



ANTS

LEAF LITTER (GROUND-DWELLING) ANTS

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Introduction

Ants are social insects classified into only a single family, Formicidae, within the Order Hymenoptera and Class Insecta. With over 13,000 described species (antbase.org; antcat.org) and a social lifestyle consisting of colonies ranging in size from just a few to millions of workers, ants are a dominant force in all terrestrial ecosystems, especially tropical rainforests (Alonso and Agosti, 2000; Lach *et al.* 2010). They are important members of terrestrial ecosystems, with high biomass and population size, and provide key ecological functions such as aerating and turning soil, dispersing plant seeds, consuming dead animals, and controlling pest insects (Perfecto 1991, Wagener *et al.* 2004, Philpott and Armbrecht 2006, Frouz and Jilkova 2008).

In addition to their ecological importance, ants have several features that make them especially useful for rapid assessment and conservation planning, including: 1) they are dominant members of most terrestrial environments, 2) they are easily sampled in sufficiently high numbers for statistical analysis in short periods of time (Agosti *et al.* 2000a), 3) they are sensitive to environmental change (Kaspari and Majer 2000), and 4) they are indicators of ecosystem health and of the presence of other organisms, due to their numerous interactions with plants and animals (Alonso 2000).

Standardized sampling of leaf litter ants: The ALL Protocol

The Ants of the Leaf Litter Protocol, commonly known as the ALL Protocol, was developed in 1996 by a group of leading ant taxonomists and ecologists based on their experiences surveying ants throughout the world. Details of the ALL Protocol are available in Agosti and Alonso (2000) with additional information on ants and ant sampling provided in Agosti *et al.* (2000a).

The ALL Protocol is used to estimate the abundance and composition of ants inhabiting a volume of leaf litter. Whole colonies of ants nesting in the litter as well as ants foraging in the litter from colonies outside the litter sample are collected. This method is appropriate for rapid assessment because it samples a high percentage of the leaf litter ant fauna in a short time.

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The ALL Protocol has been used by a wide range of ant experts and biodiversity practitioners (see Agosti *et al.* 2000b) and has been taught in several biodiversity assessment courses. It is the basis for several long-term surveys and monitoring of biodiversity in Madagascar and other sites (www.antweb.org, Fisher and Robertson 2002), by the Rapid Assessment Program (RAP) at Conservation International (Alonso *et al.* 2011), and in Guyana (Helms, Branstetter, and Alonso unpublished). Longino and collaborators have used a modified version of the ALL Protocol to study ants across Central America (<https://sites.google.com/site/longinollama/home>). Many recent studies have tested the efficacy of the ALL protocol in a variety of habitats and have found it to be an efficient and successful method for sampling the leaf litter ant fauna. A few examples include studies in montane rainforest in Ecuador (Delsinne and Arias-Penna 2012), deciduous dry forest in Brazil (Silvestre *et al.* 2012), subtropical mesoxerophile oligarchic forest in Argentina (Leponce *et al.* 2004), Borneo rainforests (Pfeiffer and Mezger 2012), Brazilian cerrado (Lopes and Vasconcelos 2008), and Papua New Guinea rainforests (Lucky *et al.* 2011).

Access to over 450 articles that cite the ALL protocol is available at Google Scholar (https://scholar.google.ch/scholar?cites=746641997506351099&as_sdt=2005&scioldt=0,5&hl=e). The ALL protocol has been translated into Spanish (<http://dx.doi.org/10.5281/zenodo.11738>) and Farsi (<http://dx.doi.org/10.5281/zenodo.16183>).

Core Methods

Overview of the ALL Protocol

The ALL Protocol starts with a minimal configuration, utilizing two ant collecting methods that have been proven to sample the largest component of the ground and leaf litter inhabiting ant fauna: the mini-Winkler extractor (Fisher 1999) and pitfall traps. The mini-Winkler extractor is highly effective in forest habitats while pitfalls are especially suitable for open areas. This combination of methods allows the standard protocol to be applied in a wide range of habitats, from forest to open grasslands (Silva *et al.* 2013).

The ALL Protocol is rapid; sampling can be completed in a total of three days per site if desired. The sample size, 20 one square meter (1 m²) samples of leaf litter and 20 pitfall traps have been found to be sufficient to sample up to 70% of the leaf litter, and up to 50% of the complete local ant fauna in a habitat (Leponce *et al.* 2004). Depending on the study objectives, other complementary methods can be added to the standard protocol in order to sample a wider range of ant species. Pitfall trapping involves placement of open containers in the ground. Surface-active animals fall unwittingly into these traps as they walk along the surface. In the mini-Winkler extraction method, a quantity of moist leaf litter is collected, usually all the litter and humus present under a 1 x 1 m quadrat, and placed in an extraction apparatus. The apparatus compels mobile ants, through disturbance to the litter or through changes in microclimate, to migrate from the litter into a collecting receptacle.

Sampling design:

Basic set-up

200 m transect (at least one)

Covered area to hang mini-Winklers

3 day time period (one day to collect samples, 48 hours for mini-Winkler extraction and pitfalls)

1–2 people (2 people recommended)

Methods employed at each sampling point

Standardized, Repeatable Techniques

Collect leaf litter within 1 square meter

(Optional: measure volume or wet weight of leaf-litter after sifting)

Sift litter

Extract ants from litter using mini-Winkler sacks

Place 1 pitfall trap

Optional Techniques to collect more species

Inspect dead wood

Scrape soil (15 x 15 cm area at 1 cm layers down to 10 cm)

Direct collecting by hand

Baiting

Placement of the sampling design: The choice of placement of the sampling transect should be determined based on the research objectives. For example, a transect may be placed randomly if an objective overview of ant diversity in the habitat is desired, or the transect can be positioned so that it transverses several microhabitats within the sampling area, thus collecting ants from a variety of habitat types. Alternatively, the transect may be placed in the same areas where mammal or reptile surveys have been done in order to make some comparisons between taxa. Furthermore, sampling need not be limited to only one transect per site. Several transects can be utilized at each site, often at different elevations. Additional samples may also be added to a transect but data should be made available so that analyses of a 20 sample transect are possible in order for comparisons between studies and sites to be made.

How often to sample: For rapid inventory, a transect is usually sampled only once, but several transects may be run either simultaneously or consecutively at a site. Analytical tools can be used throughout the study to determine the ultimate sample size needed to collect a high proportion of the leaf litter ant species in an area. For more extensive surveys, it is recommended that more than one transect be run and the species accumulation curve plotted by sample and transect if time permits. This approach evaluates the proportion of the estimated ant fauna that has been sampled and will help determine if additional transects are needed.

Time and effort: A minimum of three days is needed to carry out the standard ALL Protocol at a site. Leaf litter collections should be run through the mini-Winkler extractor (sack) for a 48-hour period. Pitfalls should also be left out for 48 hours. The number of mini-Winkler sacks will usually be the limiting factor to the efficiency of this sampling method. The ALL Protocol requires taking 20 leaf litter samples. This implies that 20 mini-Winkler sacks are needed to process all the samples at the same time. If 20 mini-Winkler sacks are available and can be run at the same time, then all samples can be processed in just over 48 hours. If less than 20 mini-Winkler sacks are available, samples may be extracted one after the other. This will prolong the sampling process, since for every set of mini-Winkler sacks used, 48 hours is needed for litter extraction. In areas of deep leaf litter, more than one mini-Winkler sack may be needed to hold the leaf litter sifted from a square meter; thus additional mini-Winkler sacks are recommended.

Leaf litter samples should be collected at the same general time period for each transect. Since this activity will take approximately three hours for two people, this should be done either in the morning (8-11 am), at midday (11 am-2 pm), or in the afternoon (1-4 pm). Leaf litter should not be sifted during heavy rains but instead at least four hours after rain has stopped. Pitfall traps should also be put in the ground at the same time for each transect. Pitfall traps and mini-Winkler samples should be collected 48 hours after they have been set up.

Personnel needed: It is recommended that two people carry out the protocol together, to provide assistance with leaf litter gathering, sifting, and other tasks. However, it is possible to carry out the protocol with a single individual. We estimate that the total time needed to sample, process, and identify ant specimens from one transect is 161 hours for a single professional.

The field sampling protocol is straightforward and does not require advanced skill. Identification of the ant species once collected takes a great deal of skill and training. However, sorting of ant specimens to morphospecies can be learned fairly quickly. Species identifications can then be done in collaboration with specialists or by using pictorial keys that are rapidly becoming available.

A team of two people works best. To start, both people can mark the transect with one holding the measuring tape and the other marking the 10 m intervals. A range finder (optical or laser) can also be used to set the transect. One person sets the pitfall trap while the other marks out the 1 m² plot for leaf litter collection. One person collects the leaf litter while the other sifts the litter. Setting up the mini-Winkler sacks in the laboratory or tent is also more efficient and quick with two people.

Materials Needed

Setting the transect

20-50 m measuring tape or range finder, 20 flags, flagging, permanent marker, compass.

Pitfall traps

25-30 plastic cups of uniform size and with smooth inside walls, pitfall trap scoop, hand trowel or shovel, Propylene glycol, water, dish detergent (liquid soap), and a tea strainer or muslin cloth, additional cups for setting and collecting traps, 50+ vials, 95% ethanol, permanent marker. Any plastic drinking cup with smooth sides can be used, but it is best to use cups with openings of the same diameter consistently to standardize samples.

Mini-Winkler extraction

Requires a litter sifter, 20 mini-Winkler sacks (some sources include pires@maxnet.com.br; www.santetraps.com), a quadrat, a ground cloth, 20 large cloth sample bags, 1+ meter measuring tape, 80 flags or flagging, 20 plastic cups, whirlpack bags, 100+ vials, 95% ethanol, leather work gloves, machete, permanent marker.

General hand collecting and soil scraping

2-3 soft forceps, 100+ vials, 95% ethanol, aspirator, machete, hand trowel, white tray or ground cloth, fine permanent marker.

Baits

Cardboard with crumbly cookies or Falcon tubes with a mixture of honey and water (1:1) and sardines in edible vegetable oil placed on the surface of the leaf litter. 6 repetitions of the two types, exposed at least one hour; 2- 3 soft forceps, vials, 95% ethanol.

Sorting and identification of specimens

2-3 petri dishes, 95% ethanol, vials, 2 #5 fine forceps, #3 entomological

TABLE 1: Recommended Time Table

Field Work

DAY ONE

Early morning:	One person	Two people
1. Mark the transect	1.5 h	1.0 h
2. Dig in the pitfall traps	1.5 h	1.0 h
3. Collect the 1 m ² leaf litter samples	5.0 h	3.0 h
Afternoon		
1. Fill in the mini-Winkler sacks	3.0 h	2.0 h
Later afternoon / Early evening		
1. Direct Collecting at night	1.0 h	1.0 h
Total	12 h	8.0 h

DAY THREE

Morning

1. Collect one log	1.0 h	1.0 h
2. Direct collecting	1.0 h	1.0 h
3. Scrape soil	1.0 h	1.0 h

Afternoon

1. Analyze soil samples	2.0 h	1.0 h
2. Collect pitfall traps	2.0 h	1.5 h
3. Collect ant samples from the mini-Winkler sacks	2.0 h	1.5 h
4. Check all labeling	0.5 h	0.5 h

Total	9.5 h	7.5 h
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Lab work, identification and analyses

Mounting, labeling and identifying ant specimens from mini-Winkler samples	60 h
Mounting, labeling and identifying ant specimens from pitfall traps	60 h
Mounting, labeling and identifying ant specimens from other samples	10 h
Entering and analyzing data	10 h

Total	140 h
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Field Methods

How to implement the method in the field

I. Setting the transect: Using a measuring tape or range finder, establish a 200 meter transect in a straight line with sampling stations marked at every 10 meters with flags or flagging.

II. Sampling stations

At each of the 20 sampling stations, two methods are conducted:

A. Leaf litter collection and sifting:

1. With the measuring tape, measure a 1 m² quadrat about 1 m from the transect line. Mark the corners of the quadrat with flags or with a flagging tied to a stick placed in the ground.
2. One person holds the sifter, which consists of an open-ended sack with a metal ring and attached handle at the top end, a mesh screen handle located about one-third the length of the sack from the top, and a bottom end that can be tied shut (see Bestelmeyer *et al.* 2000). Prior to filling the sifter, its bottom end should be tied shut so the sack does not open during the sifting process.
3. The second person should collect litter from the quadrat. The litter should be scooped from the edge of the quadrat toward the center and placed by hand into the sifter. Gloves can be used to prevent stings and bites. The litter should be removed from the top of the litter pile to the bottom and put quickly into the sifter. Twigs and clods should be broken open, decayed logs minced with a machete to expose and disturb ant nests within them. Do not collect the underlying mineral soil but do collect all leaf litter and the humus (decaying litter) layer.
4. Place the litter into the sifter and shake the sifter to separate the detritus and coarser material from the small invertebrates in the litter. To standardize your samples, it is best to time each sifting event- 20-30 seconds is likely enough time for each sift. The sifter should be shaken thoroughly both laterally and vertically. The litter in the upper section should be turned over several times in the process. When the litter is very dry, it should be shaken briefly because most of the animals will fall through the mesh quickly and extended shaking will only add more debris to the sample. When the litter is wet, it should be shaken longer so that ants that are stuck to wet leaves may fall through.
5. Remove the large excess litter from the top of the sifter and add more litter from the quadrat to be sifted. This process may need to be repeated a number of times for a 1 m² sample. After the sample has been sifted, the top of the sifter bag should be twisted (twice) shut to ensure that animals do not escape through the top.
6. When the entire 1 m² quadrat has been sifted, transfer the sifted litter from the sifter to a sample bag, which should be large enough to hold a single litter sample. Pour the contents of the sifter bag into the sample bag by opening the tie at the bottom of the sifter. Write the sample number on two labels; Place one inside the bag with the sample and attach one to the outside of the bag (may be written on flagging). The bag should be porous (to avoid suffocation of the ants) and synthetic (e.g. nylon) to prevent rot.

7. Return the excess litter from the quadrat back to its original place.
8. Keep all bags in a cool, shady place while completing the field work. Take the litter samples back to the camp or laboratory for extraction in the mini-Winkler sacks (see below). Extraction must be started the same day to avoid the death of ants in the bags.

B. Pitfall traps

1. Pitfall traps should be placed 1 meter from the transect line on the opposite side from where the leaf litter samples were taken. Traps should be placed so as to minimize the disturbance of the surface around the trap because surface texture conditions may affect ant capture rates.
2. A hand trowel that is only slightly larger than the trap should be used to dig a hole into which the plastic cup is placed.
3. The traps should be placed with the lip of the trap flush with, or just below, the soil or leaf litter surface. Soil or leaf litter should completely cover the lip of the trap.
4. When setting the trap, putting two cups in (one inside the other) is useful to catch and remove soil and litter that falls into the trap while it is placed. Once placed, remove the inside cup. This will allow for a cleaner pitfall and make for faster sorting.
5. Surface features should be returned to normal by hand once the trap is set. When possible, traps should be allowed to settle for about a week (with a lid covering the surface) before they are opened, in order to avoid the “digging in effect” that can lead to abnormally high ant capture rates due to disturbance of nest galleries in the course of setting the trap. For the purposes of a rapid survey, settling time may not be available and the possibility of this effect should be noted.
6. The killing agent is placed inside the cup after it is set and should fill about 25% of the cup’s volume. Several types of killing agents can be used. In areas of high desiccation such as open grasslands, a 70/30 mixture of 50-70% ethanol and propylene glycol (an “environmentally friendly” anti-freeze that is used in automobiles but not toxic to vertebrates) is an ideal choice because it combines a preservative (ethanol) with a liquid that is slow to evaporate (propylene glycol). Ethylene glycol (regular anti-freeze) can also be used in the place of propylene glycol but it is toxic to vertebrates (which might drink out of the cup). In forested areas, ethanol or water may be used in the pitfall traps. In some cases, water may degrade specimens of larger ants and ethanol may repel ants if the scent is strong. In all pitfall fluids, a drop of unscented detergent is recommended to break the surface tension of the liquid and prevent the ants’ escape. The detergent should not have a strong scent so that it does not attract or repel ant species.
7. If rain is likely to flood the trap, a cover (such as a large leaf or a flat piece of wood) can be suspended above it (about 3 cm), but should not be larger than the circumference of the opening to avoid changes in microclimate. Traps placed in depressions or drainages may also flood.

8. For the purposes of the ALL Protocol, the traps should be left open for 48 hours. This time should allow for an adequate sampling of ants foraging around the trap and provide a measure of forager abundance.
9. When the traps are collected, the liquid can be drained through a tea strainer into another to catch the invertebrates but remove excess liquid. The contents can be rinsed and transferred into a vial filled with 70-90% ethanol. Alternatively, the ants can be removed from the strainer or cup using forceps and placed in a labeled vial of ethanol. Collect other invertebrates and place them in a separate labeled vial.
10. Take care to look for very small ants that often stick to leaves and mud in the cups. These are often the most important ants to find so be careful not to miss any ants, many of which are almost microscopic to the naked eye. If you feel that you cannot distinguish ants in the cup, then collect the entire contents of the cup and sort it later using a microscope.
11. When done, fill in the hole with soil and cover the area with leaf litter so that it looks like it did before you dug the hole.

III. Additional methods

During the 48 hour period while the mini-Winkler sacks and pitfall traps are doing their work, it is a good idea to do some general hand collecting in the area near the sampling transect in order to collect a greater number of ant species. General collecting is not standardized, so should not be part of a monitoring program, but it is a valuable addition to an inventory. General hand collecting includes inspecting rotting logs, branches and twigs on the ground, scraping soil, and visually searching for ants. Ants can be collected with forceps or an entomological aspirator, and placed directly in vials containing 95% ethanol. When doing general collecting, be sure to record as much data as possible about where the specimens were collected, particularly distinguishing between ground and vegetation collections. The standardized protocol restricts sampling to ant species that live or forage in the leaf litter or on the ground. General collecting can add additional ant species from the vegetation.

Baiting ants is another additional method that attracts ants depending on the type of bait used. Sugar cookies (especially pecan sandies) or cotton balls soaked with sugar water, canned tuna, or dead insects are often used to attract sugar, oil and protein loving ant species. Baits may be placed on a small piece of cardstock to better view the ants at the bait, in Falcon tubes that easily can be picked up, or directly on the ground/tree/rock etc. Many ant species will recruit additional ants from their colony to baits which allows collection of multiple specimens from the same colony and often the collection of additional castes (e.g. soldiers) and sizes of workers.

IV. Extraction of ants from the leaf litter using mini-winkler sacks

1. The mini-Winkler sacks consists of a metal box frame that supports a covering made of canvas or cotton (see Besterlmeyer *et al.* 2000). Litter from each sample bag is separated into one 0.4-mm mesh bag that is suspended inside the mini-Winkler sack. Ants in the litter migrate out of the mesh bags and are collected in receptacle tied to the bottom of the mini-Winkler sack. The mesh bags should have stitches in their centers that maintain a flattened shape to the bag, which accelerates the migration of ants from the litter. The receptacle may be a twirl bag or a cup partially filled with ethanol solution.
2. The first step in using a mini-Winkler sack is to find a protected site where it can be mounted. A sack can be suspended from a nail in a wall, a beam in a shed, a pole under a tarp in the field, or from a tree branch in sites where rain is unlikely. It is important to find a location where the sack will not be tossed about by the wind or bumped by passers by, since any vibration or shock causes additional debris to fall into the receptacle. In preparation for loading the mesh bags, attach a dry receptacle (such as an empty plastic cup) to catch falling debris. Label mini-Winkler sacks according to the sample it is to receive.
3. The next step is to distribute the contents of the leaf litter sample bags into one or more mesh bags. Prior to filling the mesh bags, place a large, white, plastic cloth on the ground, prepare the mesh bags, and have a vial or two on hand in which to place escaping ants. One person should hold open the mesh bag while the other person slowly pours the sifted leaf litter in to the bag. Hold the mesh bag over the cloth so that escaping animals can be seen and collected. As each mesh bag is filled, occasionally and gently shake the bag to settle the material. Air spaces in the litter may hinder migration from the bag. Because ants crawl to the top of the litter column before falling out, it is most effective to fill each mesh bag as completely as possible. Ensure that the mesh bag is kept flat by the stitching.
4. After each mesh bag is filled with sifted leaf litter, hang it inside a mini-Winkler sack.. This should be done as quickly as possible. Each mini-Winkler sack holds one mesh bag. Maxi-Winkler sacks are larger and can accommodate up to four mesh bags. In areas of deep leaf litter, more than one mesh bag may be needed to hold the leaf litter sifted from a square meter. In these cases, additional mesh bags should be filled and either be hung individually inside several mini-Winkler sacks or hung inside one Maxi-Winkler sack. The mesh bags should not touch the walls of the mini-Winkler sack. Pour any leaf litter material that remains on the ground cloth into a cup and pour this into the mesh bag. Next, pour any material that has fallen into the collecting receptacle into the mesh bag.
5. Add about 1 inch of 95% ethanol solution to a plastic cup or whirlpack/twirl bag and attach it to the bottom of the mini-Winkler sack.
6. Finally, tie the top of the mini-Winkler sack closed to prevent animals from escaping.
7. The mini-Winkler sack should be allowed to hang undisturbed for 48 hours. Do not move or disturb the sacks or soil/litter will fall into the sample cups.

8. On conclusion of the 48 hour processing period, remove the collecting cup/bag from the bottom of each mini-Winkler sack and collect the contents with forceps. Put the ants into a labeled vial filled with 95% ethanol. Put other invertebrates into a separate labeled vial of 95% ethanol. It is current practice to use 95% ethanol to kill and store ant specimens so that genetic analyses may be done on the specimens if desired in the future.

V. Sorting samples in the laboratory

Samples from pitfall traps and mini-Winkler sacks can contain a lot of soil and debris. Ant specimens and other invertebrates can be separated from debris either manually (under a microscope) or by using the saltwater extraction method: Slowly heat water in a beaker, generously adding salt until the solution becomes saturated and no more salt will dissolve. The solution should be hot but not scolding, and never boiling. Empty the sample with specimens into a graduated cylinder no more than 4 cm in diameter and drain off the alcohol. Add the saline sample, cover and slowly turn the cylinder over. The organic material including ants, should float to the top, while inorganic material should sink to the bottom. Allow fifteen seconds for the contents to settle before quickly decanting the material over a straining apparatus and rinsing with alcohol. Using a microscope, the ant specimens can then be sorted from other organic material and other particulates that may not have been separated in the saline solution.

VI. Specimen preparation and conservation

The ants collected in biodiversity studies are valuable to taxonomists and local researchers so they should be handled with care. A reference collection of the ant species collected at the site should be established at the local field station, university, or research institution. If possible, a few representatives of each ant species should be pinned and housed in a cool, dry collection case, imaged and the digital record made globally accessible. A good alternative is to submit a reference image collection to <http://antweb.org>. The pinned specimens will serve as a reference for future ant identifications. The remaining ant specimens can be stored in vials of alcohol.

Ant specimens should also be sent to those ant taxonomists who are working on particular groups of ants, regardless of whether their taxonomic assistance is needed. These specimens may be valuable to a taxonomic revision by providing needed material on poorly known species or additional data on geographic distributions. Additional specimens should be deposited in major ant collections. Depositing ant specimens in national collections allows other researchers to examine them for taxonomic comparisons. Specimens of additional invertebrates (and occasionally amphibians, reptiles, and small mammals) that are collected in the pitfall traps or leaf litter samples should also be preserved and given to specialists working on those taxa. See other methods in this book for preservation methods for these taxa.

VII. Species identification

Level of expertise required: Perhaps the most difficult part of incorporating ants into biodiversity programs is the identification process. Few people in the world are able to identify ants to species level, largely due to the lack of training and the poor state of ant taxonomy in tropical regions. However, it is not impossible; identification to genus and morphospecies can be done by most people after a little instruction and a lot of practice.

Identification to genus or species group level is now very much improved through access to images of a large percentage of all ant species, and all ant genera (<http://antweb.org>). Furthermore, this is improved by an increasing number of local lists of ants that provide a viable start. Images in many cases allow one to compare specimens and determine if a particular species is already known.

An additional advantage is the availability of the entire taxonomic ant literature online through several websites. The most complete and helpful is <http://antbase.org> which together with the Hymenoptera Name Server provides the entire taxonomy and synonym of ants as well as a link from a particular name to the respective page in the cited publication. <http://antcat.org> is also a catalogue with no links to the species but there are more literature citations including non-taxonomic aspects. <http://Plazi.org> provides access to taxonomic treatments of ants, with increasing number of links to cited sources such as type specimens, other species, and most importantly a search function that allow searching over the entire corpus of treatment, both as full text and database search. This provides on the fly lists of taxa for a given region, by a certain author. Though far from being complete, this site is growing rapidly. Two others, <http://species-id.net> and <http://antwiki.org> are wiki sites that provide access to species information and imagery and can be edited by the user.

All of these efforts make incorporating ants into biodiversity conservation so much more efficient – but they depend on the users to add content and to point out errors, missing elements, or to provide guidance on where further developments should go.

Context Dependent Sampling Considerations

The ALL Protocol requires access to a site for at least three days and should be used when ant activity is highest, e.g. not during height of rainy or dry seasons. Ant species composition does not change seasonally since they are perennial organisms, but their activity and use of the habitat can change. Ant activity usually declines during heavy rains and in extremely dry conditions. Ant colonies may also move vertically in the soil according to moisture levels. This method should not be used alone to conduct a full inventory of ant species but in conjunction with other methods.

This method is unlikely to collect all ant species in an area. The number of individuals collected can give you an indication of the abundance of ants but does not give you a measure of relative ant abundance between species. See data analysis section for frequency measures.

The ALL method is easy to implement in the field but ants, like other insects, are not easy to identify once collected. This method assumes that the researchers will be able to identify ant species or collaborate with specialists to do so. The method is biased toward ants that move around in the litter so that they fall into the pitfalls or are collected in the leaf litter samples.

The ALL Protocol does not work as efficiently in heavy rains since the ants are less active and tend to stick to the leaf litter. Therefore, sampling should not be done during heavy rains. Sampling during the rainy season is possible as long as sifting is done during breaks in the rain, at least four hours after the rain has stopped. Some moisture is preferable so that the ants are active and the litter is moist so very dry seasons should also be avoided. Sampling at the start of the dry season or in light rainy season is best, or during breaks in rainfall during the rainy season. Pitfall traps need to be covered during rains so that the cups do not get filled with water and mud and the specimens washed out.

The ALL Protocol requires 20 leaf litter samples and pitfall traps to run for a 48 hour time period. Following this approach will allow researchers to compare their data to many other studies conducted using the same method. However, if direct comparisons are not desired, there are ways to enhance the ALL Protocol in order to collect more species and individuals from the samples. For example, the mini-Winkler sacks and pitfall traps can be left running for longer than 48 hours, but this should be weighed against the advantages of running additional transects instead. Leaf litter from Brazilian Atlantic rainforest that was allowed to process for one day collected about 90% of the species and 70% of the individuals that could be extracted from the sample, and in two days about 95% of the species and 85% of the individuals were collected (Delabie and do Nascimento, unpublished data). The rate of extraction of ants from litter samples can also be increased by removing the litter to a polyethylene bag and shaking it once every 24 hours of processing. This “shuffling” of the leaf litter has been shown to enhance the efficiency of the mini-Winkler extractor (Guénard and Lucky 2011). When the litter is shaken gently and returned to the inlet sack, ants that have settled down in the center of the litter are again agitated and begin to move, and eventually fall out. After 4 days, Delabie and do Nascimento found that samples that were agitated once per day yielded 15% more species and 70% more individuals than unagitated samples. Guénard and Lucky (2011) obtained 10% more specimens but no additional species after shuffling and 84 hours of extraction. For comparative reasons, it is recommended to use the above suggested standard protocol, and only to deviate, if there are strong local reasons to alter the protocol.

A study in northern Argentina by Leponce *et al.* (2004) found that <45% of the local ant species were documented with one ALL transect but that two transects yielded 60% and three transects about 72%. Thus multiple transects are recommended per site. Leponce *et al.* (2004) also found 50% higher species richness when ALL transects were sampled during warmer weather, thus indicating that comparisons should be made under similar weather conditions or compared by rarefaction (number of species for a given number of occurrences (Colwell *et al.* 2012).

Alternatively, a Berlese or Tullgren funnel may be used for extracting ants from the leaf litter, or the litter samples may be sorted by hand. Extraction using Berlese or Tullgren funnels should take the same length of time as the mini-Winkler sacks and hand sorting should also be completed in 48 hours. However, these methods will not be directly comparable to the ALL Protocol.

Target Organisms and Habitats

The ALL Protocol samples ants that live and forage in the leaf litter and in the soil. The method does not sample ants that primarily inhabit vegetation and the canopy or live deep in the soil. This method best surveys ants that are active in the leaf litter and often samples small, cryptic ants that are not collected by general searching or by inexperienced collectors. It can also sample ants present in the leaf litter or soil that do not move much and would therefore go undetected by other methods. The method generally targets worker ants, but occasionally collects entire colonies.

The mini-Winkler extraction technique works best with leaf litter from forests and is not quite as effective in grasslands or areas without leaf litter. However, breaking up clumps of grass and herbs above the sifter helps to increase the efficiency. Pitfall traps work well in any area but must be covered if heavy rains occur. Together, these two methods form a solid basis for the ALL Protocol that can be employed in all types of habitats.

Data Management

Data collected using the ALL Protocol primarily consist of ant species richness (number of species) and species composition. Abundance and density estimates can be obtained by using the number of samples as the measure of frequency (see data analysis below). This technique can measure the abundance and composition of ants inhabiting a volume of leaf litter.

Data to record:

For each transect, you should record a minimal set of parameters, including: name of collector, date, transect number, sample number, collection type (pitfall, mini-Winkler, or general), locality including geographic coordinates, and habitat. See the attached datasheet.

It is of the utmost importance to label all samples adequately. Most of the labeling can be done prior to the commencement of field-work. Vials used for collecting ants by hand or from logs should be labeled as well. Basic data for each label include:

Location (Country: primary administrative division (e.g., state): City/site.)

Geographic Latitude, Longitude (and error of measurement), best measured with a GPS in the field or extracted from global maps such as GoogleEarth using a standard format such as WGS 84, and elevation.

Date collected

Collector

Sample number

Each sample should receive a unique collection number that is recorded in the field notebook. The sample code is the only means by which multiple specimens may be recognized as coming from the same sample. This code should reflect the site, transect, and collection method.

In addition to standard collection information, ecological data should be recorded. Greater detail is useful and could consider some or all of the following variable environmental and ecological conditions:

1. Habitat classification by vegetation type or dominant plant species, including slope, aspect and elevation.
2. Type of ant nests (in soil, between leaves, with mound, etc.).
3. Air, soil, and litter temperatures and relative humidity.
4. The percentage of ground cover of bare ground, litter, vegetation, rocks, logs, and other potential ant nest sites.
5. The depth of leaf litter or volume/weight of the sifted litter .
6. Vertical vegetation profiles (or foliage height profiles), measured as the number of touches of vegetation on a thin rod at different height intervals above the ground.
7. An estimated amount of overhead canopy cover (use a densiometer if possible).

The use and measurement of these variables will depend on the objectives and limitations of the study. This information can be especially useful in characterizing the ecological preferences of ant species.

Data Treatment and Interpretation

The ALL Protocol will produce the following data: richness, composition, relative abundance, and frequency of occurrence among litter samples. Mini-Winkler samples can also be used to measure ant species density (# species/m²) and pitfalls can be used to measure ant activity since they can be sensitive to weather conditions.

Data from both methods allow for the estimation of ant abundance and detection of individual species, some of which may be of particular interest since they may be endangered, threatened, endemic, invasive, or restricted to a specific habitat type or set of conditions.

Since ants live in colonies, the number of individual ants of a particular species collected on a transect is not a direct measure of the abundance of that species. This is because the number of individual ants per colony varies greatly between species and also because ant distributions are extremely clumped. You may just happen to put a pitfall trap right next to a colony that has thousands of ants, and you'll get hundreds of individual ants in your trap. However, there may only be one colony there. Instead of the individual ant, the reproductive unit for ants is the colony.

Therefore, the number of colonies is the best measure of abundance. However, these two methods (and most collecting methods) cannot distinguish the number of colonies. To estimate abundance, we use frequency of collection, or the number of samples (traps) that a species is collected. This is based on the assumption that 10 m is enough distance between samples to be sampling a different set of ant colonies. Therefore, in diversity analyses, the number of traps in which an ant species is found should be used as the measure of abundance instead of the number of individual ants collected. Worker abundance may also be of interest in ecosystem or macroecology studies, e.g. counts of workers per mini-Winkler sample (Longino *et al.* 2014).

Statistical analyses will depend on the research objectives and questions. See Longino (2000) for more details on the statistical methods. Some questions that could be asked and statistical analyses that can be used to address them include:

1. Estimate ant species richness based on the data using EstimateS (<http://viceroy.eeb.uconn.edu/estimates/>) and coverage-based rarefaction and extrapolation (Chao and Jost 2012).
2. Estimate ant diversity at a site. Several diversity measures are available including Shannon index (H'), alpha index (α) the Simpson index (D) and the Berger-Parker index (d). The Shannon index (H') is useful for calculating the effective number of species (Gotelli and Chao 2013).
3. Calculate the effective number of species (Jost 2006).
4. Compare whether one site or transect has higher ant diversity than another. Compare species accumulation curves at comparable coverage (Chao and Jost 2012).
5. Assess patterns of association among samples or sites. Comparisons can be made using indices of similarity such as Jaccard's index, indices of complementarity such as the Marczewski-Steinhaus distance measure, ordination, and classification procedures.

Collecting and identifying ants provides data that can then be used to address the goals of any biodiversity project. What is done with the data is perhaps the most important part of the entire study. Careful consideration should be given to which methods of data analysis will best address the questions of each particular study.

Once the list of species for an area has been made, target species of interest may be further studied or monitored. Some of these species may be indicators of closed canopy, therefore undisturbed forest, such as ants in the genus *Strumigenys*. Others such as generalist and invasive species can indicate that an area has been disturbed. For these types of analyses, specimens must be identified to species level. If just a total count of the number of ant species in an area is needed, perhaps to compare to other areas, then identification to the morphospecies level may be satisfactory.

Conservation Implications

The ALL Protocol includes two standard methods most commonly utilized by ant researchers and provides a standardized, repeatable protocol for sampling the leaf litter ant fauna. This allows for comparisons between studies and over time, thus lending itself well to long-term monitoring and conservation planning.

Ant conservation

Ants are similar to other taxa in that they face a range of threats to their survival from habitat loss and change, climate change, habitat fragmentation, etc. One of the major threats to ants is invasive ant species that out-compete native ants for food and other resources, or kill them directly, especially on islands or in degraded habitats (Lach and Hooper-Bui 2010).

Unfortunately, ants are not generally considered “charismatic” and are usually overlooked in conservation planning. Much of conservation actions are based on the assumption that other taxa, such as plants, birds, or mammals, can serve as surrogates for the conservation needs of ants. The lack of data on ant species distributions, particularly for tropical regions, also makes identifying rare and threatened species difficult. Thus it is important that more data are collected on ant diversity and distribution through the use of the ALL Protocol as a standardized method.

Several types of ants warrant special conservation attention. These include rare or endemic species that are often found on islands or on isolated mountain tops; species dependent on other ant species such as social parasites, slave-making ants, and specialized predators; species with mutualistic interactions with plants; species with major impacts on the ecosystem such as army ants and leaf-cutting ants; ant species in older, monotypic or species-poor clades; and ant “phenomena” such as supercolonies that may be over 1000 years old (Alonso 2010). Finally the home range of an ant colony is much smaller than for vertebrates which can reveal much finer grained areas of endemics.

The data currently available on ant species distributions indicate that the Neotropical, Indomalayan, Afrotropical and Australian bioregions have the highest ant generic diversity and endemism, and are thus important areas for ant conservation (Fisher 2010, see also <http://antmaps.org>). Islands should also be a key focus due to the immense impacts of invasive ant species on the ant fauna. Steps toward ant conservation should include compiling current data, incorporating ants into broader conservation efforts, identifying and monitoring threats to ants, and promoting education and awareness of ant conservation.

Ants as indicators

Ants as an overall taxon, as well as subsets of ants such as *Strumigenys* and other ants dependent upon closed-canopy forest can be used as indicators of disturbance or to monitor progress of restoration efforts. Likewise, the presence of invasive ant species typical of disturbed or open areas can also be good indicators of the level of disturbance. Development projects are often required to monitor invasive species that may be unintentionally introduced into their project area but most such programs focus solely on invasive plants. Invasive ant species should also be included in such monitoring and control programs since early detection and eradication is essential to preventing new introductions. See Kaspari and Majer (2000), Alonso (2000), Hoffman and Andersen (2003), Andersen (2010), Philpott *et al.* (2010) for further discussions of the use of ants as indicators.

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Odontomachus hastatus. Photo © Trond H. Larsen

Literature Cited

- Agosti, D. and Alonso, L. E. 2000. The ALL Protocol: a standard protocol for the collection of ground-dwelling ants In: *Ants, Standard Methods for Measuring and Monitoring Biodiversity*, D.
- Agosti, J. Majer, L. E. Alonso and T. R. Schultz (eds.). Washington, DC: Smithsonian Institution Press. Available online at http://antbase.org/databases/publications_files/publications_20330.htm.
- Agosti, D., J.D. Majer, L.E. Alonso, T. R. Schultz (eds.). 2000a. *Ants: Standard Methods for Measuring and Monitoring Biological Diversity*. Smithsonian Institution Press. Washington, D.C.
- Agosti, D., J.D. Majer, L.E. Alonso, and T.R. Schultz (eds.). 2000b. Sampling ground-dwelling ants: Case studies from the world's rain forests. *Curtin University School of Environmental Biology Bulletin*. No. 18. Perth, Australia. 75 pp.
- Alonso, L.E. 2000. Ants as indicators of diversity. In: *Ants, Standard Methods for Measuring and Monitoring Biodiversity*, D. Agosti, J. Majer, L. E. Alonso and T. R. Schultz (eds.). Washington, DC: Smithsonian Institution Press.
- Alonso, L.E. 2010. Ant Conservation: Current Status and a Call to Action. In: L. Lach, C.L. Parr, and K.L. Abbott (eds.). *Ant Ecology*, Oxford University Press, New York, USA.
- Alonso, L.E., and D. Agosti. 2000. Biodiversity studies, monitoring, and ants: An Overview. In: *Ants, Standard Methods for Measuring and Monitoring Biodiversity*, D. Agosti, J. Majer, L. E. Alonso and T. R. Schultz (eds.). Washington, DC: Smithsonian Institution Press.
- Alonso, L.E., J.L. Deichmann, S.A. McKenna, P. Naskrecki and S.J. Richards (eds.). 2011. Still Counting...Biodiversity Exploration for Conservation – The First 20 Years of the Rapid Assessment Program. Conservation International, Arlington, VA, USA, 316 pp.
- Bestelmeyer, B. T., Agosti, D., Alonso, L. E., Brandão, C. R. F., Brown Jr., W. L., Delabie, J. H. C., and Silvestre, R. 2000. Field techniques for the study of ground-dwelling ants: an overview, description, and evaluation In: *Ants, Standard Methods for Measuring and Monitoring Biodiversity*, D. Agosti, J. Majer, L. E. Alonso and T. R. Schultz (eds.). Washington, DC: Smithsonian Institution Press.
- Chao, A., and L. Jost. 2012. Coverage-based rarefaction and extrapolation: standardizing samples by completeness rather than size. *Ecology* 93:2533-2547.
- Colwell, R.K., A. Chao, N.J. Gotelli, S.Y. Lin, C.X. Mao, R.L. Chazdon, and J.T. Longino. 2012. Models and estimators linking individual-based and sample-based rarefaction, extrapolation and comparison of assemblages. *Journal of Plant Ecology* 5: 3-21.
- Delsinne T.D., and T.M. Arias-Penna. 2012. Influence of leaf litter moisture on the efficiency of the Winkler method for extracting ants. *Journal of Insect Science* 12:57. Available online at insectscience.org/12.57.
- Fisher, B.L. 1999. Improving inventory efficiency: A case study of leaf litter diversity in Madagascar. *Ecological Applications* 9:714-731.
- Fisher, B.L. 2010. Biogeography. In: L. Lach, C.L. Parr, and K.L. Abbott (eds.). *Ant Ecology*, Oxford University Press, New York, USA.
- Fisher, B.L. and H.G. Robertson. 2002. Comparison and origin of forest and grassland ant assemblages in the High Plateau of Madagascar (Hymenoptera: Formicidae). *Biotropica* 34(1): 155-167.
- Frouz, J. and V. Jilkova. 2008. The effect of ants on soil properties and processes (Hymenoptera: Formicidae). *Myrmecological News* 11: 191-199.

- Gotelli N.J., and A. Chao. 2013. Measuring and Estimating Species Richness, Species Diversity, and Biotic Similarity from Sampling Data. In: Levin S.A. (ed.) *Encyclopedia of Biodiversity*, second edition, Volume 5, pp. 195- 211. Waltham, MA: Academic Press.
- Guénard, B. and A. Lucky. 2011. Shuffling leaf litter samples produces more accurate and precise snapshots of terrestrial arthropod community composition. *Environmental Entomology* 40: 1523-1529.
- Jost, L. 2006. Entropy and diversity. *Oikos* 113:363-375.
- Kaspari, M., and J.D. Majer. 2000. Using Ants to Monitor Environmental Change. In: *Ants, Standard Methods for Measuring and Monitoring Biodiversity*, D. Agosti, J. Majer, L.E. Alonso and T.R. Schultz (eds.). Washington, DC: Smithsonian Institution Press.
- Lach, L., and L. M. Hooper-Bui. 2010. Consequences of ant invasions. In: L. Lach, C.L. Parr, and K.L. Abbott (eds.). *Ant Ecology*, Oxford University Press, New York, USA.
- Lach, C.L. Parr, and K.L. Abbott (eds.). 2010. *Ant Ecology*, Oxford University Press, New York, USA.
- Leponce, M., L. Theunis, J.H.C. Delabie, and Y. Roisin. 2004. Scale dependence of diversity measures in a leaf-litter ant assemblage. *Ecography* 27: 253-267.
- Longino, J.T. 2000. What to do with the data. In: *Ants, Standard Methods for Measuring and Monitoring Biodiversity*, D. Agosti, J. Majer, L. E. Alonso and T. R. Schultz (eds.). Washington, DC: Smithsonian Institution Press.
- Longino, J.T., M.G. Branstetter, and R.K. Colwell. 2014. How ants drop out: ant abundance on tropical mountains. *PLoS ONE* 9:e104030.
- Lopes, C.T., and H. L. Vasconcelos. 2008. Evaluation of Three Methods for Sampling Ground-Dwelling Ants in the Brazilian Cerrado. *Neotropical Entomology* 37(4): 399-405.
- Lucky, A., E. Sarnat, and L.E. Alonso. 2011. Ants of the Muller Range, Papua New Guinea. In: Richards, S.J. and Gamui, B.G. (eds.). *Rapid Biological Assessments of the Nakanai Mountains and the upper Strickland Basin: surveying the biodiversity of Papua New Guinea's sublime karst environments*. RAP Bulletin of Biological Assessment 60. Conservation International, Arlington, VA.
- Perfecto, I. 1991. Ants (Hymenoptera: Formicidae) as natural control agents of pests in irrigated maize in Nicaragua. *Journal of Economic Entomology* 84: 65-70.
- Pfeiffer M., and D. Mezger. 2012. Biodiversity Assessment in Incomplete Inventories: Leaf Litter Ant Communities in Several Types of Bornean Rain Forest. *PLoS ONE* 7(7): e40729. doi:10.1371/journal.pone.0040729.
- Philpott, S.M. and I. Armbrecht. 2006. Biodiversity in tropical agroforests and the ecological role of ants and ant diversity in predatory function. *Ecological Entomology* 32: 360-377.
- Philpott, S.M., I. Perfecto, I. Armbrecht, and C.L. Parr. 2010. Ant Diversity and Function in disturbed and changing habitats. In: L. Lach, C.L. Parr, and K.L. Abbott (eds.). *Ant Ecology*, Oxford University Press, New York, USA.
- Silva F.H, J.H. Delabie, G.B. dos Santos, E. Meurer, and M.I. Marques Ml. 2013. Mini-winkler extractor and pitfall trap as complementary methods to sample Formicidae. *Neotropical Entomology* 42(4): 351-8. doi: 10.1007/s13744-013-0131-7.
- Silvestre, R., M.F. Demetrio, and J.H.C. Delabie. 2012. Community Structure of Leaf-Litter Ants in a Neotropical Dry Forest: A Biogeographic Approach to Explain Betadiversity. *Psyche* Volume 2012, Article ID 306925, 15 pages. doi:10.1155/2012/306925
- Wagner, D., J.B. Jones, and D.M. Gordon. 2004. Development of harvester ant colonies alters soil chemistry. *Soil Biology and Biochemistry* 36: 794-804.



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