

5. Assemble the following reaction on ice in the order indicated to a total volume of 20 µL:

Reagent	Amount to add
Linearized template (from Step 3)	0.5 µg (X µL)
DEPC-treated H_2O	(13 - X) µL
NTP labeling mixture (10X)	2 µL
Transcription buffer (10X)	2 µL
RNase inhibitor	1 µL
RNA polymerase (T7 or SP6, as appropriate)	2 µL

6. Mix the reaction gently. Centrifuge briefly. Incubate for 2-4 h at 37°C.

7. Add 2 µL of DNase I. Incubate for another 15 min at 37°C.

8. Purify the RNA using the RNeasy Mini kit according to the manufacturer's instructions. Resuspend in 50 µL of DEPC-treated H_2O.

9. Verify 2 µL of the probe on a 1% agarose gel.

   A band should appear at a size slightly lower than the original PCR DNA template.

10. Add 50 µL of ISHS to the probe.

    Store the diluted probe at -20°C, or at -80°C for longer periods.

Ovary and Embryo Fixation

11. Prepare the tissues of interest:

    For ovaries:

    i. Dissect ovaries (see **General Dissection of Female Ant Reproductive System and Brain** [Dolezal and Brent 2009]) in PBS(P) containing 0.05% Tween 20.

    Keep the tissues on ice during the dissection process.

    ii. Remove the peritoneal sheet covering each ovariole using very fine forceps.

    iii. With one pair of forceps, hold the membrane at the base of the ovariole (where large oocytes are located). Using a second pair of forceps, pull the membrane toward the fine part of the ovariole.

    The membrane should come off inside-out, like a sock.

    For embryos:

    iv. Collect the embryos in a 1.5-mL tube in PBSTx.

    v. Boil the embryos for 45 sec. Quench quickly on ice.

    vi. Remove the chorion and vitelline membrane manually using very fine forceps.

12. Fix the dissected ovarioles (from Step 11.iii) or embryos (from Step 11.vi) in a 1.5-mL tube with 200 µL of 4% paraformaldehyde supplemented with 20 µL DMSO and 600 µL heptane for 20 min at room temperature.

13. Wash fixed ovarioles or embryos three times with freezer-cold methanol.

    Store fixed tissues at -20°C in methanol or process immediately for in situ hybridization.

14. Prepare a series of methanol:PBSTw (70:30, 50:50, and 30:70). Rehydrate the tissues by washing as follows:

    i.   Methanol:PBSTw (70:30) for 5 min.

    ii. Methanol:PBSTw (50:50) for 5 min.

    iii. Methanol:PBSTw (30:70) for 5 min.

    iv. Three times in 100% PBSTw, 5 min each wash.

Hybridization

15. Wash the tissues in PBSTx to permeabilize the tissue and allow better probe penetration.

16. Prehybridize the tissues with ISHS in an oven or waterbath for 1 h at 58°C-60°C.

17. Denature 2 μL of probe (from Step 10) in 100 μL of ISHS by placing in boiling water for 2-3 min. Quench immediately in ice.

18. Replace the ISHS with the denatured probe. Incubate overnight (~16 h) at 58°C-60°C.

19. Remove the probe solution. Replace with 300-500 μL of fresh ISHS preheated to 58°C-60°C.

20. Prepare a series of ISHS:PBSTx (75:25, 50:50, 25:75), prewarmed to 58°C-60°C. Wash the tissues as follows:

    i.   0.5 mL of ISHS:PBSTx (75:25) for 15 min at 58°C-60°C.

    ii. 0.5 mL of ISHS:PBSTx (50:50) for 15 min at 58°C-60°C.

    iii. 0.5 mL of ISHS:PBSTx (25:75) for 15 min at room temperature.

    iv. Wash tissues three times with 1 mL of PBSTx at room temperature.

    v. Wash tissues three times with 1 mL of PAT at room temperature.

21. Incubate tissues for 1 h in 1 mL of PAT at room temperature.

Probe Detection

22. Dilute the anti-DIG antibodies 1:2000 with PAT. Incubate the tissues for 2 h at room temperature (or overnight at 4°C) with the diluted antibody.

23. Wash the tissues five times in 1 mL of PBSTx, 10 min each wash.

24. Transfer the tissues to three-well dishes so the reaction can be monitored under the microscope.

25. Wash the tissues twice with 0.5 mL of APS, 10 min each wash.

26. Prepare the reaction solution by adding 4.5 μL of the NBT stock and 3.5 μL of the BCIP stock per 1 mL of APS.

Prepare 300 μL reagent for each sample, plus some additional to allow for pipetting errors.

27. Incubate the tissues in 300 μL of reaction solution in the three-well dish in the dark.

28. Monitor the reaction closely by examining the color development regularly to avoid overdevelopment and formation of nonspecific background.

See Troubleshooting.

29. Stop the reaction by washing the tissues five times in PBSTx, 5 min each wash.

30. Prepare a series of glycerol:PBS(P) (30:70, 50:50, 80:20) and prepare the tissues for mounting as follows:

    i.   Wash the tissues in glycerol:PBS(P) (30:70).

    ii. Wash the tissues in glycerol:PBS(P) (50:50).

      iii. Wash the tissues in glycerol:PBS(P) (80:20).

The samples can be stored in 80% glycerol at -20°C.

31. Mount the samples on slides in 80% glycerol.

32. Observe and capture images with a microscope.

TROUBLESHOOTING

Problem: No color is obtained.
[Step 28]
Solution: Check probe orientation and quality. Sometimes the experiment works, but produces too much background (i.e., nonspecific staining). Staining can be enhanced by optimizing the amount of probe used, increasing the hybridization temperature a few degrees, or by including additional washing steps.

REFERENCES

Dolezal A, Brent C. 2009. General dissection of female ant reproductive system and brain. *Cold Spring Harb Protoc* (this issue). doi: 10.1101/pdb.prot5244.

Smith CR, Dolezal A, Eliyahu D, Holbrook CT, Gadau J. 2009. Ants (Formicidae): Models for social complexity. *Cold Spring Harb Protoc* (this issue). doi: 10.1101/pdb.emo125.

Phase-Unknown Linkage Mapping in Ants

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INTRODUCTION

To create a genetic linkage map of an ant genome, the phase of a marker (i.e., which marker/allele came from the grandmother and which came from the grandfather of the individuals used for linkage mapping) must be known. However, field colonies contain only two generations: the queen(s) and their offspring. Normally, virgin queens disperse from their nest, mate with one or multiple males who die after mating, and start a new colony. A new reproductive brood (i.e., virgin queens and males) is usually produced after colony establishment, which can take from 6 mo to 3-5 yr. Hence, determining the phase of markers from field samples is impossible because the grandparents of a reproductive queen are no longer available for genetic analysis. Ants raised in the laboratory are likewise unsuitable for such analyses, because most ants cannot be bred regularly in the laboratory, and attempts to inseminate ant queens artificially have not been successful. However, three features facilitate "phase-unknown" linkage mapping for any ant (or other social hymenopteran) species, which is described here: (1) Mature colonies usually produce many reproductive offspring during a short period of time, which allows for the collection of a good-sized mapping population (>100 males) from a field colony; (2) Ants have a haplodiploid sex determination system (i.e., males are haploid and females diploid); (3) Queens produce males parthenogenetically. Thus, because they have no father, only those markers for which the queen is heterozygous are expected to segregate 1:1 in her male offspring.

RELATED INFORMATION

For a more extensive discussion on the background and potential uses of ants as model organisms, see **Ants (Formicidae): Models for Social Complexity** (Smith et al. 2009).

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with **<!>**, and recipes for reagents marked with **<R>**.

Equipment

Computer system and linkage mapping software (e.g., MAPMAKER or Multipoint)
For an exhaustive list of linkage mapping programs, see http://linkage.rockefeller.edu/soft/.

METHOD

1. Arbitrarily assign a phase to every allele of each genetic marker.
   See Troubleshooting.

2. Double the complete data set. Switch the phase of each second marker, i.e., convert 1 to 0 and 0 to 1 (Fig. 1).
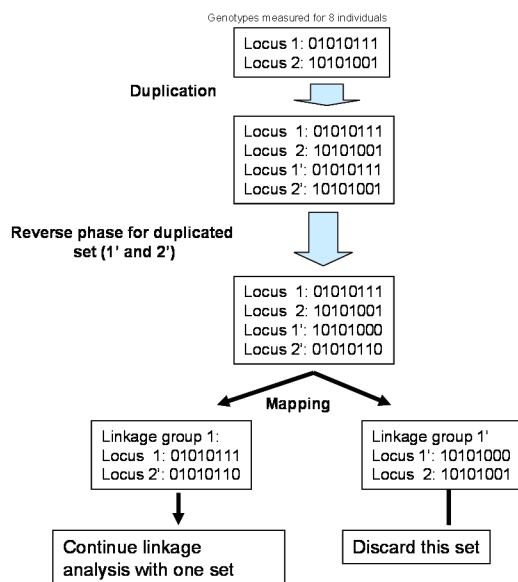   *This new data set now contains each possible linkage phase for each marker and individual.*

FIGURE 1. Procedure to generate a phase-unknown linkage map based on the genotypes of 100 males produced by a single queen.

3. Analyze the data set using a suitable mapping program capable of handling backcross data.

4. Use the estimated recombination frequencies between markers to generate linkage groups. Because the data set is doubled, each linkage group is present twice. Note that only the high progeny number allows for the determination of the phase of linked markers in this approach.

5. For all further analyses, discard one of the two identical linkage groups.

   The correct phase of a linkage group cannot be determined without further information.

TROUBLESHOOTING

Problem: The species to be mapped has multiple queens in the nest.
[Step 1]
Solution: If only a few queens are present and the progeny of each can be identified affirmatively using molecular markers, discard samples from all but one queen. Note, however, that at least 100 male progeny are still needed from that queen.

Problem: Workers in the species being mapped might have produced some of the males in the sample.
[Step 1]
Solution: Using molecular markers establishes the queen's genotype at each locus. If non-queen alleles are present in a male, it is likely that the male was produced by a worker (or that multiple queens are present in the colony). Discard all males that cannot be assigned unambiguously to the focal queen.

DISCUSSION

This simple method of phase-unknown linkage mapping enables the creation of linkage maps for any ant species that produces enough male offspring, without the need to breed the species in the laboratory or wait years until the colony produces male offspring. The validity of this approach has been tested in *Apis mellifera*, *Bombus terrestris*, and *Nasonia vitripennis*, where linkage maps were produced on mapping populations for which the phase was known (Wilfert et al. 2007; J Gadau, unpubl.).

REFERENCES

Smith CR, Dolezal A, Eliyahu D, Holbrook CT, Gadau J. 2009. Ants (Formicidae): Models for social complexity. *Cold Spring Harb Protoc* (this issue). doi: 10.1101/pdb.emo125.

Wilfert L, Gadau J, Schmid-Hempel P. 2007. Variation in genomic recombination rates among animal taxa and the case of social insects. *Heredity* **98:** 189–197.

Appendix 1: Recipes

[NOTE: This print edition of *CSH Protocols* contains only recipes for reagents requiring multiple components or non-obvious critical steps. Recipes for reagents marked with the <R> symbol not listed below can be found online at http://www.cshprotocols.org/recipes.]

0.2 M phosphate buffer

Reagent	Amount to add
$NaH_2PO_4 \cdot H_2O$	5.25 g
$Na_2HPO_4 \cdot 7\,H_2O$	23.0 g
H_2O	to 1 L

Alkaline phosphatase buffer (APS)

50 mM $MgCl_2$
100 mM NaCl
100 mM Tris, pH 9.5
0.1% Tween 20

Ant homogenization buffer

<R>10 mM EDTA
60 mM NaCl
<R>10 mM Tris-Cl (pH 7.5)

Ant solubilization buffer

<R>30 mM EDTA
<!>2.0% SDS (sodium dodecyl sulfate)
<R>200 mM Tris-Cl (pH 9.0)

Apple juice-agar plates

<!>0.6 g methyl 4-hydroxybenzoate (Sigma H5501)
10.0 g sucrose
250 mL apple juice
9.0 g Bacto agar
300 mL H_2O
Prepare the apple juice solution by adding the first two ingredients to the apple juice. Boil and then let cool.
Prepare the agar solution by adding the Bacto agar to the H_2O. Boil.
Mix the apple juice and agar solutions, cool to 65°C, and pour into the lids of 35-mm Petri plates. (Save the Petri plate bottoms for embryo collection.) This recipe makes 275 plates.

BCIP (50 mg/mL in DMF)

<!>BCIP (5-bromo-4-chloro-3-indolyl-phosphate; e.g., Roche)
<!>DMF (dimethylformamide)
Prepare a solution of 50 mg/mL BCIP in DMF and store in aliquots at −20°C.

Borate buffer

Reagent (final concentration)	For 4 L	For 500 mL	For 100 mL
<!>Boric acid (100 mM)	24.74 g	3.09 g	0.62 g
NaCl (75 mM)	17.54 g	2.19 g	0.44 g
Sodium tetraborate (25 mM)	38.14 g	4.77 g	0.95 g

Dissolve the ingredients in H_2O. Use a stir bar and low heat to ensure that the boric acid dissolves fully. Sterilize by vacuum-filtration with a 0.22-μm filter. Adjust the pH to 8.4.

Chelex solution (5%)

4 g Chelex resin, 200-400 mesh
40 mL TE buffer
40 mL H_2O (sterilized)

Diethyl pyrocarbonate (DEPC)-treated H_2O

Distilled H_2O
<!>Diethyl pyrocarbonate (DEPC)
Add 1 mL of fresh DEPC to 1 L of H_2O. Shake well to disperse the DEPC through the H_2O. Incubate at 37°C for at least 12 h and/or autoclave at 15 psi on liquid cycle for 20 min to inactivate the remaining DEPC.

EDTA

To prepare EDTA at 0.5 M (pH 8.0): Add 186.1 g of disodium EDTA•$2H_2O$ to 800 mL of H_2O. Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH (~20 g of NaOH pellets). Dispense into aliquots and sterilize by autoclaving. The disodium salt of EDTA will not go into solution until the pH of the solution is adjusted to ~8.0 by the addition of NaOH.

Embryo homogenization buffer

Reagent	Final concentration
NaCl	0.1 M
<R>Tris-Cl (pH 8.0)	0.03 M
<R>EDTA	0.05 M
<!>Triton X-100	0.5%
<!>β-Mercaptoethanol	7.7 mM

Gel electrophoresis buffer

<R>TAE (1X)
<!>Ethidium bromide (1.25 μM)

In situ hybridization solution (ISHS)

<!>Formamide (50%)
<!>Heparin (50 μg/mL)
Salmon sperm DNA solution (50 μg/mL; e.g., UltraPure; Invitrogen)

<R>SSC (5X; pH 6.5)
<!>SDS (sodium dodecyl sulfate) (0.3%)
Tween 20 (0.1%)
Store at –20°C.

King's B medium

10 g protease peptone #2 (DIFCO)
1.5 g anhydrous K_2HPO_4
15 g glycerol
5 mL $MgSO_4$ (1 M; sterile)
Antibiotics (as needed)
Add H_2O to first three ingredients to bring volume to 1 L. Adjust the pH to 7.0 with HCl. Autoclave and then add
5 mL of sterile 1 M $MgSO_4$. If $MgSO_4$ is added before autoclaving, the medium becomes cloudy.
Add antibiotics (as needed).

Low TE

<R>1 mM Tris-Cl (pH 7.5)
<R>0.1 mM EDTA

M-199 incubation medium

Ficoll 400 (Sigma)
Medium 199, modified with Hanks', L-glutamine (US Biological M2852)
1. Add 2 g of Ficoll 400 to 100 mL of Medium 199. Mix with a magnetic stir bar until the Ficoll dissolves.
2. If necessary, adjust the pH to 7.2-7.5 with NaOH.
3. Filter-sterilize with a Nalgene 115-mL sterile filter unit type S (0.20-µm) or a 10-mL syringe attached to a
0.2-µm syringe filter.
4. Store refrigerated in labeled 15-mL vials.
   After prolonged storage, refilter the medium through a 0.2-µm syringe filter.

NaCl/Triton rinsing solution

Reagent	Final concentration
NaCl	0.7%
<!>Triton X-100	0.4%

NBT stock solution (75 mg/mL)

<!>DMF (dimethylformamide)
<!>NBT (4-nitro blue tetrazolium chloride)
<R><!>Diethyl pyrocarbonate (DEPC)-treated H_2O
Prepare 70% DMF in DEPC-treated H_2O. Then prepare the NBT to a final concentration of 75 mg/mL in the
70% DMF.

NDS

Reagent	Final concentration
<R>EDTA	0.5 M
<R>Tris-Cl (pH 9.5)	0.01 M
<!>Sarkosyl	1%

Nested Patch PCR buffer (10X)

200 mM Tris-HCl (pH 8.4)
500 mM KCl

Paraformaldehyde (4%)

<!>4% paraformaldehyde
<R>1X PBS(P)
0.1% Tween 20
Add 4 g of paraformaldehyde to 100 mL of 1X PBS(P) containing 0.1% Tween 20. Heat without boiling until the paraformaldehyde is completely dissolved. Dispense into 1-mL aliquots and store at –80°C. Use fresh aliquots only.

PAT

<R>1X PBS(P)
<!>1% Triton X-100
1% bovine serum albumin (BSA)

PBS(P)

Reagent	Quantity (for 1 L)	Final concentration (10X)
NaH_2PO_4	2.56 g	18.6 mM
Na_2HPO_4	11.94 g	84.1 mM
NaCl	102.2 g	1.75 M

Adjust the pH to 7.4 using NaOH or HCl as necessary. This recipe produces a 10X stock solution; prepare 1X PBS(P) by diluting with H_2O. Both 1X and 10X PBS(P) can be kept indefinitely at room temperature.

Phosphate buffer (0.2 M)

Reagent	Amount to add
$NaH_2PO_4 \bullet H_2O$	5.25 g
$Na_2HPO_4 \bullet 7\ H_2O$	23.0 g
H_2O	to 1 L

Phosphate-buffered saline (PBS) for bacterial FISH

NaCl (130 mM)
<R>Phosphate buffer (10 mM)

SSC

For a 20X solution: Dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800 mL of H_2O. Adjust the pH to 7.0 with a few drops of a 14 N solution of HCl. Adjust the volume to 1 L with H_2O. Dispense into aliquots. Sterilize by autoclaving. The final concentrations of the ingredients are 3.0 M NaCl and 0.3 M sodium citrate.

TAE

Prepare a 50X stock solution in 1 L of H_2O:
<!>242 g of Tris base
<!>57.1 mL of acetic acid (glacial)
<R>100 mL of 0.5 M EDTA (pH 8.0)
The 1X working solution is 40 mM Tris-acetate/1 mM EDTA.

TE (pH 7.5)

Reagent	Final concentration
<R>Tris-Cl (pH 7.5)	10 mM
<R>EDTA (pH 8.0)	1 mM

Tris-Cl

<!>Tris base
<!>HCl
To prepare a 1 M solution, dissolve 121.1 g of Tris base in 800 mL of H_2O. Adjust the pH to the desired value by adding concentrated HCl.

pH	HCl
7.4	70 mL
7.6	60 mL
8.0	42 mL

Allow the solution to cool to room temperature before making final adjustments to the pH. Adjust the volume of the solution to 1 L with H_2O. Dispense into aliquots and sterilize by autoclaving.

If the 1 M solution has a yellow color, discard it and obtain Tris of better quality. The pH of Tris solutions is temperature-dependent and decreases ~0.03 pH units for each 1°C increase in temperature. For example, a 0.05 M solution has pH values of 9.5, 8.9, and 8.6 at 5°C, 25°C, and 37°C, respectively.