Ants as Naturally Long-lived Insect Models for Aging.

Joel D. Parker and Karen M. Parker

Department of Ecology and Evolution

Biology Building

University of Lausanne

CH-1015 LAUSANNE

Switzerland

Joel.Parker@unil.ch

Karen.Parker@unil.ch

Scope and Purpose

This chapter explains in what respects ants can be useful models in understanding the mechanisms of aging. It includes an introduction highlighting how ants fulfill a need for long lived model systems in aging research. Three ant model systems are then described including their relevant natural history characteristics, collection and laboratory maintenance. Practical considerations are given for molecular studies and techniques. Finally, an overview is given of the available genomic resources and types of comparative studies that are possible.

Glossary

Allate/Deallate: Queens and males with wings are called allates. After the mating flight, the queens remove their wings and are called deallate females.

Budding: The founding of a new colony when a polygynous colony splits with some workers and queens leaving to start a new colony.

Caste: Designation of a set of individuals in a colony that are distinct in regard to morphology and behavior.

Claustral colony founding: The founding of a new colony by a single queen that raises its first brood without any outside help.

Minims: The first group of sterile worker offspring in a new colony. They are smaller than the workers of a mature colony.

Monogyny: The condition when a colony is headed by a single fertile queen.

Physogastric queen: A condition in certain insects where the abdomen becomes distended by the growth of reproductive organs.

Polygyny: The condition when a colony has more than one fertile queen.

Queen: A female reproductive in an ant colony. Usually there is one or a group of related queens in a colony.

Worker: The sterile female offspring of the queen that perform all of the tasks in the colony.

Why Ants?

Humans owe their relatively long life span to living in societies that reduce the risk of extrinsic mortality. Other organisms in organized societies are also expected to exhibit a similar lengthening of life span over evolutionary time. One hundred million years before the first human stood up and walked, social insects existed in societies with cities, roads, division of labor, farming, slave making, and organized group defenses (Hölldobler & Wilson, 1990). Sociality has resulted in a 10 to 100 fold increase in the life span of queens in ants, bees, and termites; a trend that was rigorously demonstrated using phylogenetic methods to compare life span and social structure across the insects (figure 1, Keller & Genoud, 1997). The evolution of sociality and its associated increase in life span is a general trend that has independently evolved several times.

Ants represent an ideal system to ask how evolution effects the changes necessary for long life. In addition to the differences in life span among species, there are also order of magnitude differences in life span between different castes in a single species. For example, queens of the ant *Lasius niger* have been known to live nearly 29 years in a lab, while workers live for only a few years and males a few weeks (Kutter & Stumper, 1969; Hölldobler & Wilson, 1990). Given that the same egg can become either a queen or a worker, differential gene expression seems to be the key difference between those 2 castes.

The traditional model systems used to study aging (*Drosophila melanogaster, Caenborabditis elegans, Mus musculus and Saccharomycies cerivisiae*) all share a short generation time, making them ideal experimental systems in terms of laboratory rearing and experimental manipulations. Unfortunately, this has introduced a bias in

the types of life histories that have been sampled in modern aging research as almost all the mechanisms proposed for aging have been limited to these systems. Some of these mechanisms are not thoroughly understood in an evolutionary context and may not truly represent adaptations necessary for long life. Hence, these mechanisms need verification in long-lived species like humans. In addition, studies of long-lived species such as ants may also reveal new and novel life span extending processes and mechanisms that have withstood the test of evolutionary time.

The Ant Model Systems

Ants as a group share certain important life history characteristics, such as living in family groups with overlapping generations. Ants are haploid-diploid; the queens and workers are diploid and female where as males are haploid and develop from unfertilized eggs. In monogynous colonies, there is one reproductive queen per colony with sterile workers who do not contribute directly to the reproduction of the colony. Sexual winged queens and males are produced by the colony at specific times of the year. Queens of monogynous colonies remove their wings after mating and found a new colony independently (claustral founding). For polygynous species, where there is more than one reproductive queen per nest, newly mated queens are accepted back into their colonies. In these cases, new colonies are typically formed by budding.

For aging research, there are two ant species and one genus that are particularly well suited for development into model systems. The species *Lasius niger* and the genus *Pogonomyrmex* have considerable life spans and are easy to rear in laboratory settings. The third species, *Solenopsis invicta*, has a variety of modern genomic tools available such as a large microarray and a large cDNA sequence

database. These species are all very common in the Holartic, western North America, and the southern United States, respectively.

Lasius niger

Queens of the common black ant, *Lasius niger*, have the longest recorded life span in the laboratory of 28 and 3/4 years (Kutter & Stumper, 1969). This monogynous species occurs in the Holartic region in forests and farm land. In central and northern European meadows, they occur in densities up to 1 mature colony per square meter. Colonies can be marked by placing a concrete paving slab over the colony entrance. The colony places brood underneath the slab for warming during the non-winter months, which simplifies collection. The colonies do not seem to migrate, facilitating long term monitoring and collection. Colonies can be huge with 10,000 or more workers, although smaller young colonies are easily maintained under laboratory conditions. *Lasius niger* has been extensively used in ecological studies as well as in genetic studies assessing colony relatedness, mating number, and sex ratio evolution (Fjerdingstad et al., 2002; Fjerdingstad et al ., 2003; Jemielity & Keller, 2003; Fjerdingstad & Keller, 2004). A recent search of the Web of Science internet database yielded 141 publications on *L. niger*.

This species has large mating flights in mid to late summer when hundreds of newly mated deallate queens can be collected. New laboratory colonies can be started in glass test tubes filled halfway with water with a tight wad of cotton holding the water back. The founding queen is placed in the tube which is closed with a cotton plug (figure 2). The tubes are placed in the dark for approximately six weeks, until the first minims emerge. The cotton plugs are removed from the tubes which are placed inside plastic boxes with additional water tubes. The inside walls of the plastic boxes are painted with Fluon®, a fluoropolymer that most insects have difficulty

climbing (available from Whitford Worldwide). Colonies will produce full sized workers after approximately one year and can be transferred to progressively larger boxes as the colony grows. Best results are obtained when colonies are kept at 22°C with 60% constant humidity. *Lasius niger* prefers liquid food and our current food mix is 1:1:2 ground meal worms:eggs:honey plus 1% volume of liquid baby vitamins. To facilitate pipetting, the meal worms are flash frozen with liquid nitrogen and ground into a fine powder with a mortar and pestle. Aliquots of the mixture are stored frozen at –20°C and diluted 1:1 with water just before use. Colonies are fed three times a week and are given one or several drops of food, depending on their size.

Pogonomyrmex

The approximately 20 North American species of the seed harvester ant genus *Pogonomyrmex* are possibly the most studied genera of ants in the world with two books and a large body of primary literature dedicated to them (Cole, 1968; Taber, 1998; Johnson, 2000, 2001). *Pogonomyrmex* contains the longest lived ant species recorded in the field, 30 years for *P. salinus* (Porter & Jorgensen, 1988). The best candidate model species within the genus *Pogonomyrmex* are *P. rugous, P. barbatus* and *P. occidentalis*.

These monogynous, multiple mating species are extremely common in the western deserts of North America, forming large conspicuous disc or mound shaped colonies. The colonies rarely move, facilitating permanent marking and long term demographic studies. All of these species sting and can be very aggressive. The mating flights are usually rain triggered in the mid to late summer months. Queens can be collected after mating flights and colonies started in water tubes as for *L. niger*, transferring them to larger boxes with additional water tubes as needed (figure 3). Being desert ants, they should be kept at 30 to 35°C. Seed harvesters are poor

climbers, but Fluon® is recommended to help contain them within their plastic boxes. A more elegant design is a sandwich style nest with either soil or plaster (see Johnson website for pictures and details). *Pogonomyrmex* should be fed pesticide-free grass seed or small bird seed, dead insects such as frozen crickets or meal worms, and a 1:1 mixture of honey and water. Colonies can be raised to large numbers and, in many cases, can produce sexuals in 2 to 3 years.

Solenopsis invicta

From a molecular biology viewpoint, the most attractive model ant species is the red imported fire ant, *Solenopsis invicta.* Although queens live only 2 to 5 years, they have the advantage of existing in polygynous and monogynous colonies. This ant has the most extensively developed genomic tools available with a large cDNA EST database and a microarray chip nearing completion (J. Wang and L. Keller, pers. comm.) A BAC library is also available for purchase (see website list). Primary tissue culture and gene expression studies have been successfully employed using *S. invicta* (Chen, 2004). In addition, the first gene directly affecting social structure (i.e. queen number) was discovered and cloned from *S. invicta* (Krieger & Ross, 2002).

Solenopsis invicta are common where introduced and easily cultured in the laboratory. Their transient, shallow nests makes it easy, if not sometimes painful, to collect mature colonies with queens. An entire nest can be shoveled into a Fluon® - coated bucket and water added slowly. *Solenopsis invicta* form living rafts in response to flooding and the floating colony raft can be scooped from the water and placed into plastic boxes treated with Fluon® (figure 4). Newly mated queens can also be collected after mating flights in the early summer and colonies started in water tubes as for *L. niger* and *Pogonomyrmex* . Their optimal laboratory temperature is 25 to 30°C. Their dietary requirements are more demanding, requiring freshly killed

insects and a constant source of water. Specific method descriptions and food recipes can be found online (see internet section of the appendices).

There are two problems that must be considered when working with *S. invicta*. First, it is impossible to avoid being stung on a regular basis when working with these small, aggressive ants. Some people develop sensitivity to the stings and can experience life threatening anaphylactic shock (Solley et al., 2002). Secondly, this is a highly destructive and invasive species that should never be transported to, or kept in, warm moist regions of the world where they have not already been established.

Sampling at the colony level

Mature queens of single queen colonies such as *Pogonomyrmex* and *L. niger* are almost impossible to collect due to the depth and size of the colonies. Fortunately, the genotype of a queen can be determined by genotyping male allates from the colony. As males are the product of unfertilized eggs, a sample size of 6 will cover 99% of the queen's genome. The genotype of the father(s) of the colony, as well as the queen's, can also be reconstructed by sampling workers. In both cases, such sampling will not negatively impact large colonies and represents a benign way to monitor the genetic structure of extant, wild long-lived populations.

Social insects should be sampled at the level of colony or group of colonies for molecular studies. When the queen has been fertilized by only one male, all of her daughters are full sisters and share the same haploid father and diploid mother. Thus, workers from a monogynous colony headed by a singly mated queen are identical for 75% of their genome. This can lead to pseudo-replication as colonies represent closely related families with related genetic backgrounds. Measurements of a group of 25 workers consisting of 5 workers from 5 different colonies can not be considered

25 independent samples because of within colony relatedness. Instead, this example contains measurements of 5 independent colonies, each consisting of the average from 5 workers. Thus, in most cases, worker colony samples should be averaged and one value taken per colony for statistical tests

Molecular Methods

There are some practical considerations one should keep in mind when isolating DNA, RNA and protein from ants. Queens tend to be very high in fat content before their mating flight as well as in their physogastric form. Supplementary extractions with ether to remove this fat can be helpful for DNA and RNA isolation. Ants also tend to have much harder exoskeleton and higher surface to mass ratio than *Drosophila* and complete homogenization of tissue can be difficult without a bead type shaker homogenizer.

Some experiments are better conducted on dissected tissues or body sections. Physogastric queens lay large quantities of eggs, and the mRNA profiles of the queens can become dominated by that of the eggs. Males contain a large amount of sperm and subsequently large amounts of DNA for their body weight. Beginning a DNA extraction with dissected vas deferens containing sperm represents an immediate 10 fold purification. The formic acid in Formicine ants can also be problematic as was found in protein preparations from *L. niger* workers (Parker et al., 2004a). The acidity can effect isolation and gel loading buffers as well as interfering with enzyme activity assays. The efficiency of phenol extractions in DNA isolations can also be adversely affected by the acid.

Genetics

Recently, the standard of proof in molecular biology has required demonstration of gene function by knocking out or overexpressing a gene in an experimental system. This comparison is not yet possible at the organismal level in ants either through selective breeding or by gene transfer, but has been successfully achieved for primary tissue culture in *S. invicta* (Cônsoli, 2002; Chen, 2004). To date, no one has developed an immortal cell line for any social insect. Eventually, one may find a way to breed the ant model systems above, either by simulating the natural mating conditions or by artificial insemination as is done for honey bees, but neither have yet been accomplished.

The lack of a genome sequence for ants does not pose as much of a practical problem as one might think. Although *S. invicta* is the only ant species with a very large cDNA sequence database, differential gene expression studies with specifically targeted genes or suppressive subtractive hybridization are still possible using genomes already sequenced. Specific genes can be cloned by aligning sequences from the honeybee, fruit fly, mosquito, silk worm and fire ant sequence databases using the program CODEHOP (Rose et al., 1998) with the *Lasius niger* codon usage table selected. This technique has been successfully employed to clone numerous *L. niger* genes, including an extracellular SOD which was not thought to exist in insects (Parker et al., 2004b).

Comparative Studies

Ants as a group are extremely diverse and this diversity lends itself to many types of comparative studies across species and across populations. There are also

exploitable differences across caste and sex within a single species. Some of these represent reversals of typical life span correlations with size and longevity as observed in humans and birds. For example, there are two sizes of workers in the weaver ant, *Oecophylla smaragdina.* The major (large) workers perform the dangerous tasks outside the colony and have shorter life spans than the minor (small) workers who remain within the highly protected nest. But this difference in life span remains intact even in a protected laboratory environment (Chapuisat & Keller, 2002). It would be interesting to investigate if there are metabolic differences between these two sizes of worker ants.

The phenomena of monogynous/polygynous colonies have evolved independently many times across a wide range of ants and other social insect taxa. There is evidence that, within the same species, queens of monogynous populations live longer than queens of polygynous populations as predicted by evolutionary theory (Keller & Genoud, 1997). *Solenopsis invicta* is an excellent system to investigate lifespan variation within a single species but across populations with different social structure as it has both polygynous and monogynous populations. In addition, the genetic basis for this difference in queen number is already known (Krieger & Ross, 2002).

One possibility for cross species comparisons are the many cases of social parasites. Socially parasitic ants lack a worker caste and invade a host colony where their reproductive brood are raised by the host colony workers. In the genus *Pogonomyrex,* there are socially parasitic ants who live only 1-3 years and have recently evolved from a shared common ancestor of their longer lived hosts (*P. barbatus* and *P. rugous* (Johnson et al., 1996; Parker & Rissing, 2002). They

represent an opportunity to investigate the evolution to a shorter life span once the relevant processes are discovered.

Reproductive rate correlates with life span such that highly fertile individuals have shorter life spans than less fertile members of the same species (Williams, 1957; Charlesworth, 1980; Partridge & Gems, 2002). Yet this fecundity/longevity tradeoff is reversed for queens and workers. The queen of a large social insect nest must lay eggs at a rapid rate to maintain the number of sterile workers in the colony and yet she has an extremely long life span (Rueppell et al., 2004). This reversal of the fecundity/longevity tradeoff has been shown true even without the morphological and physiological differences which are present between queen and worker ants. In the Ponerine ant *Platytyrea punctata ,* all workers are capable of producing diploid workers yet the reproductive workers live significantly longer than their nonreproductive counterparts (Hartmann & Heinze, 2003).

Given that castes share the same genome, the differences in life span must be based on differential gene expression at some point(s) in their life history. This provides the foundation to test gene expression differences already associated with aging in model systems. If the mechanisms hypothesized for life span extension in model systems are truly general, then there should be evidence of these same mechanisms being used in queen ants. One such mechanism, the resistance to oxidative stress conferred by superoxide dismutase (SOD) was recently tested in a comparison of cytoplasmic SOD activity and expression levels across all castes (Parker et al., 2004a). The study found that a high level of cytoplasmic SOD does not correlate with life span as for previous work in *Drosophila* (Orr & Sohal, 1994; Sohal et al., 1995; Hari et al., 1998; Sun & Tower, 1999; Arking et al., 2002; Spencer et al.,

2003) and is in agreement with previous comparative studies (Perez-Campo et. al, 1998; Barja, 2002).

These small, highly fecund and extraordinarily long lived queens must overcome all of the physiological problems of maintaining mitochondria, proteins which are degraded, damaged and/or incorrectly folded, as well as accumulative damage to DNA and membranes that are associated with long life. Queens in particular must do all of these things an order of magnitude longer than workers, and while reproducing at a high enough rate to sustain a colony. All of these individual molecular processes represent potentially fruitful lines of inquiry that could reveal novel aging resistance mechanisms.

Conclusions

It should not be overlooked that only those processes verified by both unnatural lifespan manipulations in the laboratory and naturally occurring evolved differences can be considered as true proven causes of life span variation. Without verification, one will never know whether the mechanisms uncovered in the traditional model systems are artifacts of laboratory stress or specific to short-lived organisms. Testing in the laboratory will guard against making up "just-so" stories based solely on observed natural correlations. The challenge is to combine studies across both types of systems to discover what processes truly underlie the human aging process. Ants offer one of the best insect models to build this bridge.

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Figure legends

Figure 1. Sociality causes an increase in longevity. Redrawing of figure 2 from Keller and Genoud 1997 showing how extreme life spans evolved multiple times in association with the evolution of sociality.

Figure 2. Founding *Lasius niger* queens. Queens being placed into water tubes after collection and stored in the dark until the first minims emerge.

Figure 3. *Pogonomyrmex rugosus* laboratory colony. The colonies are kept in plastic boxes normally used for rodent housing. There is an additional small box with water tubes for the area where the queen and brood live.

Figure 4. *Solenopsis invicta* laboratory colony. The colonies are kept in plastic boxes used for small rodent housing.

Table 1. Internet resources.

Description	Address
Codehop program and degereneate PCR recommendations	http://bioinformatics.weizmann.ac.il/blocks/codehop.html
Fireant BAC	https://www.genome.clemson.edu/cgi-bin/orders?page=productGroup&service=bacrc&productGroup=133

library	
Fluon® manufacturer	http://www.whitfordww.com
Formis ant literature database	http://cmave.usda.ufl.edu/~formis/
Robert Johnson's Pogonomyrmex display nests	http://lsweb.la.asu.edu/sirg/pogonomyrmex/culturingPogonomymrexqueens.htm
Robert Johnson's North American Pogonomyrmex distribution maps and pictures	http://lsweb.la.asu.edu/sirg/pogonomyrmex/NORTHAMERICANPOGOS.htm
Texas A & M fire ant collection and maintenance	http://fireant.tamu.edu/materials/factsheets/FAPFS008.2002rev.pdf
Texas A & M fireant research and management project	http://fireant.tamu.edu/
General ant information and images	http://www.antweb.org/index.jsp http://myrmecology.info/portal/news.php

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