CORE STANDARDIZED METHODS

FOR RAPID BIOLOGICAL FIELD ASSESSMENT

EDITED BY TROND H. LARSEN

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INTRODUCTION

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Trond H. Larsen and Leonardo Viana

Conservation International, 2011 Crystal Drive, Suite 500, Arlington, VA 22202, USA

Reliable, standardized and replicable methodologies for quickly assessing key ecosystem values in the field are essential for conservation planning and decision-making at the local to regional scale at which most threats occur. Rapid biological assessments are a cost-effective solution to this problem, providing data in a timely manner to address a wide range of conservation needs, and in particular to establish a baseline that can be used to detect changes over time.

A great deal of high level methodological guidance exists, but most lack practical details. A few books describe relatively comprehensive sampling methods but do not focus on a core set of standardized methods, making it difficult to decide which protocols to adopt. Other publications are available with lengthy, detailed guidance on sampling individual taxa. We believe this is the first book that focuses exclusively on a concise, practical set of standardized protocols for a wide range of taxa. This is no simple task. Many scientists tend to employ their own individualized, often opportunistic, approaches for finding as many species as possible in a short time, sometimes honed through decades of personal experience. These contributions are invaluable, yet do not address many conservation requirements.

While not intended to replace these methods, the identification of a core, at-a-minimum set of standardized methods, including innovative and automated approaches where applicable, is of great importance for making the results of rapid surveys comparable and replicable across sites and over time. New technologies and automated equipment make rapid surveys increasingly more cost-effective and unbiased. These methods also move beyond presence-absence records to record relative or absolute abundance, which is crucial for assessing threat and monitoring change.

Typically, rapid assessments require at least one week per site. A critical and often unanswered question in baseline assessments is how to know when sampling effort is sufficient. We have addressed this question with representative species accumulation curves and analyses in each individual chapter. Regional differences in ecosystems, climates, and evolutionary histories also mean that methods for some taxa need to be tailored to particular geographies. The focus of this book is on tropical terrestrial and freshwater ecosystems worldwide, although most methods should be applicable in temperate zones as well.

It is not possible to sample all taxonomic groups during a rapid survey. In this book, we describe methods for major taxonomic groups (plants, vertebrates), as well as a select set of invertebrates that represent cost-effective indicator taxa and play important ecological roles. This book represents a consensus of multiple experts for each taxonomic group, including intensive peer review. We expect that a future edition of this book will include methods for marine taxa, various ecosystem services, as well as social assessments.

The adoption of standardized methods provides the following benefits:

1. Methods can be more easily replicated when the same site is sampled at a later date, which is especially important if different researchers are involved, making it possible to understand how biodiversity has changed over time

2. Biodiversity data from a particular site can be placed within a regional or global context because it can be easily compared with data from other sites where the same methods were employed

3. Sampling completeness can be estimated, which allows interpretation of how many species occur at a given site. Estimating sampling completeness relies on statistical approaches to determine the actual number of species occurring at a site based on standardized sampling effort

4. Standardized sampling provides population-level abundance data as opposed to other opportunistic sampling that can yield only presence-absence data; the former is much more powerful for understanding changes in biodiversity over time and for identifying rare species that may be more vulnerable to environmental change

5. Standardized methods allow even amateurs to be trained effectively in many cases, reducing the dependence on only a handful of experts globally. However, this book is still targeted towards professional biologists and is not intended to provide a sufficient level of detail for novices to apply in the field.

We do not include a separate chapter on analytical approaches or data management, as these are already well covered in other publications (e.g., Hill et al. 2006; Sutherland 2006; Eymann et al. 2010).

Conservation International's Rapid Assessment Program (RAP) is just one example of how rapid surveys can influence conservation. Since the first expedition in 1991, CI's RAP teams have conducted biodiversity surveys in more than 90 terrestrial, freshwater and marine environments, leading to the discovery of more than 1,500 species new to science and the protection of 21 million hectares (5.2 million acres) of land and seascapes. Other organizations and institutions have used similar rapid assessment approaches to achieve tremendous conservation outcomes. We hope this book will help to unite the broad range of institutions and researchers who continue to advance knowledge building through field assessments.

Utility of Standardized Sampling Methods for Mining and Energy

The extractive sector is required by government regulations and/or lender requirements such as the IFC Performance Standards to evaluate impacts and risks of their activities. A part of that compliance includes conducting baseline studies of areas to be developed to assess pre-development conditions. However the methodologies used often vary widely and lack minimal best practice standards. This greatly impacts the initial evaluation of a site leading to potential risks to companies that fail to adequately identify critical aspects of the ecosystems in which they work. This also precludes one of the primary purposes of the data which is to track sustainable development goals (e.g., assuring "no net loss" or a return of the area to predevelopment status following project closure). The need for reliable, standardized and replicable methodologies that can quickly assess the key critical components of an area for decision-making purposes is of paramount importance for the sector.

Field assessments of biodiversity provide essential information for decision-making at the local to regional scale for a wide variety of stakeholders, including communities, governments, conservation organizations, and companies. Mining and energy companies are especially in need of robust data from biological assessments as development activities sometimes occur in remote and/or ecologically sensitive areas (Cunningham *et al*. 2000; Carter 2008; World Bank 2012). Companies are responding by developing environmental management systems to assess and mitigate potential biodiversity impacts. Governments, project financiers, and civil society stakeholders are increasingly providing strong signs that they seek to avoid, mitigate and where appropriate, offset impacts to biodiversity in areas of conservation value.

Despite a growing body of high level guidance, to date there are no detailed protocols describing a core set of standardized methods for rapid field assessments of biodiversity for the extractive sector. The methodologies currently used by companies in baseline assessments often vary widely in their comprehensiveness and reproducibility. The selection of a particular methodology may depend on a field biologist's preferences and may include non-comparable methods for sampling their focal taxon. Articulating which methods to use for baseline assessment at the project level, when, where and how they are to be applied so that there is consistency and reproducibility across space and time supports better informed management decisions. These factors collectively point to the benefits for mining and energy companies to apply standardized sampling methods to respond to stakeholders and best understand the long-term impact of their operations on biodiversity.

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VASCULAR PLANTS (NON-EPIPHYTES)

VASCULAR PLANTS (NON-EPIPHYTES)

Abel Monteagudo[1,2], Tatiana E. Boza E.[3], Erickson G. Urquiaga F.[3], Patricia Alvarez-Loayza[4]

Introduction

Taxon Definition – Vascular Plants: plants with a vascular system of xylem and phloem, including trees, treelets, vines, shrubs, epiphytes and herbaceous vegetation (Bailey 2003). Due to distinctive sampling techniques, vascular epiphytes are primarily covered in a separate chapter of this handbook. Vegetation on earth is of critical importance. Plants comprise about 98% of the earth's biomass and they create our oxygen-rich atmosphere via the light reactions of photosynthesis. They are important primary producers, providing the basis for the food web and habitat for numerous and often highly specialized animal, fungi, bacteria, and lichen communities. There are numerous plant species of conservation concern, due to high endemism rates, habitat loss, etc. Many plant species can be used to characterize forest quality and are indicators of forest degradation due to anthropogenic impacts (Terborgh, *et al*., 2008). Trees, treelets, vines and shrubs with vascular tissue have permanent woody tissue (Bailey 2003). Herbaceous plants are short, have less woody tissue and are usually green (Bailey 2003).

Core Methods

We recommend the Modified Gentry Plot (MGP), which are 0.1 ha in size, as the core standardized approach for rapid biodiversity inventories of woody plants (trees, treelets, vines and shrubs) (Fig. 1). There are several alternative methodologies such as the establishment of 1 ha or 50 ha plots, but even with this increased sampling effort, none of them record all plant species at a site, and they are less suitable for rapid surveys (Condit 1996). MGP 0.1 ha plots provide fast and reliable data on the composition and relative diversity of the plant community from the inventoried area (Phillips & Miller 2002). As the core standardized methods for rapid surveys of herbaceous plots, we have further modified the MGP to add small square plots, each 1 m^2 and line point intercepts (Fig. 1) which are useful for study replication and comparisons, as well as to estimate diversity, coverage, frequency, abundance, mortality and biomass (Perovic. *et al*. 2008).

The square method is used to measure grassland productivity, determine mortality rate and perform an inventory by counting number of tillers, stems, number of species (richness). It can also be used to develop the grassland species distribution maps (Tapia, 1954).

[1] Jardín Botánico de Missouri, Oxapampa, Perú

[2] Universidad Nacional de San Antonio Abad del Cusco, Cusco, Perú

[3] Institute of Systematic Botany, University of Zurich, Zurich, Switzerland

[4] Center for Tropical Conservation, Duke University, Durham, North Carolina, USA

The Line Point Intercept is a rapid and accurate method to measure vegetation, soil and litter coverage, frequency and density of vegetation, as well as to to calculate canopy, basal area, composition and vegetation structure and to have a better representation of the surrounding environment (Herrick. *et al*. 2009; Elzinga, *et al*. 2001).

The MGP methodology is recommended for rapid surveys, especially in large areas with one or more ecological gradients, e.g., humidity, sun exposure, edaphic condition, altitude, etc. (Eymann *et al*. 2010). The plots are based on numbers of individuals to be sampled and can be modified for use with forests, savannas, clonal plants, epiphytes, floating aquatics, etc. (Foster *et al*. unpublished). Studies demonstrate high predictability of species richness in 0.1 ha samples along environmental gradients, as well as patterns of dominant families and life forms within the same life zones and phytogeographic regions (Gentry 1988, 1995). Gentry applied this methodology in 13 Holdridge vegetation zones, including tropical dry forest, warm temperate dry forest, tropical montane forest, etc. (Phillips & Miller 2002). 0.1 ha plots permit a high number of samples in a short time and allow researchers more time to identify important plant species than methods using larger plots. Additionally, the MGP, which is long and rectangular in shape (see Plot Establishment below), can be adapted to different topographies and conditions (e.g., montane forest, swamps, grasslands, cloud forest), unlike traditional square plots used for woody plants (Fig. 2).

One hectare plots, on average, record more species than 0.1 ha plots. However, individual 1 ha samples also require much more effort than individual 0.1 ha samples. Phillips's 0.1 ha inventories were substantially more efficient in terms of floristic data gathered per effort invested; the 0.1 ha protocol is about twice as efficient as the 1 ha protocol in shrub-rich forests and about three times as efficient in shrub-poor forests (Phillips *et al*. 2003). With an MGP the number of individuals and species, presence-absence, and area of survey are recorded, allowing estimates of abundance and density. The MGP approach is ideal for describing and comparing areas at the site to landscape level, and for informing management and conservation decisions.

Site Selection – Sampling site selection should be determined before going into the field by distinguishing the main ecosystem or habitat types of interest using Landsat images, topographic maps, LIDAR images, etc. MGP should then be set up in each habitat type (Fig. 1), although these can be modified as needed if habitat area or topography do not permit (Fig. 2). Each plot should be randomly located, to avoid the effect of "Majestic trees" (RAINFOR manual, see link below).

Plot Establishment – Our proposed 0.1 ha inventories represent modified versions of the Gentry method (Gentry 1982; Phillips & Miller 2002). For woody plants, in each site, ten 2 × 50 m subplots should be established, totaling 0.1 ha located within a 100 × 180 m sampling grid, which equals 1.8 ha of forest (Fig. 1). All subplots should be oriented in the same randomly chosen direction so as to minimize possible sampling biases (Phillips 2003; Fig. 1). Each plot should be described using maps, compass, direction and coordinates using a GPS device, with the goal that the same transect could be located or replicated by an independent person in the future (Foster *et al*. 1998). Further details on these protocols are available from additional sources (http://www.geog.leeds.ac.uk/projects/pbc/; Phillips *et al*. 2003a, 2003b).

To sample herbaceous plants, five square plots (1 x 1 m) should be set up adjacent to each of the 10 subplots of 5 x 20 m of the transect, totaling 50 m² (or 50 square plots) (Fig. 1). These plots are easy to set up and are ideal for grasslands, prairies, lowland and montane forests (Mostacedo, 2000).

MODIFIED GENTRY PLOT - MGP

Sampling System: 10 Subplots of 100 m²

MGP - Plot Base Line 180 m
Line point intercept 50 m
2 m
50 m
20 m

Subplots of 2 x 50 m.
Plot Base Line 180 m.
Line Point Intercept 50 m.
Square to herbaceous and grasses 1 m²
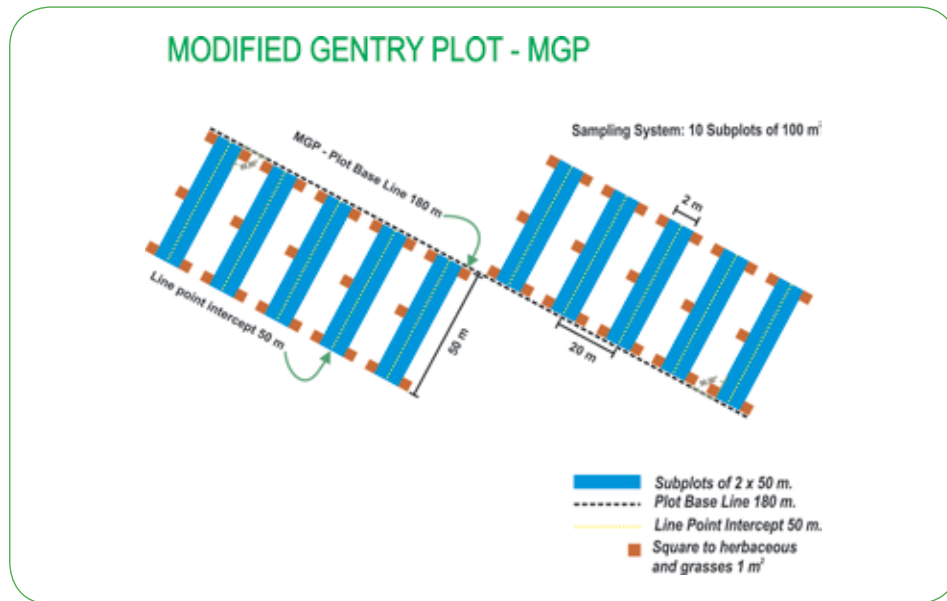
Figure 1
Modified Gentry Plot for a rapid inventory, modified from http://www.geog.leeds.ac.uk/projects/pbc/manual.pdf.
Shaded square plots represent additional sampling for herbaceous plants 1 x 1 m.

Other examples of modifications this method.

Rivers
Barriers
Slopes
Rocky massifs
Different plant community

Figure 2
Examples of MGP modifications at different topographies or habitats. Each straight dark blue line represents a 2 x 50 m subplot for woody plants located near different geographical characteristics such as rivers, slopes, and other habitats.

Data Recording – Woody plants: Trees, treelets, and shrubs

Each woody plant rooted within the subplot area and with diameter at breast height (DBH) of ≥2.5 cm at the Point of Measurement (POM) should be included (Fig. 3). Every individual plant should be identified or classified at least to the "morphospecies" level. Voucher specimens are collected for each unique species and whenever there is any uncertainty to its identification (see Voucher Collections below). Data is recorded using a tree, treelet and shrub data collection sheet printed on waterproof paper (see Appendix 1 as an example). When necessary, diameters will need to be measured 50 cm above buttresses and other stem irregularities (Fig. 3). Detailed information can be found at http://www.rainfor.org/upload/ManualsEnglish/RAINFOR_field_manual_versionNov2015_EN.pdf

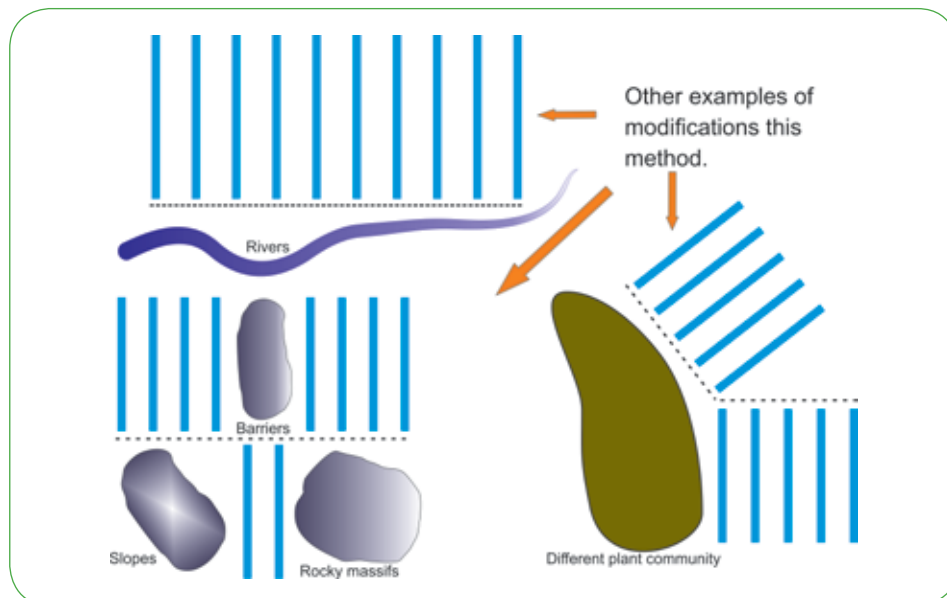In addition, a subset of trees within each MGP should be measured for tree height, in order to estimate carbon stocks and to establish plot level diameter/height relationships for accurate modeling. Three main methods are used to measure tree height: mechanical hypsometer, electronic hypsometer and laser. For this type of rapid inventory, we recommend a Suunto clinometer. Trees coded as leaning, rotten, broken, forked below 5 m, fallen or resprouted should be excluded from height measurements. We recommend selecting the following range of trees for height measurements when possible:

- 5 individuals, 2.5-10 cm DBH
- 5 individuals, 10-20 cm DBH
- 5 individuals, 20-30 cm DBH
- 5 individuals, 30-50 cm DBH
- 5 individuals greater than 50 cm DBH

Detailed information on height measurements can be found at http://www.rainfor.org/upload/ManualsEnglish/TreeHeight_english[1].pdf

Materials and supplies

Item	Qty
Clipboard	2
Suunto Clino Master Clinometer	2
Swedish Sectional Ladder	4
50 m measuring tape (e.g., Lufkin brand)	3
5 m metric fabric diameter tape	2
Refill for 5 m metric fabric diameter tape	6
Diameter tape Domaine "La Bruyere" (www.zimmersa.com)	6
Tree pruner with aluminum poles	2
Rite in Rain Copy Paper	1 box/site
Pocket Field Book (hard cover)	4
Compass (e.g., Suunto Sight Master)	6
Pink Flagging tape	10
Hand Pruner with case	6
Duct Tape	6
12" ruler	4
Cotton cord	2 rolls
Binoculars (10x12)	2
GPS (e.g., Garmin CSX 60)	1
Digital Camera	1
Hand Lens (magnifier)	2
Machete with sharpener	2
Belt Pack to carry material in the field	6
Newspaper to carry vouchers	30 kg
Alcohol to preserve vouchers	15 kg
Permanent black marker (e.g., Sharpie)	20
Pencil	20
Paper envelopes to store DNA samples	200
Ziplock bags (medium size) for DNA samples	200
Woven plastic sacks to carry specimens	10
Plastic bags (30 x 60 cm)	2 packs
Tablet (to carry the digital herbarium)	1
Laptop (to input data)	1
Silica Gel or Drierite	5 lb
Calipers (for herbaceous specimens)	2
Scale (100 gr)	2
Scale (10 gr)	2
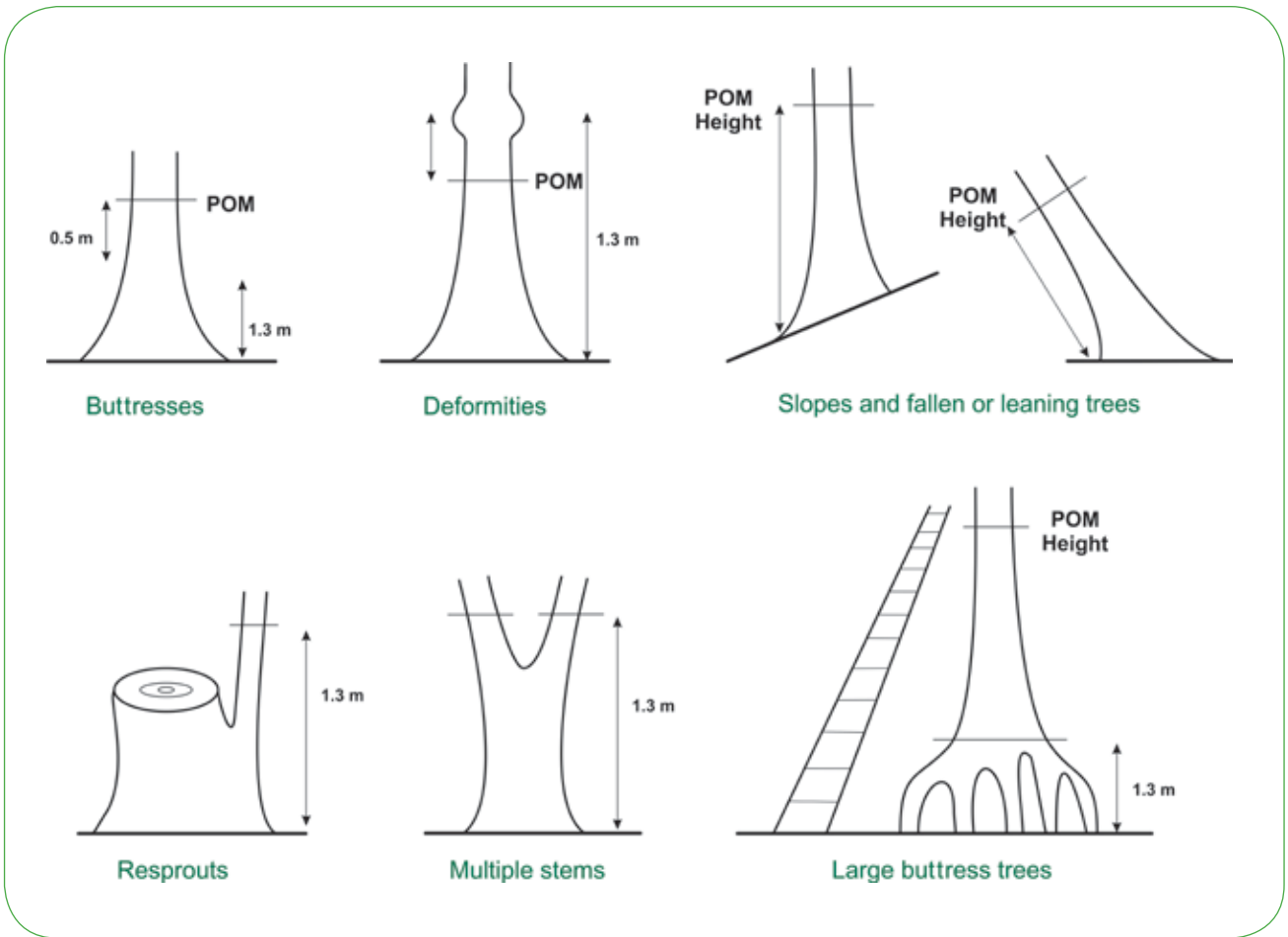
Figure 3
Examples of modifications for DBH measurements

Photo © Trond H. Larsen

Figure 4
Left: Vegetation clumps that need to be measured using interception lines; Right: Herbaceous vegetation measured using the square plots. (Photos: Project "The dynamics and carbon implications of fires in the Andes". Photos by Erickson Urquiaga)
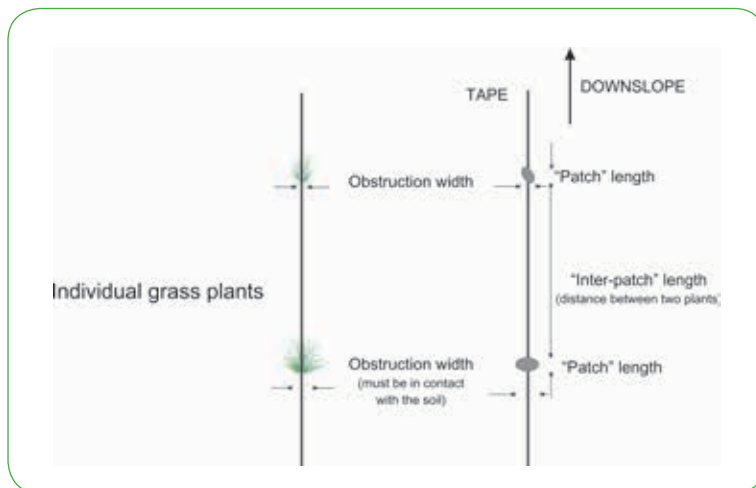
Figure 5
Interception line structure (Figure modified from EPES 2011)
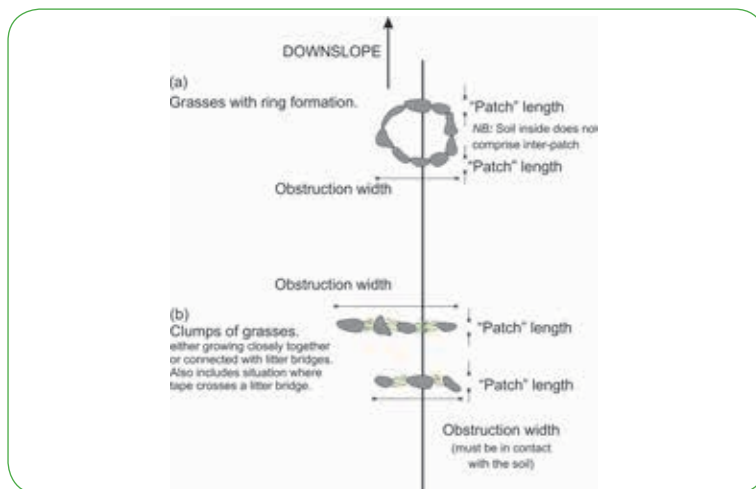
Figure 6
Interception line methodology examples (Figure modified from EPES 2011)

Herbaceous plants (terrestrial and climbers) – As with woody plants, each individual herbaceous plant within each 1 x 1 m subplot should be identified or classified at least to the "morphospecies" level, with voucher specimens collected for each unique species and whenever there is any uncertainty to its identification. Data is recorded using an herbaceous plant data collection sheet printed on waterproof paper (see Appendix 2a as an example). For grasses, the diameter of the grass cluster should be measured 5 cm above the ground. Grass vouchers should be collected that include the root system.

In some landscapes such as wetlands, grasslands and shrublands, "interception lines" should be established as a baseline for each subplot. The interception line (Canfield, 1941; Cuello, et al., 1991 Smith, 1980) estimates the area of herbaceous clumps, bare soils and rock outcrops (Fig. 4). The interception line should be established perpendicular to the baseline of the MGP. At each vegetation clump along the interception line, the distance from the line and the distance among clumps should be measured (Figs. 5, 6). Height and diameter (minimum and maximum) should be recorded for all clumps and rosettes (Appendix 2b).

Sampling Effort and People Required:
The typical sampling time required to complete one MGP at a single site in the field, including the herbaceous subplots, is about 5 days, but ranges from 3-5 depending on the habitat type and floristic diversity.

- 1 expert botanist capable of identifying at least 50%, to the genus level, of the flora in the area
- 1 additional plant collector (junior botanist or forester) to assist with sampling in general
- 1 local assistant or junior botanist to assist with plant measurements and to learn identification skills Optional:
- 1 tree climber to collect plant samples
- 1 person to record data and record GPS points (junior botanist or forester)

Voucher collection – We recommend collection of plant individuals and specimens following the Missouri Botanical Garden collection protocol (e.g., http://www.jbmperu.org/hoxa/cbotanica.pdf). This generally requires two weeks of work, with one botanist to identify the plants and one assistant to dry and organize the plants.

1. Selection of vouchers: Only fertile samples (branches with leaves and reproductive organs (flowers and fruits)), should be mounted for the herbarium. Non-fertile specimens can be collected for storage, but are not usually accepted in herbariums. In unique cases (e.g., rare plant species) sterile specimens should be collected for herbariums.

2. Number of vouchers: Three sets should be collected for each fertile species, preferably from the same individual. Only one set of sterile collections should generally be made, although repeated collections of sterile plants may be needed to reliably distinguish morphospecies. For DNA sequencing (national law permitting), collect two leaves from each individual, cut them with clean scissors (disinfected with alcohol or bleach), store them in paper envelopes, and put them inside ziplock bags with silica gel.

3. Collection types: Before storing the plant sample in the newspaper or envelope, record plant characteristics, e.g., size, shape of the branch, branching pattern, location of flowers, color, etc.

4. Fixation and preservation: Plant species should be preserved within 24 hours of collection with alcohol at 50%.

5. Drying and pressing: Plants can be prepared either with or without a stove, depending on availability in the field (see protocol specified by the Missouri Botanical Garden above).

6. Mounting: The dry specimen is placed on dry white paper and is fixed with special tape and thread and needle (see supplies above).

7. Labeling, data recording and cataloging: For label production and data storage, all data recorded in the field should be transferred to a digital database (e.g., Access).

Supplemental methods — In addition to the core methods described with the MGP approach above, it is useful to explore as much of the target area as possible to opportunistically collect species that appear to be rare, unknown to science, or of conservation importance. Fertile samples are a priority for opportunistic collecting.

Photographic records of each collection — Ideally, photographic records should be taken of all fertile and sterile samples (for each morphospecies) in the field. Photos should include an object (e.g., coins, pens, metric ruler) that illustrates the size of the plant sample. Additionally, vertical photos of tree trunks should be taken from a distance. Some tree species contain latex, which can be photographed after making an incision in the bark. All details of fruits and seeds should be photographed (various aspects, aril, and other characteristic details).

Soil samples — For each site, one soil sample (250 g) should be collected (0-15 cm below the organic layer) in each subplot (2 x 50 m), at a random point. To represent soil conditions at each site, all samples are then mixed together, since all tropical soils are variable at small spatial scales (Jetten *et al*. 1993 in Duivenvoorden 2001).

Supplies and Equipment

Fieldwork — All material for fieldwork is listed in Box 1 above.

Herbarium Collections — Newspaper, permanent markers, field notebooks, alcohol at 50% or 70% and polyethylene plastic bags. Flowers and fruits should be preserved in wide mouth plastic containers with tight lids in alcohol, with glycerin drops added to 50% alcohol under dry conditions. Once at the herbarium, specimens should be moved from plastic containers to glass containers with a commercial solution of formaldehyde at 10% and alcohol at 50%.

Samples to be dried require a botanical press with accessories, straps, nylon webbing, heavy white dryers, corrugated aluminum ventilators, and an electrical stove with resistance of 750 W. Specimen mounting requires mounting paper (none acidic, white, 43 x 32 cm, 300 g), adhesives, thread, and needle. Seeds and fruits are kept in coin envelopes. Mounted specimens should include a detailed data label (11 x 10 cm) with the institution name.

Biomass and Carbon Stocks – MGP plots can be used to calculate above-ground biomass and carbon stocks.

- **Trees:** The biomass and carbon stocks of trees are estimated using allometric equations applied to the tree measurements, typically DBH, height and tree density (Brown 1997, Parresol 1999, Chave 2005).

- **Palms:** If palms are present, only the height should be used since biomass for palms is related to height rather than to diameter.

- **Lianas:** Liana biomass is difficult to measure because they are often long and cross the plot in several places, and additionally often lack allometric biomass equations. Unless they form a significant component of the ecosystem, we do not recommend including them.

- **Non-Tree Vegetation:** Biomass of non-tree vegetation is measured by simple harvesting techniques. For herbaceous plants, all vegetation inside a square frame (50 cm x 50 cm) should be harvested and weighed in situ. The samples should then be dried in an oven of 60º C for at least 72 hours (using the same oven for drying herbarium plant samples). Once the dry weight has stabilized, the difference in weight provides the biomass. For shrubs and other large non-tree vegetation, larger frames should be used (about 1–2 m2, depending on the size of the vegetation). Detailed information can be found at:

  http://www.rainfor.org/en/manuals

  http://gem.tropicalforests.ox.ac.uk/files/rainfor-gemmanual.v3.0.pdf

Conservation Implications and Limitations

Plants form the foundation of the ecosystem and plant communities are often extremely diverse, including many endemic and endangered plant species (Leon, 2006, Leon-Yañez 2011). Many communities and species have not been surveyed or recorded. Density of endemic species usually peaks at mid-elevation (often 10–15 times higher from 2000–3500 m than in the lowlands, indicating the need for special conservation attention). Total numbers of endemic species often peak from 1500–3000 m for herbs, shrubs, and epiphytes, while endemic trees, vines, and lianas tend to be richest in the lowlands from 0–500 m.

Rapid surveys of plant communities are limited in that it is usually not possible to sample the majority of species or to survey a large area. Due to their high diversity, species identification usually requires skilled experts with local knowledge of the region. However the survey methods described here are ideal for maximizing the number of species recorded in a short period of time and in a standardized manner (Van Der Weff & Consiglio, 2004). With the precision of new GPS technology and equipment, it is now much easier to relocate and resurvey the same small vegetation plots in the future, increasing our understanding of how plant communities are changing and which factors may be driving these changes (e.g., anthropogenic disturbances, fire, etc.). For example, climate change and carbon dioxide availability are known to be altering overall forest biomass and changing species composition and community structure.

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Appendix 1: TREES, TREELETS, SHRUBS and PALMS
(Plot 100 x 180 m, subplot 2 x 50m)
(Plot No. _____ Subplot No. _____)
Trees, palms and tree-ferns with DAP > 2.5 cm and lianas with POM > 2.5 cm

LOCATION:		
LOCALITY:		
Lat. S	**Long. W**	**Elevation (m)**
Vegetation Type:		
Soil Description:		
Plant Description:		

Date:						
Tree No.	**Collection No.**	**Fam.**	**Scientific Name**	**Height (m)**	**DBH (cm)**	**Specimen condition (Phenology)**

Appendix 2a: LINE POINT INTERCEPT
Intercept Line No _____
Herbs

LOCATION:

LOCALITY:

Lat. S	Long. W	Elevation (m)

Vegetation Type:

Soil Description:

Plant Description:

Date:

Point No.	Distance from start	List Form	Fam.	Scientific name	Diam. 1 (cm)	Diam. 2 (cm)	Width (cm)	Height (cm)	Notes

Appendix 2b: SQUARE

Plot N°_____ Square N°_____

Herbs

Point N° is the place where the sample is taken; Diameter 1 and 2, perpendicular to each other up to the highest leaf; Width tiller diameter at soil level; Height at the tallest leaf from the soil; Cover percentage of vegetation at the determined area.

LOCATION:

LOCALITY:

Lat. S	Long. W	Elevation (m)

Vegetation Type:

Soil Description:

Plant Description:

Date:

Point No.	Life form	Fam.	Scientific name	Cover (%)	Diam. 1 (cm)	Diam. 2 (cm)	Width (cm)	Height (cm)	Notes

VASCULAR EPIPHYTES

VASCULAR EPIPHYTES

Thorsten Krömer[1] and S. Robbert Gradstein[2]

Introduction

Taxon Definition – Vascular epiphytes are plants that germinate and live upon another plant without parasitic-roots and at least for a part of their life cycle do not take nutrients from the soil. Over 27.600 species of plants, in 73 families and 913 genera are epiphytes accounting for about 9% of all plant species. The majority of vascular epiphytes are ferns and monocots - especially orchids, bromeliads and aroids - relatively few are dicots (e.g., ericads, gesneriads, *Peperomia*), and virtually none are gymnosperms.

Why include Vascular Epiphytes in rapid biodiversity assessment? Vascular epiphytes as a study group are particularly appropriate for rapid baseline surveys because they are relatively small (allowing for high species richness on fairly small plots), physiognomically distinctive (making them easy to survey), have high species numbers (allowing for quantitative analyses) and are comparatively easy to identify.

The high diversity of vascular epiphytes is one of the most striking characteristics of tropical rain forests and humid montane forests. These organisms are of major significance for a great number of reasons: 1) they contribute substantially to ecosystem diversity, production and nutrient cycles; 2) they provide appreciable nutrient and energy sources to associated organisms such as pollinating birds, bats and mutualistic ants; 3) they act as global indicators for climate change; 4) they are of major horticultural and, hence, of economic value; and 5) they create an arena for observational and experimental studies on a wide range of biological questions including diversity patterns, systematics, plant interactions, ecophysiology and mechanisms of evolutionary change.

There is growing recognition that vascular epiphytes are increasingly threatened. The main causes for epiphyte extirpation and population reduction are overcollecting of horticulturally valuable species for commercial purposes and habitat loss due to deforestation and land use changes. Because epiphytes, especially orchids, may occupy very narrow ranges and often occur in regions of rapid development, many tropical plant species listed as "endangered" by conservationists are epiphytes.

[1] Centro de Investigaciones Tropicales, Universidad Veracruzana Interior de la Ex-hacienda Lucas Martín, Privada de Araucarias s/n, Col. Periodistas, C.P. 91019 Xalapa, Veracruz, Mexico

Tel: + 52-228-8421700 ext. 12653
Fax: + 52-228-8108263
Email: tkromer@uv.mx

[2] Muséum National d'Histoire Naturelle Dept. Systématique et Evolution / Cryptogamie Case Postale 39, 57 rue Cuvier, 75231 Paris cedex 05, France
Tel: +33-(0)140793178
Email: sgradst@gwdg.de

Due to their life high up in the forest canopy and their strong dependence on atmospheric water and minerals, vascular epiphyte species are very sensitive indicators of environmental disturbance and climate change.

Core Methods

The method of the Rapid and Representative Analysis of Epiphyte diversity (RRED-analysis), recommended here, has been specifically designed and tested for standardized, rapid assessment of the biodiversity of vascular epiphytes.

Key Publications of method:

Gradstein, S.R., Nadkarni, N.M., Krömer, T., Holz, I. & N. Nöske. 2003. A protocol for rapid and representative sampling of vascular and non-vascular epiphyte diversity of tropical rain forests. Selbyana 24: 105-111.

Krömer, T. & S.R. Gradstein. 2003. Species richness of vascular epiphytes in two primary forests and fallows in the Bolivian Andes. Selbyana 24: 190-195.

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Target organisms: Vascular epiphytes

Target habitats: All forest types, but especially tropical rain forests and humid montane forests

Biodiversity data provided: Species lists, richness (alpha diversity), frequency and vertical distribution

Time and personnel needed: 8 working days are needed for a complete inventory, including tree climbing and processing of specimens of eight 20 x 20 m subplots by two persons (one specialist and one field assistant)

Skills required: Regional experience on epiphyte identification and tree climbing training, preferably the single rope technique

Equipment and costs:
Tree climbing equipment: ca. 1.000 US$

Standardized sampling protocol for rapid survey – Vascular epiphyte diversity in natural or secondary tropical forest is measured based on sampling of one hectare of homogeneous forest. Forest margins should be excluded. Eight mature canopy trees >10 cm DBH are sampled from the base to the outer portions of the crown using the single rope tree climbing technique, in order to provide a large sample of the upper canopy community from a variety of microhabitats. These methods will allow the collection of data and plant material from the entire tree without sawing branches.

Presence/absence of vascular epiphyte species is recorded in five vertical tree zones:

Zone 1. Basal part of trunk (0–2 m high);

Zone 2. Trunk up to the first ramification and excluding isolated branches originating on the trunk zone. Zone 2 is often subdivided into a humid lower part of the trunk (zone 2a) and a dryer upper part (zone 2b);

Zone 3. Basal part of the large branches, up to the second ramifications (about a third of total branch length);

Zone 4. Second third of branch length; and

Zone 5. Outer third of branch length.

Epiphyte diversity on shrubs and understory trees is additionally sampled in eight 20 x 20 m subplots (zone U), one around each sampled canopy tree, using collecting poles and binoculars.

The method is based on the observation, obtained by means of species-accumulation curves and diversity estimates in natural and secondary lowland and montane forests in different tropical regions and climate zones, that sampling of eight mature canopy trees and a 20 x 20 m subplot that follows the terrain around each tree yields a representative inventory (ca. 80% of the total estimated number) of vascular epiphyte species within one hectare of forest (Fig. 1).

RRED-analysis was developed and tested by the authors and their associates in Bolivian and Mexican rain forests and humid montane forests, in the framework of doctoral and postdoctoral research of the first author and in consultation with the Global Canopy Programme [www.globalcanopy.org] and the International Canopy Network [http://internationalcanopynetwork.org].

A standard method for rapid sampling of vascular epiphyte diversity has been lacking and the one presented has been newly and specifically developed for the purpose. This is necessary, because haphazard collecting only gives a rough impression of the species richness of a forest, but it does not provide robust data for comparing biodiversity of different habitats. Vascular epiphyte inventories based solely on observations from the ground are also incomplete and biased, as many small species growing in the canopy cannot be detected from the forest floor. Therefore, inventory of the canopy must be conducted with access from canopy climbing. Furthermore, vascular epiphytes on shrubs and small trees must be sampled/recorded additionally, because the epiphyte flora in the forest understory is usually different from that on the large canopy trees.

RRED-analysis focuses on species richness and frequency, but does allow for assessment of species abundance and biomass (for that see Wolf *et al*., 2009). Completeness of the sampling is influenced by the observer's knowledge of vascular epiphytes. Lack of knowledge of these plants may result in overlooking of species that are difficult to recognize for small size, lack of flowers or fruits, or other reasons. Therefore, the team carrying out RRED-analysis should contain at least one vascular epiphyte specialist.

RRED-analysis is the most recent and comprehensive standard method available, including suggestions of the following papers on epiphyte sampling published before:

Gradstein, S.R., Hietz, P., Lücking, R., Lücking, A., Sipman, H.J.M., Vester, H.F.M., Wolf, J.H.D. & E. Gardette. 1996. How to sample epiphytic diversity of tropical rain forests. Ecotropica 2: 59-72.

Nieder, J. & G. Zotz. 1998. Methods of analyzing the structure and dynamics of vascular epiphyte communities. Ecotropica 4: 33-39.

Shaw, J.D. & D.M. Bergstrom. 1997. A rapid assessment technique of vascular epiphyte diversity at forest and regional levels. Selbyana 18: 195-199.

Research Design

Sampling design:
Basic Set-up: Select eight mature canopy trees with a high epiphyte load, each surrounded by a 20 x 20 m subplot, within a 1.0 ha plot of homogeneous forest.

Placement of the sampling design: Trees in close vicinity of each other tend to have similar vascular epiphyte flora resulting from clumped distribution of many epiphyte species. Therefore, canopy trees standing well apart (separated by at least 25 m) and with crowns not overlapping should be selected. Trees at forest margins should be avoided because of edge effects. However, natural edges, as those along rivers, should be used, because the epiphyte diversity can peak or different species are found in riparian trees. In habitats that are not very rich in epiphyte species, the number of sampled trees and surrounding 20 x 20 m subplots can be reduced in accordance with the leveling-off of the species-accumulation curve. Tree-climbing might be dispensable in some dry forests, young secondary forests, scrub or mangrove, e.g., in many locations where species richness and canopies are low and when good binoculars are available.

Time and effort: About eight working days are needed by an experienced working group for a complete inventory, including tree climbing and processing of specimens of eight 20 x 20 m subplots.

Each canopy tree and 20 x 20 m subplot is sampled once. RRED-analysis is carried out during the daytime. RRED-analysis should not be attempted during heavy rain for safety reasons. However, if possible one or several visits are recommended in different seasons to detect or collect fertile plant material necessary for plant identification (e.g., bromeliads, orchids).

It is recommended that at least two people carry out this protocol. The vascular epiphyte specialist does the collecting of species data by tree climbing, the field assistant takes care of securing the climbing operation at ground level and the recording of the species data.

Field Methods
How to implement the protocol in the field

Basics Steps:
1. Selection of homogenous 1-ha forest plot.
2. Selection of eight canopy trees to be climbed by Single Rope Technique (SRT).
3. Carrying out of the inventory, usually by sampling of one tree and its surrounding 20 x 20 m subplot per day.

Sampling:
Species diversity of vascular epiphytes is scored by recording presence-absence of all species in each of the five vertical tree zones and in the understory subplots. Small trees and shrubs in the 20 x 20 m plot should be inspected during the climbing of the mature tree. To avoid damaging the vascular epiphyte populations within the sampled trees, all species encountered in the 1.0 ha plot (but not in every single tree or subplot) should be collected only once in triplicate.

Data to Record:
Species presence in each tree zone and in the understory plot is recorded on the data sheet (see attached). For any specimens collected, label each with collection number and provisional scientific name. Minimally, presence/absence of individual species must be recorded for each of the five tree zones and the understory plot.

To document the habitat of the epiphytes, characteristics of the host tree such as tree height, trunk diameter (diameter at breast height or DBH), bark structure (rough, smooth, flaking) and crown architecture (main branches horizontal or oblique, etc.) should preferably be recorded (see attached data sheet).

Voucher specimens are dried in newspaper, in a plant press. Collection number is written on the paper containing the voucher specimen and, preferably, on small field labels attached to the specimen. Succulent materials are sliced to enhance drying.

The following additional voucher data are recorded in the field note book under the collection number: Scientific name, Name of the person responsible for the scientific identification, Location, Habitat, Field characters of the species (e.g., growth form, color of flower, fruit, etc.), Collector(s) name(s), Collection number, Date of collection.

Data Management

Identifying Specimens — Vouchers should preferably be collected in triplicates, one for the local herbarium, one for the collector's herbarium and one for mailing to a specialist for final identification (if needed). Furthermore, cultivation of vouchers may be necessary for species identification when flowers are lacking, especially sterile material of orchids.

With basic botanical knowledge, specimens with flowers may usually be fairly easily identified to family or genus, occasionally even to species, by using published keys or by comparison with dried reference material. This will allow you to seek experts on specific epiphyte groups for help with species identification.

For identification of species, the following experts may be contacted for help: T.B. Croat, Missouri Botanical Garden (aroids); E. Gouda, Utrecht University and W. Till, University of Vienna (bromeliads); R.L. Dressler, University of Costa Rica, Lankester Botanical Gardens (orchids); G. Mathieu, Ghent University (*Peperomia*); and J.T. Mickel, New York Botanical Garden and A.R. Smith, Jepson Herbarium Berkeley (ferns). Furthermore, the Bromeliad and Orchid Research Centers of the Marie Selby Botanical Gardens may provide help with identification of orchids and bromeliads.

Data treatment and interpretation — Single RRED-analysis provides species lists, richness (alpha diversity) and frequency for one hectare of forest. Multiple RRED-analyses allow for biodiversity comparisons across habitats and regions. The data can be analyzed with conventional techniques for diversity comparisons and richness estimation, as those based on frequency records (e.g., Chao 2).

Species frequencies, e.g., in the various height zones, may be statistically analyzed in various ways. A simple and effective approach would be by using contingency tables (see Krömer *et al.*, 2007, Vergara-Torres *et al.*, 2010).

Results of RRED-analysis can be interpreted at different levels: at the level of individual trees or height zones, canopy versus understory, single hectares of forest, etc.

Context-Dependent Sampling Considerations

RRED-analysis is impeded by heavy rain or storms, therefore is best carried out during dry spells in the rainy season. Sampling during the dry season may underestimate epiphyte richness because some epiphyte species lost their leaves (e.g., ferns) or lack reproductive structures; however, the cultivation of sterile plants to obtain herbaria vouchers or several visits to the sampling locality might complete the inventory.

The forest canopy can be a hazardous environment to work in, but need not be when appropriate safety precautions are taken. For a detailed safety protocol the website of Tree Climbers International [http://treeclimbing.com/index.php/climbing/rules] should be consulted. Potential hazards are the following: Hazardous trees and branches-- Branch or tree falls pose a danger to RRED-analysis. Inspection should be made of the area around the tree prior to access of the canopy for any hazardous neighboring trees or branches that might impede safe climbing. Also, RRED-analysis should not be carried out on instable fast-growing pioneer trees, and under conditions of high winds or storms.

Hazards from other organisms – In many forest hazardous species of plants and animals may occur. Plant hazards include thorns, stings, and poison (e.g., *Rhus radicans*). Personnel should be trained to identify these hazardous species (e.g., avoiding trees with vines, honeycombs or dry branches with bee activity). Hazards from animals include feral attacks, snake bites, and insect stings. Bees can be especially dangerous in the canopy. Although most snakes are harmless, they should be treated with caution and avoided. Where possible appropriate anti-venom should be available on site and arrangements should be made for quick evacuation of a casualty.

Heat exhaustion and stroke – Working in the canopy for extended periods of time under hot and humid conditions may cause risk of heat exhaustion or heat stroke. Precautions to be taken include wearing of hats and carrying of adequate water supplies.

In RRED-analysis there is always a second person at ground level for safety (see above!).

The method is already established and has therefore been widely applied. The method has been tested in different tropical forest types, including lowland and montane forests as well as primary and secondary forests, and its robustness for analysis of species richness and frequency in one hectare plots has been shown. We envisage further development of the method towards representative sampling of abundance and biomass of vascular epiphytes, which is not yet facilitated by RRED-analysis.

Conservation Implications and Limitations

RRED-analysis can be applied to the study of the responses of vascular epiphytes to forest disturbance and environmental change, by comparing species richness in natural vegetation and sites with anthropogenic influence. Numerous studies have shown a drastic decrease of epiphyte species richness in secondary vegetation (see Köster *et al*., 2009) This decrease is especially notable among orchids and certain groups of ferns (filmy ferns, grammitid ferns), which are extremely sensitive to human disturbance.

Richness of epiphytes in terms of species number per given area can thus be a good indicator of environmental quality, and a useful measure for determining the conservation status of an area (see Krömer *et al*., 2014). It goes without saying that the use of epiphyte richness as a bioindicator requires a rigidly uniform protocol for sampling of epiphyte richness data. RRED analysis was exactly designed for this purpose and has shown its usefulness.

This method can target rare and uncommon species of conservation importance. The authors have discovered several new species to science and many new species records on the regional level by using RRED-analysis.

Long-term monitoring of changes in epiphytic species richness and community structure over time, in permanent sites or plots, is highly desirable but is beyond the scope of RRED-analysis. However, these kinds of studies can provide useful information about the resistance and resilience of epiphytes to the loss, alteration and deterioration of their natural habitat as well as the effects of climate change.

Acknowledgments

Helpful References

There are several websites of general interest for epiphyte researchers:

The Global Canopy Programme [http://www.globalcanopy.org]
The International Canopy Network [http://internationalcanopynetwork.org/]
The Big Canopy Database Project [http://canopy.evergreen.edu/BCD/]

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Equipment

The following list includes the basic tree-climbing equipment (and its cost in July 2015), which can be ordered online at http://www.newtribe.com/store, http://www.sherrilltree.com/tree-climbing-gear. However, there are other providers, where similar equipment from other brands can be obtained (e.g., https://www.forestry-suppliers.com/).

Product Name	Quantity	Price per unit (US $)	Sum (US $)
New Tribe TWIST Adjustable Saddle	1	149	149
CMI Large Left & Right Ultrascenders	1	140	140
1" Nylon Tubular Webbing (to connect Ascenders)	10 ft	10	10
Petzl Rig Self-Braking Descender	1	185	185
Petzl Carabiner	5	20	100
Petzl Elios Adult Tree Climbing Helmet	1	65	65
Grippy Rappel Gloves	1	20	20
New Tribe Nifty Throwing Kit	1	42	42
New Tribe Shot Pouch 12 oz	1	15	15
New England 11 mm Static Kernmantle Rope	150 ft	132	132
NewTribe Medium Ropebag	1	49	49
Big Shot line launcher two 4' poles Kit Specs	1	120	120
		Total (US$)	**1.027**
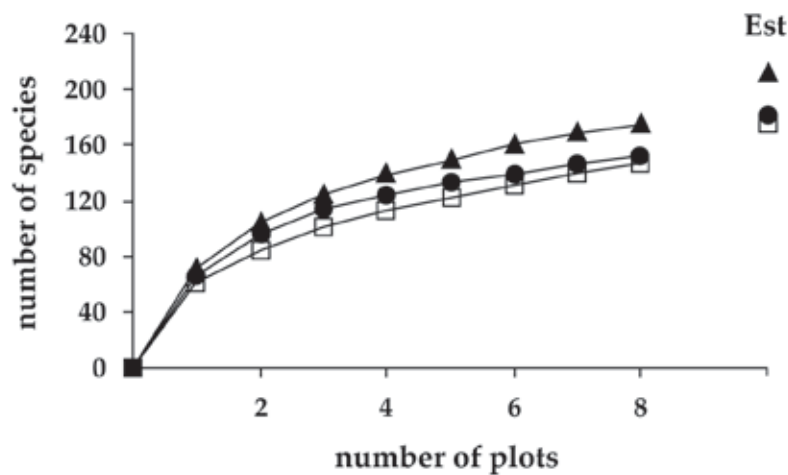
Figure 1
Species accumulation curves and estimated total number of species (Est) of vascular epiphytes in three 1 ha plots in a montane forest of Bolivia, using the Michaelis-Menten richness estimator. In each hectare plot, up to eight trees were sampled, as was a 20 x 20-m plot around each sampled tree (taken from Gradstein *et al.*, 2003).

Data Sheet

Collection Number	(Provisional) Scientific Name	Tree Zone						Additional Notes
		U	1	2	3	4	5	

LARGE MAMMALS

MEDIUM AND LARGE-SIZED MAMMALS

Maíra Benchimol

Universidade Estadual de Santa Cruz, PPG Ecologia e Conservação da Biodiversidade, Applied Ecology & Conservation Lab. Ilhéus, Bahia, Brazil.
Email: mairabs02@gmail.com

Introduction

Taxon Definition – Medium to large-bodied mammals, encompassing all terrestrial and arboreal non-volant species but excluding small terrestrial mammals.

Suitability of the Taxa for Rapid Surveys – Large mammals are considered good bioindicators of intact tropical landscapes and have therefore been increasingly used in large-scale monitoring programs worldwide (Ahumada *et al*. 2011; Luzar *et al*. 2011; Nobre *et al*. 2013). They play vital roles in ecosystem structure and functioning, participating in different trophic levels in food webs, contributing to herbivore regulation, and acting as important seed dispersers for many tree species (Terborgh 1992). They are also a vital economic resource for local human populations through their use as food, pets, artefacts and tourism (Peres 2000; Costa *et al*. 2005). Indeed, mammals comprise an important source of protein and income to local communities, especially the large-bodied species given their great amount of meat (Redford 1992; Peres 2000). Moreover, they are widely hailed as regional conservation icons (e.g., pandas), as many species are charismatic and benefit from popular sympathy, which contributes to promote conservation to the wider public (Cuaron 2000; Dirzo *et al*. 2014). Regardless of their appeal, many mammal populations have gone extinct and many others are declining, requiring surveys and monitoring that can inform conservation action to hinder continued population declines.

Methodology – The diurnal line-transect census is a well-recognized and cost-effective methodology for surveying medium and large vertebrates in tropical forests and savannas (White 1994; Peres 1999; Carrillo *et al*. 2000; Haugaasen and Peres 2005; Ogutu *et al*. 2006; Effion *et al*. 2013). It has the great advantage of low financial costs, as only few items are required for surveys (see in 'Equipment for surveys' below). Additionally, species identification is obtained directly during the surveys, without requiring collection of any type of material for processing. The sampling protocol is simple, easily replicated, results comparable among different sites, and provides reliable data for density estimates for several species (Peres 1999; Haugaasen and Peres 2005). Line-transect census methodology is considered the only appropriate technique to survey arboreal species (such as primates), yet provide low number of records for elusive and rare species (Munari *et al*. 2011). Hence, combining line-transect censuses with indirect surveys (including fresh tracks, feces, hair, burrows, digging and partly consumed fruits/seeds) can enhance detectability for many mammal species, contributing to maximize the species lists.

This chapter provides an easy, replicable and standardized sampling protocol to survey medium to large-bodied mammals for rapid biological assessments in tropical forests and savannas, based on diurnal line-transect censuses and indirect surveys. Additionally, it is recommended to use this protocol concomitantly with the camera trapping protocol, to enable estimates of population-level abundances and richness for a greater number of medium and large-bodied mammal species.

Core Methods

Sampling Protocols

Line-transect preparation — within each major habitat type (site), three 4-km long and 1-m wide linear transects should be cut before the start date of surveys. The number of sampling sites and distances to each other may have to vary according to the total area of the study landscape. Ideally, transects should be established at least a week before the start of rapid assessments so that human disturbances do not affect mammal behavior and results. At this step, transects should be measured (using a Hip-Chain® or a 50-m tape) and marked (using a biodegradable flagging tape) every 50 m. Within each sampling site, transects should be separated from each other by at least 1 km, and their location should take into account accessibility, including the existence of rivers, streams and topography, that might hamper the surveys. It is best to open transects more than 300 m from the base camp to avoid biased data due to any species behavioral responses to camp activity. Shorter transects may be necessary in fragmented forest sites where space constraints prevent long trails. Within fragmented forest landscapes, the length and arrangement of transects should consider both area and shape of each forest patch, aiming to cover a representative area (50% of a patch would be adequate).

Prior to the start of the surveys, a field sheet should be prepared to enable data records during the data collection (Appendix I).

Diurnal line-transect census — two observers, preferably one trained researcher accompanied by a local inhabitant with knowledge of species present, should walk at a constant speed (~1 km/h), with brief stops (10 s) every 500 m, along each of the three transects established at each site (Peres 1999; Peres and Cunha 2011). Transects should be walked in both directions, for a total of 24 km of sampling effort per day (3 transects walked simultaneously x 8 km). In savannas, surveys can also be conducted using a vehicle, at approximately 10 km/h. Surveys should be conducted in the morning (~6:15 – 10:30) and afternoon (~14:00 – 17:30), and discontinued during rainy periods since these can affect results.

At the start of each survey, the lead observer should record the date, transect identity, name of observers, general weather condition (sunny, overcast or cloudy) and start time. Observers will then start walking along the transect, keeping a distance of ~15 m from each other, looking for target species in all strata (in case of forest habitats) and on both sides of the transect (Fig. 1).

Upon a visual detection event, observers should record: the time, species name, number of individuals, sighting location along the transect, and the perpendicular distance from the animal (or first detected individual, in case of groups) to the trail, which needs to be accurately measured (Hip-Chain® or a 50-m tape; Fig. 1 and Appendix I). It is important that the observers see or hear the animal(s) before they detect the observers – otherwise, the perpendicular distance may be inflated, directly affecting density estimates. For each detection event, observers should remain on the transect line and spend no more than 10 minutes to count individuals and record the data. The end time of each walk should be recorded at the end of each morning and afternoon survey. In order to minimize biases related to the probability of detection, each pair of observers should be rotated on a daily basis between different transects. The number of sightings per km walked (sighting rate) should be used as a measure of abundance (both for groups and individuals), and density estimates can be calculated using specific programs such as Distance (Buckland *et al*. 1993). Probability of occupancy can be assessed by using a matrix of presence-absence data per survey for each species, using programs such as Presence (Hines 2006; see Box I for parameter terminology).
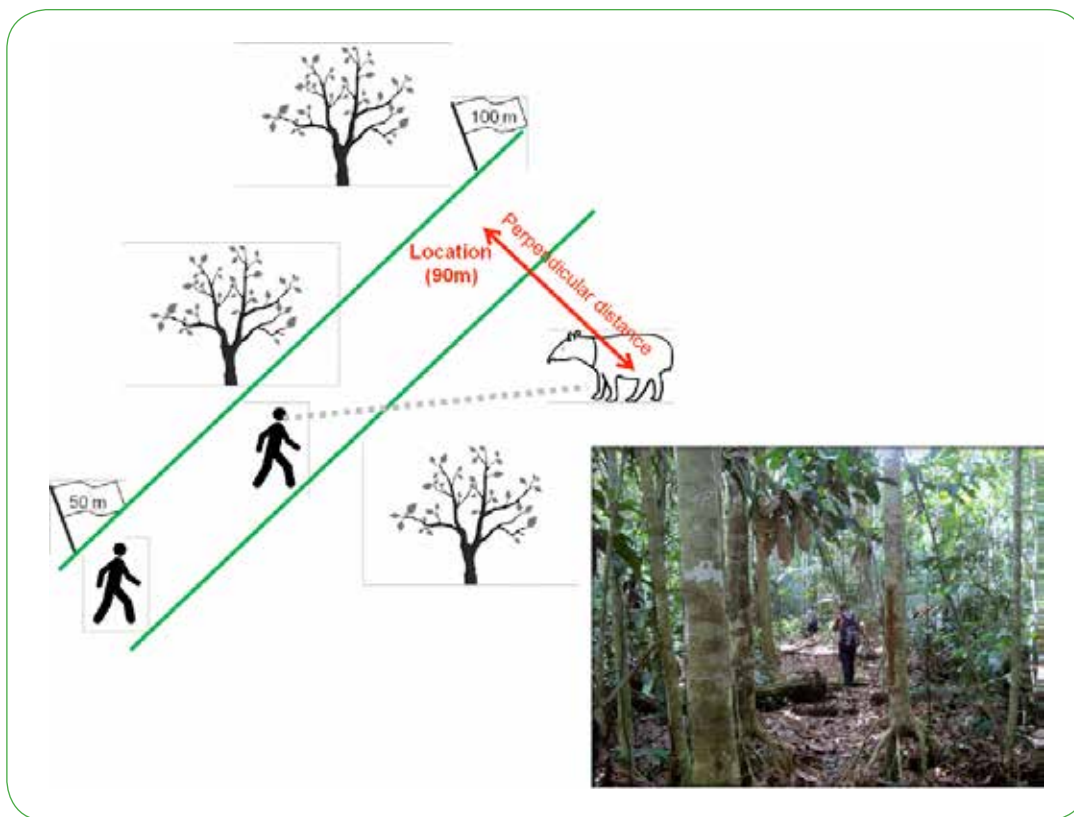

Figure 1
Demonstration of a line-transect census methodology conducted by two observers upon an animal detection, showing in red some of the parameters to be recorded on the field sheet.

Indirect surveys – concomitantly to the diurnal line-transect surveys, the same two observers should search for any indirect evidence of target species (Fig. 2), along and up to 5 m from the transect. Local field guides should be used to identify mammal tracks. Acoustic records of identifiable species could also be recorded. The field sheet can be the same as that used to conduct line-transect surveys, but the perpendicular distance is not recorded as this methodology cannot discern accurate density estimates (see Appendix I). However, indirect surveys may enhance the number of mammal species recorded within a site, and enable occupancy estimates that can be used to detect changes over time.

BOX I: Terminology

Abundance – number of individuals (or groups) per distance walked
Density – number of individuals (or groups) per area
Richness – total number of species
Occupancy – presence of a focal species (also known as occurrence)

Equipment for surveys

Item	Quantity
Field sheets	Several
Watch	01 per pair
Optical range finder or a 50-m tape	01 per pair
Binoculars	02 per pair
Digital camera	01 per pair*
Field guides (color plates), if available	01 per pair*

* Suggested but not essential.

Figure 2
Examples of indirect signs: an armadillo burrow, feces, hair and a footprint of different mammal species in an Amazonian forest site.

Selecting sampling sites – The location of sampling sites should cover a representative area of the study landscape. A first required step is to obtain satellite images of the study landscape to acquire knowledge of habitat distribution, existence of rivers, local villages and other site characteristics. Next, a visit to the area should be performed prior to the survey in order to select the sampling sites. This visit should be used to assess logistical challenges (feasibility for surveys), habitat heterogeneity (focus on the most representative habitat types), anthropogenic disturbance (depending on the goals of the survey, which may be to focus on intact sites, or to assess disturbances such as logging, fire and hunting), and accessibility (if the access occurs by boat, transects should start close to rivers and streams in order to reduce time walking prior to each survey). All transects should not traverse aquatic realms inaccessible by foot.

Effort required – A minimum of seven days of two-way surveys (i.e., morning and afternoon) along each transect at each sampling site is required. This will provide a total of 168 km of cumulative effort. This effort is expected to provide robust species richness for each sampling site – previous studies in Neotropical forests recorded up to 93% of all species (extrapolated richness) considering a total survey effort of 80-90 km (de Thoisy *et al.* 2008). However, some cryptic species are difficult to detect even with higher sampling efforts, although abundances can be obtained with such effort, and provide a good proxy of communities status. For occupancy models, one week of surveys will provide 14 'visits' on the presence-absence matrix, which is potentially adequate for analyses of site occupancy for most species.

For density estimates, however, a minimum of 40 detection events are recommended for robust estimates, although 20 sightings may provide sufficient estimates (Peres 1999). If small sample sizes were obtained at the end of rapid surveys, data from different sites can be pooled together to enable density estimates using the Distance software. Sighting rates can also be calculated and compared among different landscapes independent of sample size.

Context-Dependent Sampling Considerations

Environmental disturbances – Anthropogenic forms of disturbances such as hunting, logging and fire are likely to affect the distribution, occurrence, density and detectability of several species within a site (Peres 2001, Benchimol and Peres 2015). Hence, it is recommended to consider these factors during the selection of sampling sites and establishment of transects, which can be used to focus on undisturbed areas or to assess environmental impacts. The level of hunting pressure on mammals in the study landscape should be assessed through interviews with local people. This can be done by showing color plates or photographs of all target species to local residents that might have access to the study sites, and assign a level of hunting pressure to each site into three classes: [1] non-hunted; [2] lightly or occasionally hunted; and [3] heavily hunted. Additionally, information on local hunting patterns, including name of hunted species, number of hunters per family, and frequency of hunting events should be also assessed. These categories of hunting pressure may be used for comparisons with other sites.

Habitat considerations – For indirect surveys, the detectability of signs varies on different substrates and biophysical conditions. Sandy soils can easily retain footprints, whereas seasonally-flooded and rocky habitats are unfavorable for conducting sign surveys. Additionally, lowland habitats are prone to contain higher number of signs for several species in tropical forests than plateau habitats. Weather condition is also an important factor likely to affect this sampling performance, given that heavy rains can rapidly remove footprints.

Seasonal considerations – If only one rapid assessment can be conducted within the study landscape, surveys should be conducted in the dry season (ideally less than 100 mm of rain for sites exhibiting high seasonality). If feasible, two rapid assessments should be conducted during a year, one in the peak of the dry season and another one in the peak of the wet season. Seasonality can determine spatiotemporal shifts in mammal faunas, directly affecting detection probabilities for different species (Bodmer 1990; Peres 1997; Lehman 2006). This occurs because a greater number of tree species is producing fruits during the rainy season (Haugaasen and Peres 2005), which results in higher fruit exploitations and therefore higher daily activity of frugivores at that period of the year, likely enhancing detectability of several species. For instance, a lemur primate species was not recorded during the dry season in Madagascar and most species showed significantly higher density estimates in the rainy season compared to the same line-transect census protocol in the dry season (Lehman 2006).

In several Amazonian forests subjected to intense flooding regime, the prolonged seasonal inundation strongly affects patterns of habitat use for different mammal species, directly affecting patterns of species abundance (Branch 1983; Peres 1997; Haugaasen and Peres 2005). It is therefore expected that surveys conducted in the dry season provide lower abundance estimates for several species and may also reduce the detection of others. However, during the rainy season it is more difficult to perform rapid surveys by conducting line-transect surveys, given the great probability of losing days of collection due to the weather conditions.

Supplementary methods – Nocturnal line-transect census methodology is frequently used as a complementary technique to survey nocturnal species. It has the disadvantage of visual limitation for the observers during data collection, usually providing poor information for most night-time species (Munari *et al*. 2011). However, this sampling technique can provide reliable density estimates for some arboreal nocturnal species (Nekaris *et al*. 2008; Thornton *et al*. 2011) and should therefore be incorporated into the sampling protocol for sites harboring a high diversity of arboreal nocturnal species. The sampling protocol is similar to diurnal line-transect surveys, except that walking pace should be reduced to 400 m/h, and flashlights/headlamps are required. One-directional surveys are sufficient at night (4 km per transect per day), starting at 18:00 hs, which will provide 84 km of sampling effort per site.

Footprint trap stations are another non-invasive method commonly used to survey medium and large mammals (Wemmer *et al*. 1996; Lyra-Jorge *et al*. 2008). This permits both presence-absence and abundance data for several terrestrial, cryptic species. Sandy soils should be disposed at each 2m^2 (1 x 2 m) track plot, set out at 500 m intervals along each transect. Once a day, each track plot should be checked, footprints should be photographed to scale and measured, and then should be carefully cleared.

Additionally, rapid interview surveys can provide information on species richness at a study landscape. It is a cost-effective methodology, which enable acquisition of species occupancy and enhance community participation (Parry and Peres 2015). Standardized questions on species presence/absence likely to occur in the study area are asked to those local inhabitants with many years of local knowledge of wildlife. This can be done concomitantly with the interviews on hunting pressure.

The protocol presented here should be used in combination with the camera trapping protocol for obtaining a reliable biological assessment of an area for the target taxa. A more complete list of species composition within the study landscape is likely to be obtained if diurnal line-transect surveys, indirect surveys, footprint trap stations, interviews, and camera trapping are simultaneously adopted.

Data Management

The species identifications are obtained directly from the surveys. Data should be carefully recorded and stored in an electronic data file, which needs to contain the following information for each survey:
- date
- observers name
- site name
- unique transect identifier
- general weather condition
  - name of each recorded species and associated data
  - time of record
  - type of record (direct or indirect)
  - number of individuals
  - location along the transect
  - perpendicular distance of the animal to the transect

Conservation Implications

Medium and large mammals are key components of forest and savanna communities and are therefore considered good indicators of ecosystem health. Hence, data obtained from rapid surveys can provide information on the current quality of the study landscape for current mammal populations. Because anthropogenic disturbances are likely to affect occurrence and abundance of mammal species, these surveys will also contribute to understanding the human impacts on mammal assemblages, identifying local and global patterns of change. Additionally, data will contribute to assessments of the conservation status of individual species, providing data on distribution, mapping the occurrence of endemic species, and helping in the assessments of local and global threat of many species. Results are intended to support programs related to conservation strategies – contributing, for instance, to the creation of protected areas and development of new projects focused on protection/management of threatened species.

Limitations

Rapid assessment techniques provide means of accelerating the acquisition of scientific data in understudied areas. For medium and large mammals, however, this is not an easy task. The group requires a high survey effort to achieve comprehensive sampling, due to the distribution, occupancy and detectability of different species. A combination of different techniques is therefore required to enhance detectability of different species and obtain an adequate estimation of the magnitude of species richness and abundance. Nevertheless, all sampling techniques for the group present limitations for rapid surveys. Diurnal line transect censuses are likely to provide good data for diurnal primates, but might provide few records for many terrestrial species in rapid surveys. The inclusion of indirect surveys in the protocol contributes towards gathering occupancy data for these terrestrial species, although the method fails in providing density estimates.

Several assumptions of the line-transect census methodology are challenging (Buckland *et al*. 1993, 2001; Marshall *et al*. 2008). Firstly, sufficient and independent sightings are required for the estimation of a detection function necessary for generating density analyses. Sampling effort needed can vary with species and location, depending on their detectability, variation in group size and abundance. Secondly, individuals should be detected with certainty, and measured accurately from their initial location to enable density estimates. Lastly, good weather conditions are essential for census walks, thus rainy days preclude data acquisition. For indirect surveys, detectability is related to the local substrate and the ability of surveyors to detect and identify signs. Again, weather conditions might compromise data acquisition.

Acknowledgments

Photo © Trond H. Larsen

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Appendix I – Example of field sheet for line-transect census and indirect surveys for surveying large mammals

Observers: _____ Site:_____ Transect:_____

Date: ___/____/____ Start time:_____ Final time:_____ ☀ ⛅ ☁

Time	Species	Record type	N inds	Location	Distance	Obs

CAMERA TRAPS

CAMERA TRAPPING

Trond H. Larsen[1], Leonardo Viana[1], Travis Thyberg[2] and Jorge Ahumada[1]

Introduction

Background – Ground-dwelling medium to large-sized (> 1 kg) terrestrial mammals and birds (henceforth terrestrial vertebrates) are a key component of tropical forest communities, providing important ecosystem functions. They influence vegetation in numerous ways, such as through seed dispersal, seed predation and herbivory, through redistribution of nutrients and physical alteration of abiotic conditions, and by controlling abundance of primary consumers through predation (Dobson *et al*. 2006; Power *et al*. 1996). Larger species have an especially important role in the composition and structure of vegetation, which is apparent for example in forest–grassland transitions mediated by large herbivores (Ritchie and Olff 1999).

Terrestrial vertebrates are also disproportionally affected by humans. Land use change and overexploitation threaten hundreds of terrestrial vertebrate species – particularly species with low reproductive rates and large home ranges – and induce major shifts in the composition of animal communities (Peres and Palacios 2007; IUCN Red List). In addition, climate change may have major and much broader impacts on terrestrial vertebrate communities. Synergistic interactions between land use and climate change form the greatest threat to biodiversity (Brodie *et al*. 2012). This is problematic, for example, for mammals because direct and indirect responses of mammal communities to these factors co-determine the state of ecosystems and the services that ecosystems provide to people (Brodie and Gibbs 2009; Dobson *et al*. 2006; Jansen *et al*. 2010).

Further, disproportionate influence in combination with vulnerability makes large mammals strong indicators of ecosystem health (Power *et al*. 1996). However, there is surprisingly little quantitative information about how terrestrial vertebrate communities respond to local, regional and global threats, especially in the tropics where most biodiversity is found. One major reason for this is that terrestrial vertebrate communities have been relatively hard and expensive to survey, due to rarity and nocturnal or otherwise secretive habits of many species. One particular difficulty that arises in observing species is caused by human observers themselves; often, the presence of a survey team in the study area alters the behavior of the species being observed, creating a bias in the results. For all these reasons, it is difficult to objectively assess the abundance and distribution of large vertebrate species in a community during a rapid survey, let alone determine whether and how species are at risk and whether protection measures are effective.

[1] Conservation International
 2011 Crystal Drive, Suite 500
 Arlington, VA 22202, USA

[2] 1400 E West Hwy, Apt 801
 Silver Spring, MD 20910

Camera Traps – Camera trapping is an excellent tool that helps to avoid the difficulties described above and is complementary to line transects for assessing and monitoring terrestrial vertebrate communities (O'Brien 2008; O'Brien *et al*. 2010; Tobler *et al*. 2008, Ahumada *et al*. 2013). Camera traps have several advantages: they are automated and standardized, helping to eliminate individual sampler bias and reducing researcher hours required in the field; and they operate 24 hours per day and can be left in place even when researchers are not present, increasing detection rates even for highly elusive species. Arrays of camera traps act as visual sensor networks to detect and monitor the variation of terrestrial vertebrate relative abundances in space and time (Kays *et al*. 2011), where the rate and the proportion of points at which species are photographed (occupancy) can be used as an indicator of their abundance. Camera trap data can also be used to estimate population densities (Rowcliffe *et al*. 2008).

Core Methods

The core, standardized protocol, adapted from the TEAM protocol*, involves deployment of at least 20-30 camera trap sampling points at each site. Selection of two to three arrays of 20 or 30 points each, sampled sequentially at multiple sites, is ideal for encompassing a wider range of ecosystems and habitat types. The total number of and spacing between camera trap points will largely depend on the survey objectives, but a good spacing that works for most species is one camera trap point per 2 km^2, ideally arranged in a grid. Sampling design is an iterative process and is ultimately based on spatial data relevant to the specific project. For example, the occurrence of certain types of land cover, roads, rivers and settlements act as determinants of final suitability of point locations, with some locations deemed inappropriate (Fig. 1).

*TEAM 2011; http://www.teamnetwork.org/protocols/bio/terrestrial-vertebrate

Example of sampling design process
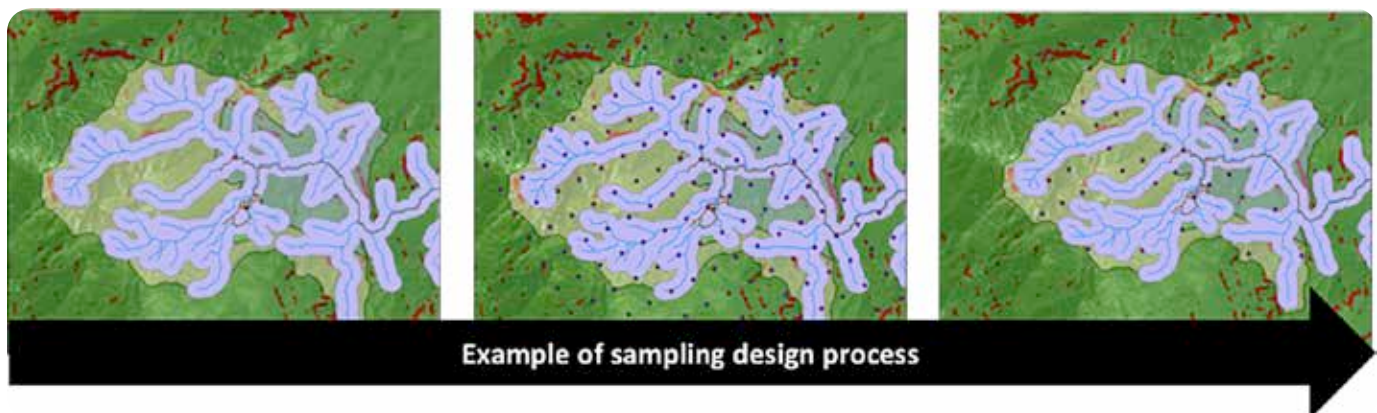
Figure 1
Selection of camera trap point locations should be based upon a desktop review of the study area (left image, including watersheds, roads, land-use, etc.), overlaid with a 'best case' array of equidistant points in a grid (center image), and then a final selection of camera locations modified by characteristics of the site (right image; Box 1).

Camera traps are deployed by a field team typically consisting of one team leader and one to three technicians in systematic grids at pseudo-randomized locations, with standardized settings and without bait (although bait can increase detection rates, it also results in biased data). The cameras are placed as close as possible to predefined locations in this grid, at locations that have a suitable tree for the mounting of the cameras and where the understory is sufficiently open for the camera to have a clear view unobstructed by vegetation, such as along small game/animal trails. Low vegetation blocking the camera can be cleared with a machete. Cameras are placed at a height of 30-50 centimeters off the ground, although this can be modified where very small or very large vertebrates are present (e.g., elephants). The angle of the camera should be adjusted to ensure direct field of view (i.e. parallel to the ground). Each camera should be checked prior to and following deployment to make sure they are operational.

BOX 1: Sampling design

- Acquire all necessary spatial data layers (i.e., Satellite Imagery, DEM, Land Cover, Rivers, Roads, Trails, Administrative and Project Boundaries, Settlements)

- Generate a grid of regularly spaced points that cover the entire monitoring area at a density of one point per 2 km²

- Remove any points that fall outside the project or monitoring area

- Remove any points that are in unusable areas (e.g., areas prone to flooding, rocky outcroppings, or areas with a slope greater than 45 degrees)

- Check if an elevation gradient exists at the project site and try to distribute the remaining proposed camera trap locations so that each elevation range is represented equally
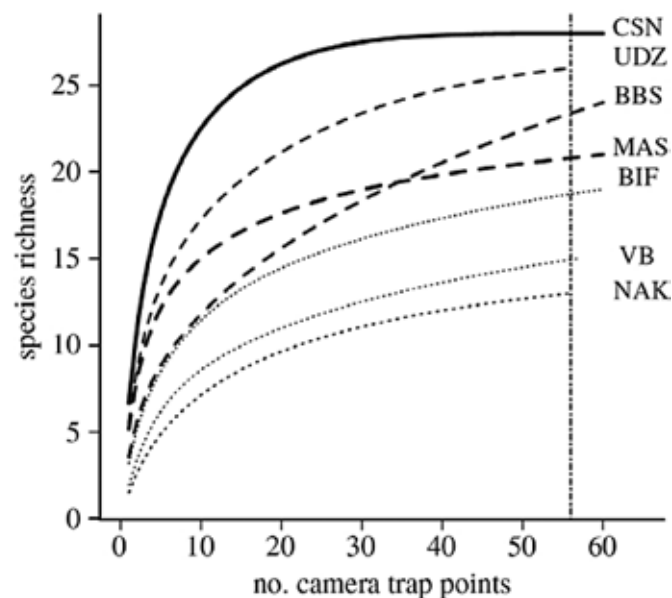

Figure 2
Species accumulation curves for large terrestrial vertebrate communities at seven sites based upon TEAM camera trapping data. Each camera trap point represents a 30 day sample. 10-20 camera trap points are suitable for 50-75% of species richness, while 60 camera trap points approach a comprehensive asymptote for the area in most studies. Reproduced from Ahumada *et al*. 2011.

To maximize the number of species recorded and improve density estimates, cameras should be deployed for a minimum of 30 days if possible, ideally during the driest season of the year. This sampling effort is much greater than that required for other taxa and sampling methods during rapid surveys, but is recommended due to the relatively low detection rate of camera traps. To maximize the time cameras are deployed in the field, it is ideal if the camera traps can be set up by the reconnaissance team or the people who are in the field to explore and cut trails prior to the visit by the full biological survey team. Alternatively, camera traps can be left in place after the team has departed and collected at a later date. Even if camera traps can only be left in place for a week, they can still detect species that might otherwise not be observed and in some areas, can record the majority of species present (Fig. 2).

Camera trap sensors detect a heat differential with the background environment when animals move in front of them, and do so in a non-invasive manner, largely independent of the activity patterns and shyness of species. Abundance and density estimates can be calculated for species with unique patterns or markings that are photographed multiple times in what is known as capture-recapture analysis (Amstrup *et al*. 2005). Occurrence (or the proportion of points where the species is sampled) is a preferred metric for camera trap data because detection can be independently estimated and corrected (MacKenzie *et al*. 2003). Protocol calls for the use of high quality camera traps (currently Reconyx™ RM45 and Hyperfire™, Reconyx Inc., Holmen WI, USA, although other more affordable rainproof models are adequate such as Bushnell Trophy Cam) with fast trigger speed (1/10th of a second) that can take multiple photos upon each triggering. Color photographs are taken during the day and black-and-white photographs illuminated by infrared flash during the night, although models that use white flash for nighttime color photos are also acceptable.

BOX 2: Supplies/equipment needed

- GPS Unit (x2)
- Compass (x2)
- Maps
- Machete
- Camera Traps (x32)
- Cable Locks (x32)
- Memory Cards (x60)
- Zip-lock bags
- Batteries (x720, depending on camera requirements), preferably rechargeable
- Battery charger (x10)
- Desiccant Pellets (x100 packs)

Context-Dependent Sampling Considerations

Sampling considerations for assessing environmental change

The TEAM protocol was designed to optimize the probability of photographing an adequate sample of tropical forest terrestrial vertebrate species. While the methods presented here are well suited to establishing a baseline, the broader TEAM protocol is intended to be repeated annually in order to monitor changes in the community of terrestrial vertebrates over time and for early detection of population trends. The sampling design detects species with large ranges, such as elephants, tigers, and jaguars, as well as species with smaller home ranges, such as terrestrial birds and small carnivores. The number of points and deployment duration were carefully estimated to be large enough to allow for quantitative analyses and sufficient to detect a 5% difference in occupancy between subsequent years for all species for which the detection probability (probability that a species that is present is actually detected by at least one unit in a survey) exceeds 0.08 (O'Brien *et al*. 2010).

Habitat considerations affecting methods

While the focus here is on forest habitats, camera traps can be used in many different habitats including grasslands. In open habitats, care should be given to not place cameras in direct line of sight to either the sunrise or sunset to avoid falsely triggering the apparatus. In open areas, cameras may be restricted to isolated trees, although where no trees are present, can be attached to poles in the ground. Exact placement of cameras can vary depending on the particular characteristics of a site. Local knowledge of animal behavior and requirements is important. Identifying signs of animal presence (i.e. tracks, scat or game trails) is useful for placing cameras in optimal locations to increase detection rate. Cameras can also be placed at points which naturally constrain animal movement and force animals to move in a predictable direction, such as bottleneck locations along game trails (rock ledges, fallen trees, etc.). This approach differs from protocols that maximize encounters by placing cameras subjectively at high-use locations such as human trails, mineral licks or waterholes, or those that use bait to attract animals.

Seasonal considerations

While most terrestrial bird and mammal species are active year-round in tropical environments, seasonality can affect activity as well as camera performance. Even cameras marketed as waterproof are prone to fail due to humidity, especially during heavy rains. Sampling during the dry season maximizes equipment life and also ensures consistency among studies and sampling periods. Even during the dry season, humidity can affect camera performance, and a desiccant should be placed inside each camera.

Data Management

Each camera trap, memory card, and cable lock should be labeled with a unique ID, and the location of each camera should be recorded with a GPS unit. The cameras should automatically record the time and date that each photograph is taken. Upon collection of the memory cards and/or cameras, the images should be saved onto a computer or hard drive. A database should be created that includes information relevant to each photograph. Specialized free software, Wild.ID, has been designed by the TEAM network to manage camera trap data (https://wildlifeinsights.org/WMS/#/shareData). It is highly recommended to use this type of software rather than entering data manually, for example in Microsoft EXCEL. The TEAM network and its partners have also developed minimum standards for camera trap data (https://wildlifeinsights.org/WMS/#/resources/standards).

Limitations For Rapid Survey

Rapid surveys typically occur over the span of one to two weeks at each site and involve an entire research team in the field at the same time. Detection of large vertebrates by camera traps will be greatest when fewer people are active at the site. Furthermore, species accumulation curves indicate that three to four weeks are ideal for sampling each site – considerably longer than most rapid assessments. As mentioned earlier, cameras can be deployed before or after the full survey team is present. Another potential constraint is the relatively high cost of purchasing 32 high performance camera traps (depending on quality, ranging from $2,880 to $38,400).
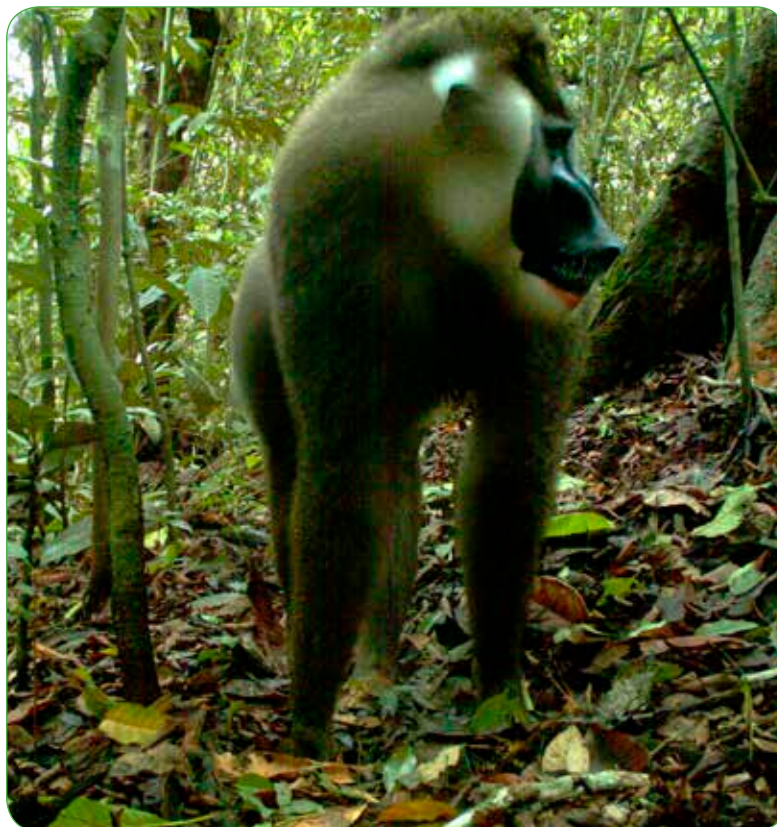
Large male Drill (*Mandrillus leucophaeus*) © Courtesy of TEAM Network and Smithsonian Tropical Research Institute

Conservation Implications

The camera trapping protocols proposed here effectively detect differences in species diversity, richness, evenness, dominance, and functional diversity among sites and over time. Population levels can be assessed using occupancy (MacKenzie *et al*. 2002; MacKenzie *et al*. 2006; Rovero and Marshall 2009), defined as the proportion of camera trap points where a species occurs. This metric does not require identification of individual animals and allows for estimation of trends in population size. Despite the longer time required and potentially high cost of cameras, camera trapping is invaluable for providing data on the entire community of large terrestrial vertebrates in an automated and unbiased way.

Ahumada *et al*. (2011) demonstrate these results using TEAM data to compare species community structure, species diversity and functional diversity between seven sites in Africa, Asia and Latin America. The sites differed in total forested area and in connectivity, ranging from unfragmented (0–20% fragmentation of the surrounding area) to partially fragmented (20–50%) to highly fragmented (50–100%). Species accumulation curves were less steep for fragmented sites, indicating significantly fewer species in fragmented forest compared to continuous forest. Species richness, species diversity and functional diversity also declined with decreasing forest area.

Camera trap sampling is an important tool to evaluate the effectiveness of protected areas, wildlife corridors and others areas where wildlife is likely to be impacted. A recent paper used the TEAM global camera trap network to measure the effectiveness of protected areas, showing that there are no consistent declines in species at any of the 15 protected areas sampled, rebutting earlier claims on the effectiveness of tropical forest protected areas (Beaudrot *et al*. 2016).

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BATS

BATS

Tigga Kingston

Department of Biological Sciences, Texas Tech University, Lubbock TX 79409-3131, USA. Email: tigga.kingston@ttu.edu

Introduction

Definition of taxon & suitability for rapid survey – With over 1360 species, bats (Order Chiroptera) comprise about 20% of mammalian species, and alpha diversity in lowland tropical forests can reach 70 species in the Paleotropics (Kingston *et al*. 2006) and as high as 110 species in the Neotropics (Voss and Emmons 1996). Nonetheless, bats are proving highly vulnerable to land use change and habitat degradation (Voigt and Kingston 2015) and their local diversity is thus a reliable indicator of habitat quality (Jones *et al*. 2009).

Local bat assemblages comprise diverse ensembles, commonly defined by trophic group and foraging space (Denzinger and Schnitzler 2013, for review). Ensemble representation differs between the Paleotropics and Neotropics, and ensembles differ in their susceptibility to capture methods (e.g., Kingston 2013). This precludes implementation of a single core method that can capture the diversity of all bats in all localities. Moreover, not all methods lend themselves to standardized protocols.

Here I focus on three species-rich groups of bats that can be reliably surveyed with standardized methods that generate abundance data. Qualitative methods that can contribute to a species list are given in Supplementary Methods.

1. Forest interior understory bats of the Neotropics – Predominantly members of the Phyllostomidae, but occasional captures of Furipteridae, Emballonuridae, Thyropteridae, Vespertilionidae, and Mormoopidae. The group includes multiple trophic groups and ensembles (frugivores, nectarivores, insectivores, omnivores, and sanguinivores) and can be effectively captured with mist nets. The Phyllostomidae is a good indicator family because of the diversity of ensembles and their differential responses to habitat change (Farneda *et al*. 2015). Members of other families may be too infrequent for comparisons among rapid surveys.

2. Forest interior understory insectivorous bats of the Paleotropics – This ensemble includes members of the Rhinolophidae, Hipposideridae and the vespertilionid subfamilies Kerivoulinae and, in Asia, the Murininae. Megadermatidae and Nycteridae are also members of this group but low capture rates complicate statistical comparisons among rapid surveys. The ensemble is proving highly vulnerable to forest loss and degradation (Kingston 2013) and can be captured in forests with a standardized protocol based on harp traps (Kingston *et al*. 2003).

3. Aerial insectivorous bats – These are globally-distributed and commonly comprise two ensembles - open-space and edge/gap bats based on tolerance (and hence proximity) to background clutter when foraging. These are primarily members of the pan-tropical Molossidae, Emballonuridae, Vespertilionidae, and the Old World Miniopteridae. This is the most effective group to sample in open, non-forest habitat where trapping methods may not be effective, as well as in the forest canopy (Marques *et al*. 2015). They are the dominant or only ensembles in temperate habitats. Sensitivity of this group to land-use change in the forested tropics is largely unstudied (Kingston 2013, but see Bader *et al*. 2015), and some species may be somewhat able to adapt to human-modified landscapes if key habitat features and roosting opportunities persist, as in the temperate zones. Nearly all species use high intensity echolocation calls, encompassing a frequency range typically < 100 kHz (Denzinger and Schnitzler 2013) and are thus amenable to acoustic surveys (Hayes *et al*: 2009).

The forest interior bats of Neotropics and Palaeotropics (Groups 1 & 2 above) use low-intensity and/or high frequency echolocation calls (commonly >120 kHz) and are thus not suitable candidates for acoustic monitoring.

Core Methods

Standardized sampling protocols for rapid survey

1. Neotropical forest interior bats of the understory

- Mist nets set along existing trails or transect lines (see Large Mammal protocol). Divide trails into 100 m segments, and, within each segment, locate a suitable site for 3 mist nets set across the trail, within 20 m of each other, representing a mist net station. Configurations that maximize captures should be deployed e.g., T's, combinations of nets set high and low to the ground (Kunz *et al*. 2009). Unlike birds, bats fly along trails so nets should have most of the trapping area perpendicular to the trail. The objective is to maximize captures per segment with three nets, so net lengths should be selected as fits the setting (but combinations of 6 m, 9 m, and 12 m probably suitable for most situations, and 12 m as a standard desirable).

- For analytical flexibility, net lengths should be recorded, and captures allocated to nets.

- Set up a minimum of 3 mist net stations per night, and move nets to new stations each day, as bats learn positions and capture rates drop off rapidly.

- Nets open from sunset to sunset+6 hrs.

- Check nets at least once per hour, and every 15 minutes during periods of high activity (Sikes *et al*. 2011).

- Person power – 1 person per station, so three people minimum.

- The 3-net station within 100 m segment open for 6 hours is the basic Sampling Unit. This protocol generates 21 Sampling Units in 7 days minimum (need a minimum of 2-3 km of trails). More stations (SUs) should be set if more people are available.

- **Analysis Considerations:** Mist net meter hours. The net station (3 nets/100 m) for 6 hours is the basic Sampling Unit. This approach assumes that net length does not influence the number of captures/net, or that nets are all 12 m, which may not be the case. We further assume a standard 6 hrs of trapping, but nets may need to be closed early, for example in heavy rain. Net effort should always be recorded and include the length of the net and time it was opened and closed, time of rain events etc. This can then be used to describe captures (or species) per mist net meter hour (Kingston 2009), and mist net meter hours used in accumulation curves. However, this approach precludes sample-based randomization techniques (rarefaction and extrapolation) because there is no consistent sample unit. This restricts analysis of species richness to individual-based estimators.

- **Planning Consideration:** Mist net catch rates diminish rapidly if nets are lit by moonlight. This may be due to lunar phobia in bats, or they may be able to see and avoid the net (if you can see the net, the bats probably can too!). Plan trips to avoid moon rise (especially full moon) during the first 6 hrs of night. Avoid netting when full moon falls in the first 6 hrs of the night.

2. Paleotropical forest interior insectivorous bats

- 4-bank harp traps set along forest trails. There are various designs, but we recommend those based on/modified from Francis (1989).

- 1 harp trap (HT) within every 100 m segment along a trail. Pick the best spot with over-hanging and close side vegetation, and fill in sides with cut palms/vegetation to prevent bats going around or under the trap (Fig 1). 10 traps per night, move to new 100 m segments each night. In areas with high capture rates, near caves for example, researchers should start with 5 traps per night until they are familiar with the abundant species. As effort increases, processing of abundant species can be reduced to recording species, sex, age, with a biopsy punch to identify recaptures. Traps can be set at any time during the day if trails are not in use, but must be in place and open 30 minutes before sunset.

- Check traps once at sunset +1-2 hours and at first light, following peaks in insect activity, bat commuting and bat captures. If traps are set near known roosts or cave systems (e.g., limestone outcrops), they should be checked regularly (every 1-2 hours) on the first night to assess the risk of high abundance in traps and associated injuries if the traps are not checked frequently.

- Person power – 1-2 people can collect and process bats, but trap moving and setting each day requires a minimum of 2 people, and with additional field assistants if terrain is challenging.

- One harp trap open all night is the basic Sampling Unit (harp trap night HTN). This protocol generates 35-70 Sampling Units in 7 days. Instead of HTN Sampling Units, HT hours can be used for some analyses if traps are closed early due to rain or ants. As with mist nets, trap effort (time from sunset that the trap is open) for each trap must be recorded, and captures allocated to individual traps (be sure to number the traps each night).

- If ants are seen anywhere near a harp trap during the first check, take the trap down as ants in the trap may kill the pre-dawn captures.

Recaptures, releases and rain

For species richness and other biodiversity estimators, it is assumed that individuals are only represented in the dataset once. To identify recaptures, a wing biopsy punch (3 mm) can be taken from each individual (which can then be stored used for genetic analysis to confirm identification or for systematics, population genetics etc). Take the punch from a consistent part of the wing (e.g. plagiopatagium between 4th and 5th finger of right wing, so you know where to check for recaptures).

Release bats at point of capture (trap or mist net), once processed – some bats have very small home ranges and navigational abilities of tropical forest-dwelling bats are unknown. Don't add extra energetic demands.

Rain biases (reduces) capture rates and presents a welfare issue (hypothermia) if bats are left exposed too long. Close nets or harp traps in heavy rain and/or discard data from rain nights for comparative analyses.

Figure 1
Four-bank harp trap set across a forest trail in Peninsular Malaysia. Note use of rattan (large palm-like leaves) and small cut vegetation to "close up" the trap so that bats are unable to fly round. Photo Regen Jamieson.

3. Aerial insectivorous bats

- Full spectrum direct recording with devices designed for monitoring set at a single point (stationary) each night. Detector should have a flat (even) frequency response from the lowest (10 kHz) to highest (120 kHz) expected frequencies (or above, but not necessary for this group). Sampling rate minimum 384 kHz (16 bit). Omnidirectional microphones tend to record more sequences, but quality for species identification is greater with directional microphones, such as that of the Petterson D500X (Chenger and Tyburec 2014).

- Record in an open area (clearing, middle of tree-fall gap, etc.), with detector or microphone on cable elevated 5 m above ground on a secured pole and microphone oriented slightly less than horizontal so that moisture does not pool on the microphone membrane (Chenger and Tyburec 2014).

- Record from sunset -30 minutes to sunrise +60 minutes. Continuous, rather than triggered recordings, are recommended as this facilitates the use of the acoustic activity index (AI) of Miller (2001) (see below).

- Four detectors, a minimum of 200 m apart, sampling representatives of common microhabitats: i) vegetation gaps (large-tree fall) within dominant vegetation e.g., large-tree fall forest gap; ii) habitat boundary such as forest edge; iii) large open areas --- agricultural clearings, savannahs, grasslands, as appropriate; iv) water bodies --- streams, rivers, lakes, ponds, (set by water's edge, or on bridges over water if present). Deploy detectors in the same location for the 7 nights (Chenger and Tyburec 2014). Be sure to record habitat details of recording site, weather conditions, and any technical problems with the equipment that might influence measures of effort.

Species identification necessitates a call library; automatic identification systems for many bat species in temperate zones are commercially available, but this is many years away in most tropical regions because automatic classifiers require a large number of recordings of identified individuals flying in multiple contexts. Manual identification, by matching call parameters from reference calls to those of free-flying bats, can take manual or statistical approaches (e.g., discriminant analysis neural networks trained with call library).

Call libraries can take years to develop, especially for the open space, edge species that are difficult to catch in ground net or trap systems. Efforts to build the library often depend on captures at roosts (caves, tree-hollows, buildings, bridges, etc.), over water, or flick-netting at foraging sites (Kunz *et al*. 2009). Recording context for the reference calls is critical and should mimic the bat's natural search phase flight space as much as possible, so that calls are representative of search phase. For the open-space, edge species this means recording on release in large spaces (camp ground, soccer fields, etc.). Commentary files should record flight path, distance to background vegetation, height above ground. At night, the bat being released can be tracked through the use of light tags (e.g., Cyalume fishing lures) attached with non-toxic glue to the underside. Elmer's glue is a non-toxic option that secures the tag long enough for recording, and the tag is then quickly lost from the bat). Alternatively, a small powerful torch (e.g., INOVA T series, beam 400-600 feet) can be used to trace the bat in flight. Daylight recordings make

descriptions of flight paths easier, but bats may be dehydrated and hungry, and daylight exposes bats to risk of predation. Daylight recordings may be acceptable if the bat is robust (e.g., species of Molossidae), and can be kept until just before dusk to allow immediate foraging and minimal predation risk. Hold the bat in a cool place so it can utilize torpor, and add a ball of wetted cotton wool to the bat bag to maintain humidity.

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Acoustic Analyses

Caution: An advantage of acoustic recording is that it is simple to apply in the field, and avoids any researcher bias. However, processing and identifying species from recordings requires substantial time commitment after the survey. Automated solutions (classifiers) for analyzing calls are improving, but call libraries are urgently needed for the tropics.

Phonic type diversity: Given the difficulties of identifying species, another approach while call libraries are under development is to classify calls to phonic type and to assess diversity of phonic types. SEABCRU phonic type naming system combines call types (of 5 major types) with peak frequency of the call. For example, if two bat species using FM-QCF calls (Frequency Modulated – Quasi Constant Frequency) with FmaxE of 50 kHz and 72 kHz, we can simply define their phonic types as FM-QCF-50k and FM-QCF-72k (see also Bader et al. 2015 "sonotypes").

</div>

Data:

i) Presence of species/phonic types generates species (or phonic type) richness for a site. With 1-min block samples described below, one can also develop species accumulation curves.

ii) Index of activity for each species. Because individual identity cannot be determined from recordings, abundance data cannot be derived. 10 bat passes could come from one bat repeatedly passing over the detector, or 10 individuals passing once. Consequently, the convention is to describe an index of activity derived from the number of bat passes. The definition of a pass varies, here – a sequence of 2 or more sequential calls, separated from the next sequence by 1 s.

- Bat activity metrics include mean bat passes per detector hour (Kunz *et al*. 2007).

- Another approach, facilitated by continuous, recording is the acoustic activity index (AI) of Miller (2001), which does not require identification of passes. Rather it uses presence of a species in one-minute time blocks. The index is the sum of these presences divided by the total number of one-minute time blocks (i.e., divided by the unit of effort).

Caution: Bats and Diseases

- Pre-exposure rabies vaccinations are required for all people working with bats. If bitten – clean bite site with ethanol immediately. Post-exposure vaccinations advised if bitten.

- Bats are implicated as reservoir hosts for a number of emerging infectious diseases (EIDs) (Schneeberger and Voigt 2015) for which there are no vaccines. Leather gloves can be useful to avoid being bitten, but can make it difficult to manipulate and handle bats during net extraction and measurements. Latex or nitrile gloves provide a barrier during handling. Personal Protection Equipment (PPE) should be considered during the preparation of specimens.

- Always wash hands thoroughly with soap after handling bats, and it is recommended that researchers change out of field clothes before eating etc.

- Cave surveys are not part of the RAP protocol, but a supplementary method. Histoplasmosis is an infection caused by the fungus Histoplasma which can grow on bat and bird guano in caves in many parts of the world. If spores are aerosolized (hot dry caves) they can be inhaled. Use of an effective face mask (respirator) is advised.

SEABCRU provides recommendations on the use of PPE for different bat-related activities:
http://www.seabcru.org/wp-content/uploads/2013/10/SEABCRU_Disease-guidelines-revised_13Aug2015.pdf

4. Supplies and Equipment

- Mist nets – Avinet bat nets (or equivalent)
    - 38 mm mesh
    - Less of a pocket than bird nets
    - 4 shelves
    - 75/2 denier/ply
    - Best lengths – 6 m, 9 m, 12 m.

- Harp traps – 4 bank, x 10. Based on design of Charles Francis (Francis 1989), suitable for trails of rainforests. Can be purchased commercially or made locally in welding shops (Appendix 1). Fishing line should be no thicker than 6lb breaking point.

- Bat detectors: The bat detector market is rapidly evolving with new products each year, core specifications needed are given below. A good resource http://batdetecting.blogspot.com.

- Regular reviews of bat detectors and side-by-side trials of monitoring devices are regularly and rigorously conducted by Bat Conservation and Management http://www.batmanagement.com/reviews/bdReviewHome.html. Systems have diverged with those designed specifically for passive monitoring (to be left overnight/up to several weeks) and those for active sampling which are needed to build the call library.
    - a) passive monitoring --- full spectrum direct recording, flat response to at least 120 kHz, sampling rate 384 kHz minimum, e.g., SM4BAT, Petterson D500X, Batlogger C.

b) active sampling/monitoring for call library -- full spectrum direct recording, flat response to 120 kHz or above, sampling rate 384 kHz or above, e.g., Petterson D240, Batlogger M (which can also be used for monitoring). For full utility, detectors exceeding these specifications (> 120 kHz, higher sampling rates) allow for recordings of bats using high frequencies (e.g., small hipposiderids, Kerivoulinae, Murininae) e.g., Petterson D1000X, Anabat Walkabout.

- Author recommendation (November 2015): Pettersson D500x Special Edition FD. Originally designed just for passive monitoring, now has the capability to record voucher calls and be used for active monitoring tasks.

- Notes:

  - Most detectors have on-board storage of recordings, but typically need SD cards, and batteries as specified by manufacturer.

  - Software for analysis – depends on system purchased, most companies have their own products for purchase (sometimes included).

  - If detectors are to be placed at 5 m, cables and external mics may be needed.

- Bat bags – good source in bulk are geology bags or small-parts tool bags.
  The researcher may wish to put in longer cord for carrying around neck. E.g.
  http://www.uline.com/Product/Detail/S-876/Cloth-and-Burlap-Bags/8-x-12-Cloth-Parts-Bags

- Spring balances (30 g, 100 g, 200 g—depends on anticipated bat size)

- Weighing bags – cut-off ladies stockings are ideal for most bats < 100g. Larger bats should be weighed in the bat bags.

- Dial (or digital) calipers – plastic dial more robust in wet tropics.

- Small ruler can be easier for measurements of soft parts (ear length, foot length). Ideally, "0" on rule corresponds to true edge of ruler.

- Flashlight -- INOVA T series (beam 400-600 feet) for tracking bats on release during recording (optional).

- Biopsy punches (3 mm).

- Eppendorf tubes (or equivalent).

- Medium for wing tissue storage (molecular grade 100% ethanol, NaCl-saturated 20% DMSO, indicating silica). A recent comparison found that wing tissues preserved in silica beads yielded significantly more total and nuclear DNA than those preserved in DMSO or ethanol (Corthals et al. 2015). Given the ease with which silica can be transported to the field we recommend this approach. Individual punches should be preserved in ~0.7 g of 3 mm indicator silica beads in an Eppendorf tube or equivalent.

5. Sampling sites – Existing trail systems or cut-in transect lines (see Large Mammal protocol). Should be at least 1.5 m wide, with straight sections that will funnel bats into traps and nets. Habitat heterogeneity along trails or transects will be common – trail is likely to pass through swamps, up small hills, tree fall gaps, closed canopy. Traps and nets should be deployed based on the 100 m segment system regardless of this variability, with capture success the main consideration (where can good net combinations or traps be set within the 100 m). If the rapid assessment intends to capture diversity of distinct habitats within the survey site, transects should be distributed among them proportional to their availability. Survey effort per habitat will likely be too low for comparisons among them, so data will need to be pooled to characterize the site as a whole.

6. Typical sampling effort required – The minimum sampling effort advocated will be too low to generate complete species lists for most habitats. It is intended as the bare minimum sufficient for comparisons among sites of estimated species richness and assemblage composition. Specifically, observed species richness should be c. 70% of species, and fall around the point where species accumulation curves start to decelerate and approach an asymptote. The harp trap protocol in the most speciose rainforest of the Paleotropics (Krau Wildlife Reserve, Peninsular Malaysia) required c. 200 HTNs to reach an asymptote. The recommended minimum of 70 HTNs yielded around 70% of species (20/28) species (Fig 2). In a study of fragments in surrounding landscape in Malaysia, there were detectable differences in species richness and composition using 15 HTNs, although richness ranged from 40-95% of that estimated for a sample of 200 individuals (Struebig *et al*. 2008). 125 HTNs in logged forest in Sabah approached an asymptote (Struebig *et al*. 2013), and samples of 42 HTNs recorded 71-99% of estimated species richness for all bats (at 200 individuals), and 72-100% of forest-interior species. For these reasons, 70 HTNs should be appropriate and comparable for rapid surveys.
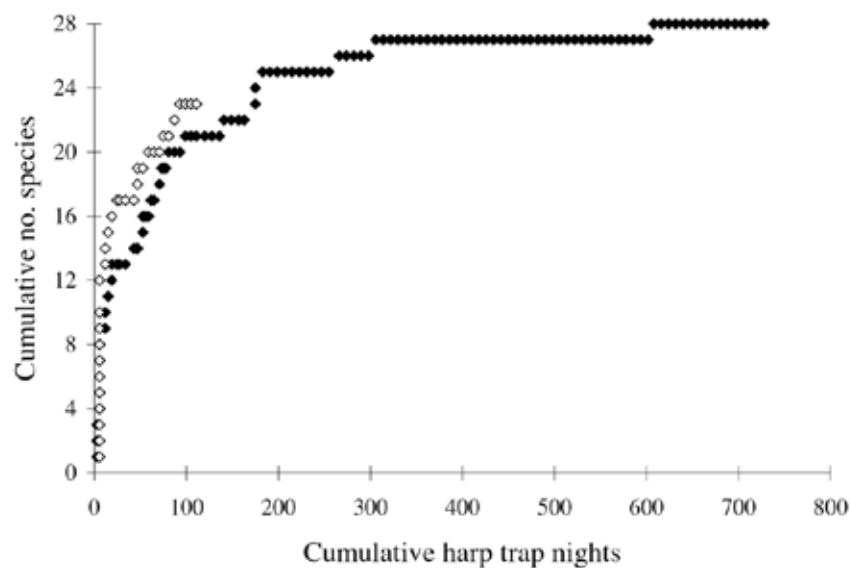

Figure 2
Species accumulation curves for all bat species captured in harp traps in the forest interior of Kuala Lompat, Krau Wildlife Reserve, Peninsular Malaysia. Symbols denote different capture years – closed 1995-1997, open 1999. From Kingston *et al*. 2003.
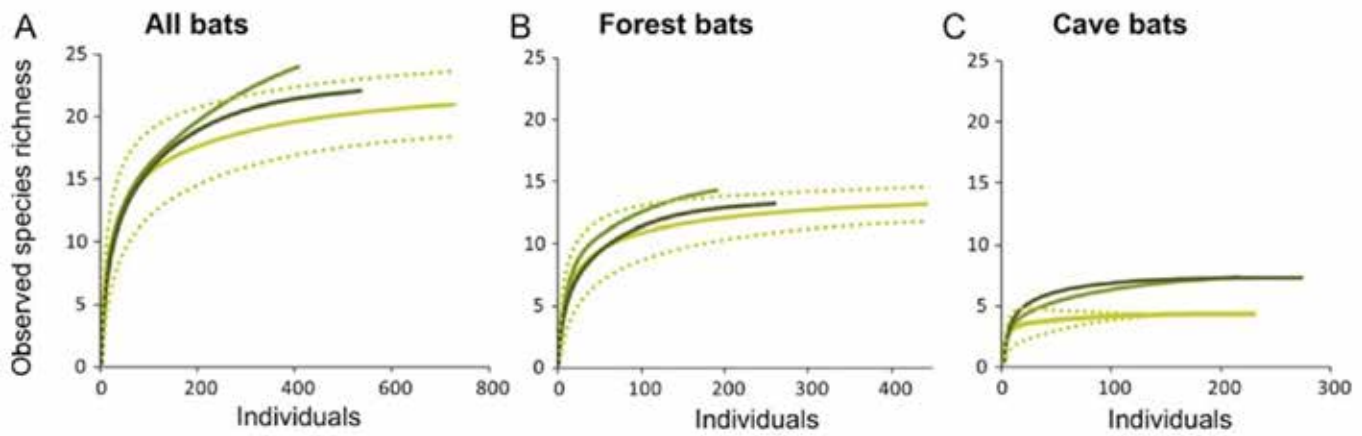
Figure 3
Rarified species-accumulation curves for bat assemblages in old-growth (dark shading; n = 3 sites), twice-logged (intermediate shading; n = 3) and repeatedly logged (light shading n = 6) forest. Curves are derived from sample-based rarefaction rescaled to individuals using pooled data from sites within each forest type (42 HTN per site = 126-252 HTNs per type). Dashed lines indicate the upper and lower 95% confidence limits of the curves for repeatedly logged forest, in which the most sites were sampled. Numbers of individuals at each of the 12 sites ranged from 107 to 214 for all bats and 53 to 97 for forest bats, positioning site-level observed richness around the point where species accumulation starts to decelerate, allowing for between site comparisons. From Struebig et al. 2013.

For mist nets in the Neotropics, 21 SUs (63 mist net nights) is a bare minimum, but should also bring most studies to a point on a rarefied or jackknifed accumulation curve suitable for comparisons of phyllostomid assemblages (Fig. 4).
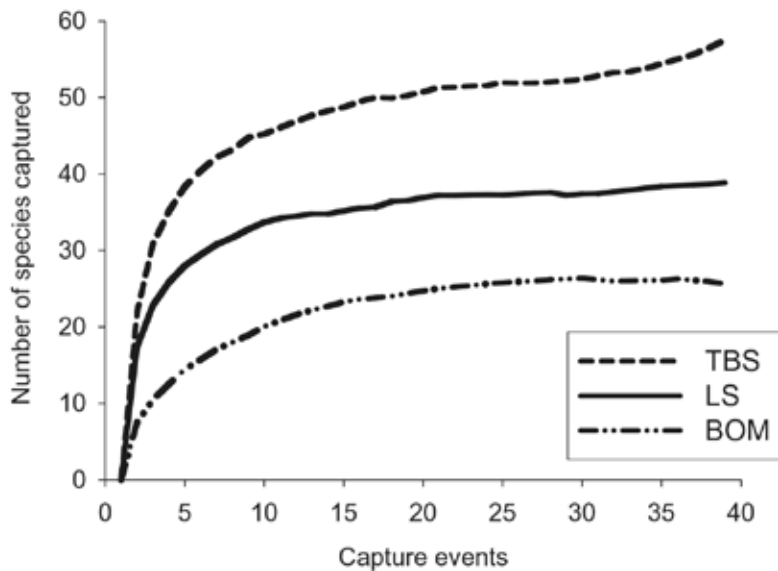

Figure 4
Species accumulation curves for three phyllostomids bat assemblages (La Selva Biological Station, Costa Rica, LS; Tiputini Biodiversity Station, Ecuador, TBS and Bombuscaro River, Podocarpus National Park, Ecuador, BOM) based on Jackknife 2 estimation (from Rex et al. 2008). Each capture event comprises 36m of ground nets (e.g. 2 x 9 m nets + 3 x 6 m nets) and a 9 m vertical canopy/subcanopy net), open for c. 12 hrs, so is somewhat comparable to the 3 SU per night (6 hrs) in this protocol). The 7 nights of 3 SUs of the protocol therefore should fall around the 7 capture events of Rex et al. 2008, as accumulation starts to asymptote.

Context-Dependent Sampling Considerations

7. Sampling considerations – The core methods recommended here are based on best practice in large tracts of lowland tropical rainforest. In fragments of forest or degraded (logged) forest, it may not be possible to physically fit the same number of SUs, and if trails do not exist then they would need to be cut and allowed to settle (4-6 weeks) ahead of trapping.

8. Habitat considerations – Harp traps and mist nets should be set across fly ways. In habitats that are naturally open (savannahs, grasslands) and in human-modified landscapes with more open habitat, utilizing topography (ridges) and any existing vegetation (savannah trees, planted fruit or shade trees) the crop itself (e.g., coffee, cacao) can improve capture rates by funneling bats into the trap/net. Acoustic monitoring is more robust to structural changes in the landscape, although detectability varies among species and can vary within species across habitats (Bader *et al*. 2015).

9. Biogeographic or regional considerations – The core methods are responsive to the evolutionary history of bats in the tropics and reflect the different radiations that have populated bat assemblages in the Old World vs. New World tropics. The Phyllostomidae are restricted to the New World where they radiated from a single insectivorous ancestor to over 200 species in just 28 million years (Agnarsson *et al*. 2011), exhibiting the most rapid trophic diversification ever seen in mammals, with species deploying sanguinivory, insectivory, carnivory, omnivory, nectarivory, palynivory and frugivory (Dumont *et al*. 2012). Echolocation calls are typically multi-harmonic FM sweeps of low intensity and short duration, and species can fail to detect and avoid mist nets. In the Paleotropics, there was no equivalent radiation of echolocating forest-interior plant-visiting bats, but rapid diversification of the insect-eating bats in the Rhinolophidae, Hipposideridae, Murininae (Asia) and Kerivoulinae (Anwarali Khan *et al*. 2010; Agnarsson *et al*. 2011). These lineages solved the problem of prey detection in acoustically cluttered environments, and as a consequence have sophisticated echolocation signal designs that enable them to better detect and avoid mist nets. However, provided harp trap lines are thin (0.18-20 mm, typically 6lb breaking strain) they are readily captured in HT traps. Conversely, harp traps set in neotropical forest capture few phyllostomids.

Aerial insectivores include pantropical families (Emballonuridae, Molossidae), global families (Vespertilionidae) and families restricted to each of the tropics (e.g., Old World– some large species of Hipposideridae and Rhinolophidae, New World – Mormoopidae). There is less biogeographic sampling bias in aerial insectivores when compared to forest interior bats between the Old and New World regions.

10. Seasonal variation – Avoid pronounced rainy seasons, primarily for logistical and bias reasons. Rain can reduce capture success substantially (bats may not fly in heavy rain; nets and traps become detectable because of rain drops), (e.g., de la Peña-Cuéllar *et al*. 2015) and necessitate early net closure for welfare reasons. Moreover, in the Neotropics, the composition of phyllostomid assemblages may differ seasonally (Klingbeil and Willig 2010). Persistent heavy rain can also damage bat detectors and microphones, even for equipment that promises to be weather-proof.

11. Supplementary Methods

- Mist nets

    - Vertical canopy nets (at fruiting/flowering trees), stacked nets (2-8 nets depending on objectives), to capture canopy/mid canopy phyllostomids. The main captures in the Old World will be fruit bats (Pteropodidae), but the most abundant captures are typically disturbance-tolerant species e.g. *Cynopterus* spp. in Asia, *Epomorphorus* spp. in Africa. Species associated with unmodified forest habitats are rare in space and time, reflecting large-scale spatio-temporal variability in fruiting trees (see Kingston 2013).

    - Mist nets and mist-net harp trap combinations over slow-moving shallow rivers and over ponds or water reservoirs (good for getting molossids, river-foraging *Myotis* and other open-space bats needed for call library).

    - Net in local villages and farm gardens for common Old World fruit bats

- Have a hand net for ad-hoc captures at roosts.

- Roost surveys. Review the ecological literature and field guides on possible roost structures used by bats in the survey area (starting with Kunz and Lumsden 2003) and then conduct roost surveys (Simmons and Voss 1998). For example, check hollows of standing and fallen trees (live and dead), cavities behind large strangler-figs, furled leaves, tent-roosts, bamboo stands. Ask local people (if present) if they know of bat roosts in their buildings, farms, nearby caves.

- Cave surveys. Anything from a collection of boulders to a large subterranean system within a karst landscape may harbor bats. A cave survey protocol is provided by SEABCRU http://www.seabcru.org/wp-content/uploads/2013/10/SEABCRU-Cave-Survey-Protocol-FINAL.pdf

- Acoustic methods

    - If an active monitoring detector and an extra person are available, acoustic transects can be walked with spot counts at habitats or microhabitats of interest e.g., streams, forest edges, caves, villages, agricultural lands (e.g., Wordley *et al*. 2015).

Data Management

12. Species identification – Bats are more accurately identifiable than non-volant mammals in the field, and as a charismatic group, keys and illustrated field guides exist for many countries and regions (e.g., Reid 2009 – Central America and Southeast Mexico; Kingston *et al*. 2006 -- Malaysia). However, other countries, especially within Africa, may have no or very outdated keys, and the chances of registering rare or new species in remote or poorly known areas are high. Voucher specimens are then to be obtained in the field to confirm identification and have a physical documentation of the species. This is important even in better-known areas as cryptic bat species are being discovered regularly. A minimum of 1 individual per species per site is recommended, for multi-site surveys this can be 4-10 individuals per species total.

Captured bats are preferably identified and processed the same night. Bats should be carefully taken out from the mist net using gloves or cloth bags and put inside cloth bags. Bats to be released should have good photos showing diagnostic characteristics, for example noseleaf and/or ear structure, and echolocation recordings made in the hand or upon release to contribute to a call library. Standard measurements for bats to be released are: forearm length and body mass, with additional measures such as tibia length useful for discrimination among some species. Age (juvenile or adult) and reproductive condition should also be recorded. Released individuals could be marked by wing punches to avoid inclusion of recaptures in abundance data. Preservation of tissue samples should be considered as well for DNA identification (e.g., barcoding). To avoid stress and dehydration on the animals they should be released the same night of capture.

For voucher specimens, individuals must be euthanized in the fastest and least distressing way possible to the animals (Sikes *et al*. 2011). Several inhaled (e.g., chloroform, ether, isoflorane) and non-inhaled products (e.g., ketamin) in the veterinary field are commercially available, and the American Veterinary Medical Association (AVMA) discusses them at length (Leary *et al*. 2013). AVMA also considers that injectable euthanasia agents are one of the most rapid and reliable methods of performing euthanasia. For example, Halatal (Ketamin 10%), injected directly in the heart in a proper dose euthanizes the specimen almost immediately. In several places these products are restricted, in that case American Society of Mammalogists (ASM) considers acceptable chest compression and cervical dislocation as options (Sikes *et al*. 2011)

If vouchers are taken, they must be fixed properly in formalin (10%) for 7 to 10 days, after sampling a piece of soft tissue (e.g., muscle, liver, kidney) for future DNA analyses (such as DNA barcoding), or prepared as dry skin with preservation in ethanol of the skull and carcass for cleaning of the skeleton, ideally with a dermestid beetle colony. Long-term preservation in ethanol is not recommended because specimens are not properly fixed against enzymatic decomposition and will deteriorate in the long run with the potential loss of valuable material. Specimens in formalin are then washed off after the 7 to 10 days, and preserved in a 70% solution of ethanol. Standard measurements, photos, and other data such as ectoparasites, if needed, are taken before specimen fixation. In the field, dry skin vouchers should be kept safe from insects, domestic dogs and cats, and other potential species or agents (e.g., rain) that can damage them. Further details on collection and specimen preparation are found in Simmons and Voss (2009).

Handling and shipping specimens are as important as other parts of the study. Collecting permits must accompany the specimens, outside and inside the boxes, which must be marked as fragile and a tag of NON-COMMERCIAL VALUE. Formalin and alcohol should be to a minimum - specimens kept wet with cotton and gauze in sealed bags can last many days during shipping.

13. Types of data collected and data management – In the field, specimens should be accompanied by basic habitat data (e.g., shrub, grassland, secondary forest) and/or microhabitat of collection (e.g., ecotone, creek). Standard measurements, age estimation, and reproductive condition (e.g., lactating, pregnant) are recorded. Data should be recorded in water-resistant notebooks (e.g., Rite-In-The-Rain)

with pencil or permanent ink, or on field spreadsheets printed on water-resistant paper. Photos of the specimen, ectoparasites, feces, pollen and other ancillaries are to be collected wherever possible. Sampling effort must be recorded. Otherwise spatial (site-site) or temporal (year-year) comparisons are severely compromised. This requires information about the trap or net dimensions, times opened, closed, weather/moon conditions, locality (geographic coordinates and elevation), proximity to local habitat feature if relevant (e.g., in a swamp).

Sampling sites require a minimum of habitat description, with emphasis on the kind of vegetation, cover, ecotone, etc., and a qualitative estimation of disturbance. Elevation and geographic coordinates should also be taken for each mist net or harp trap site. Photos of the trap sites are also recommended to document the habitat description.

Long term storage of data requires conversion to a digital format (spreadsheet). Researchers should consider adopting Darwin Core Standard so that data can be easily shared with repositories (e.g. GBIF). Darwin Core Terms can be found here: http://rs.tdwg.org/dwc/terms/index.htm, and a "crib sheet" and spreadsheet used by the SEABCRU can be found here http://seabcru.org/seabcru-resources.

Conservation Implications and Limitations

14. Conservation implications of results and utility for detecting change over time and responses to disturbance/environmental change – Bats comprise about 20% of mammalian species diversity (intrinsic value) and provide key ecosystem services as agents of pest suppression, seed dispersal and pollination, and guano is a nutrient-rich fertilizer. Bats are diverse, long-lived, but have low reproductive rates and are globally highly vulnerable to human modification of habitats (Voigt and Kingston 2015). Studies of unmodified habitats are critical for evaluating responses to different forms of anthropogenic disturbance and assessing success of management approaches intended to maximize diversity in human-dominated landscapes. Caution is warranted, however, because bat assemblages can show substantial spatial and temporal variability within unmodified habitats (Kingston 2013). This background spatio-temporal variability can make it difficult to attribute modest differences in assemblage composition to specific treatments or processes with any certainty. Multiple, spatially independent, rapid surveys of the same focal baseline area can capture this natural variability and allow for more certain interpretations of differences among assemblages.

Although bat research is thriving in some countries, with country-level keys and good national and international museum collections, in others rapid surveys will make a direct contribution to knowledge of bat diversity, taxonomy and systematics. This is especially true for much of Africa, where capacity is limited or lacking and existing knowledge often pre-dates current survey techniques (mist-nets, harp traps, acoustics etc.). Species discovery through rapid surveys is likely to be substantial. For example, in Vietnam 51 bat species were known in 1994, but with development of new capacity and international interest, by 2014 125 species including 21 new species were reported, and at least 3 bat PhDs are in institutional positions (Vu Dinh Thong, personal communication).

15. Constraints and limitations for rapid survey – Given the great diversity of bats in tropical habitats, the survey effort possible in 7 days of a rapid survey is close to the minimum required to establish baselines suitable for comparisons among sites and through time. In much of SE Asia, rain is probable even in "dry" seasons, so some of the 70 HTNs may be lost. Comparisons at 40-50 HTNs are probably still valid, but further loss of effort would likely confound comparisons. Neotropical and African dry seasons are less subject to severe rain, so this is likely less a problem.

Harp traps can be rather cumbersome to transport if the study site is very remote. Trail systems are needed for mist nets and harp traps. While these commonly exist because of local people's use of the forest, if there are no trails these will need to be cut in, ideally ahead of time. This problem is resolved if large mammal survey transects (3 x 4-km transects) are available. All bat researchers on project need pre-exposure rabies vaccination. Costs vary enormously across world and need c. 1 month ahead of survey for the 3-shot series. It is important that local counterpart students/scientists are vaccinated.

Acknowledgments

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Appendix I – Harp trap manual

PURPOSE

- To capture species that can detect and avoid mist-nets; typically forest interior species with high-frequency and/or low intensity echolocation (e.g. Kerivoulinae, Hipposideridae, Rhinolophidae).

- Can be set across forest trails and streams, at cave exits, and across larger rivers in combination with mist nets.

CONSTRUCTION

- Harp traps can be bought commercially, but can be costly and heavy. The MBCRU uses a light-weight collapsible four-bank harp trap (which maximizes the capture rate) based on the design of Francis (1990), which consists of 7 components:

- Harp trap assembled viewed from the front side (see Figs 12 & 13).

THE BANKS (quantity 8 – forming 4 banks)

- These are the horizontal bars between which the fishing line is tied.

- Made from aluminum tubing 48 cm long and 1.5 cm in diameter.

- The holes in the bars to accommodate the fishing line are spaced 2.5 cm apart (see Fig 14).

- At each end is a protruding part which fits into the cross piece of the top and bottom corners. They extend 2.8 cm past the end of the tubing (see Fig 15a,b).

- At one end they must be made with a sliding action (see Fig 15a).

THE LEGS (quantity 4)

- The legs are adjustable (Fig 16). This is achieved by having a rod inserted inside a tube and fixed in position by a wing (thumb) screw, with the nut (screw thread) mounted on the outer tube (see Fig 16a).

- Outer tube 76 cm in length and 1.8cm in diameter.

- Inner rod (solid) 75 cm in length and 1.5 cm in diameter.

BOTTOM CORNERS (quantity 2)

- This piece is the most complicated to make (and to explain!).

- The banks are secured to the cross-piece nearest the bottom of the corner-piece (see Fig 17 & 18).

- The harp trap bag is held in position on the higher cross piece (see Fig 17 & 18).

- Leg holders. These should be splayed to give the trap stability (see Fig 17).

- A horizontal pole is attached to the horizontal section and held in place with a nut and bolt. (see Fig 19)

- A vertical pole sits over the solid rod protruding from the vertical section (see Fig 20).

DIMENSIONS FOR BOTTOM CORNERS

- Horizontal section; Length 8.5 cm Diameter 2.5 cm. Receives solid rod of lower horizontal pole.

- Upright section; made up of a solid rod, which sits in a vertical tube. The visible length of the rod is 13 cm (but extends further down into the tube) with a diameter of 2.2 cm. The solid rod is long to allow the vertical poles to be set at different distances from the base to maintain the tension of the fishing line banks as they age and stretch. The length of the hollow tube is 21 cm with a diameter of 2.5 cm. Inserts into vertical pole.

- Bank cross piece. Length 27 cm, width 0.6 cm, height 2.5 cm. Holes spaced 6 cm apart and 1.6 cm diameter

- Bag holder cross piece. Length 40.5 cm, width 0.6 cm, height 2.5 cm. The 4 indentations are 1.3 cm across and spaced 1 cm apart. The first indentation (closest to vertical pole) is 11 cm from the vertical pole.

- Leg holders. Length 18 cm, diameter 2.5 cm, angle up to you, but it has to be stable.

TOP CORNERS (quantity 2)

- The banks are secured at the top of the frame in the same way as at the bottom (see Fig 21).
- Vertical section; made up of a solid rod inserted into a hollow tube. The exposed portion of the rod measures 6 cm with a diameter of 2.2 cm and the tube measures 10 cm with a diameter of 2.5 cm (see Fig 21 & 22).
- Horizontal section. Length 8.5 cm, diameter 2.5 cm (see Fig 22). Receives solid rod of upper horizontal pole.
- Bank cross piece. Length 27 cm, width 0.6 cm, height 2.5 cm. Holes spaced 6 cm apart and 1.6 cm diameter.

VERTICAL SIDES - together with the horizontal sides these make up the rectangular frame of the trap (quantity 2)

- These are tubes with wing nuts mounted at each end (see Fig 23). Receive the solid rods of the top and bottom corner pieces.
- Length 154 cm ,and diameter 2.5 cm.

HORIZONTAL SIDES (quantity 2)

- These again are tubes but have solid aluminum rods in the ends, and are connected to the upper and lower corner pieces with a nut and bolt (see Fig 24, Fig 25).
- Length 133 cm and diameter 2.5 cm. The visible part of the rods measures about 6 cm with a diameter of 2.2 cm (Fig 25).

THE BAG (see Fig 26)

- Should be made from light man-made fiber (so it dries quickly).
- The bag is supported on the trap by 2 rods threaded through the top edge (see Fig 27, 28), that then sit on the bag holder notches of the bottom corner pieces.
- On the inside the bag has a clear plastic (polythene) flap that extends about 2/3 of the depth of the bag. It is sewn at the top to ensure the bats do not climb out, but the bottom edge is left open so the bats can crawl up under the flaps for shelter (Fig 27).
- The bag should be made to fit the trap. Our design has a panel at each end with various slits to accommodate the corner piece.

USER'S TIPS

CHOICE OF MATERIALS

- Aluminum is light, relatively strong and doesn't rust. However, aluminum welding is difficult and we have found very few good welders in Malaysia who don't charge expensive rates.
- Steel is the most likely alternatively (although someone recently mentioned a trap made of PVC piping) and the main disadvantage with this is the weight. However, it isn't that much heavier and one person can still carry a steel trap with relative ease, but it depends on the distances you are likely to cover. Steel is cheaper, easier to weld and in good supply pretty much everywhere. It does rust, but regular coatings of WD40 help minimize this.

CHOICE OF TRAP SITE

- Place traps across trails that have been cut at least 2 months previously - the longer the better. Bats use trails as fly-ways so they learn where they are.
- Picking the sites for the traps is crucial. The site should have vegetation at both sides (the denser the better) and above.
- Always block in your trap using vegetation (rattan, palm, small trees) so the bat is forced to go into the trap (see trap position photo).
- Move the traps every night, catch rates drop dramatically after the first night.

BITS AND BOBS

- Always have lots of wing-nut and nut and bolt spares as they go missing inexplicably!

- The fishing line should have 6lb breakage - thin enough so the bats don't detect it, but strong enough that you are not restringing every day.

- The fishing line should have just the right amount of tension – it should be tight as possible but without bowing the banks. It stretches over time, so to adjust tension the vertical poles should be extended by sliding up along the solid rod of the bottom corner pieces and clamped into place with the wing-nuts. Usually about 5-8 cm extension will increase the tension enough.

- Strings break for all sorts of reasons - bats, birds, head torches, during transport. Try to keep up with repairs.

- When the trap is set put leaves over the ends of the bags to stop the craftier bats from escaping (some of the more agile flyers can) – there is a slight gap between the end of the banks and the bag edge.

- Torque – when the traps are being set they may twist because of the tension of all the strings being uneven, and the rotation about the vertical axes of the vertical poles (because using wing-nuts) (can see this in Fig 13). This is normal (a slight design flaw) and can be countered when you set the trap by adjusting the leg lengths until all the torque is reduced and the banks are all in line.

- We usually rest the harp trap bag poles on the second notch/indentation (second in from the middle). This means that bats sliding down the outer bank of strings will escape BUT in our experience, they usually pass through this bank and are trapped between the other banks. Many of the tropical forest bats are such manoeuvrable fliers that they will be able escape if a gap is left between the bag and the outer bank.

Photo © Trond H. Larsen

Figure 12a

Figure 12b

Figure 13

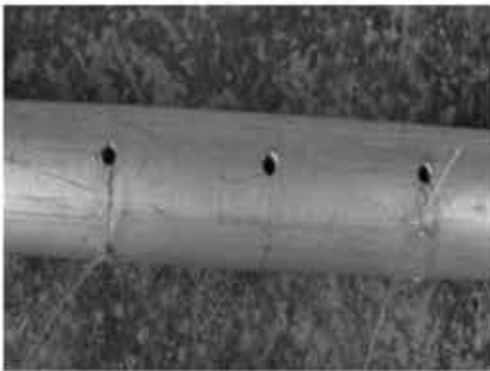Figure 14

Figure 15a

Figure 15b
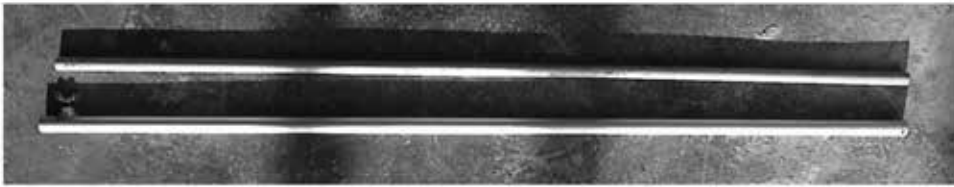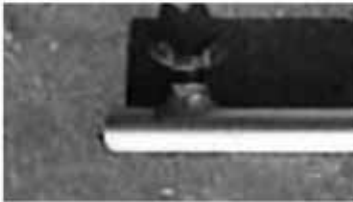
THE LEGS

Figure 16
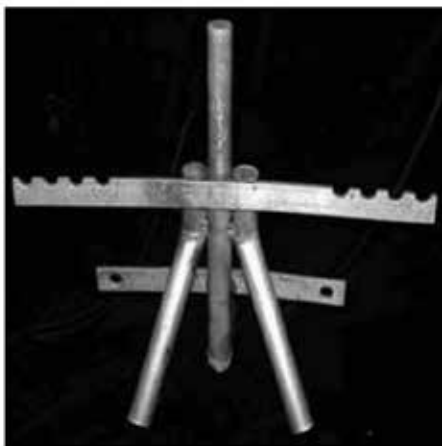

Figure 16a

Figure 17

Bag cross piece

Bank cross piece

leg holders

Figure 18
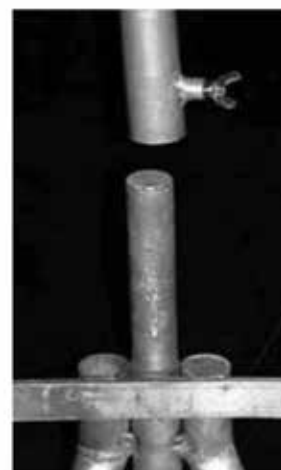

Figure 19

Figure 20

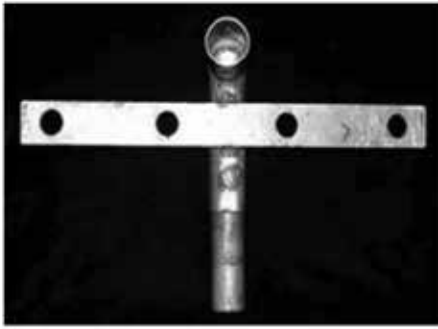## TOP CORNERS

Figure 21

Figure 22

VERTICAL POLES

Figure 23
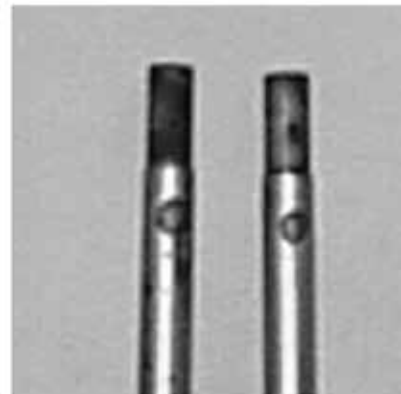
wing nuts

HORIZONTAL POLES

Figure 24

Figure 25

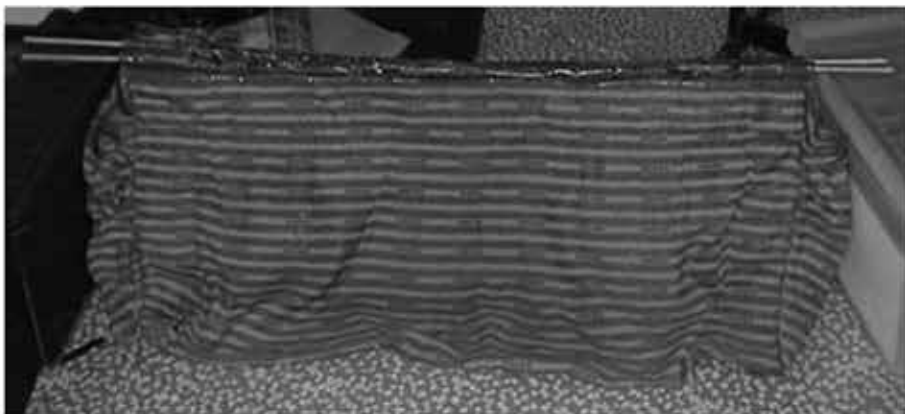## THE BAG

Figure 26
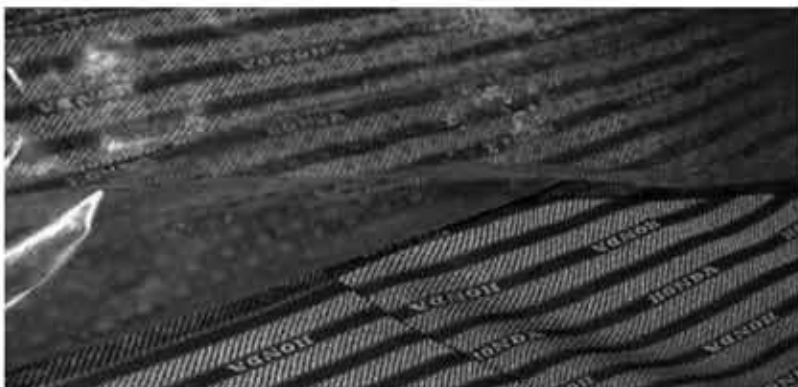
Figure 27
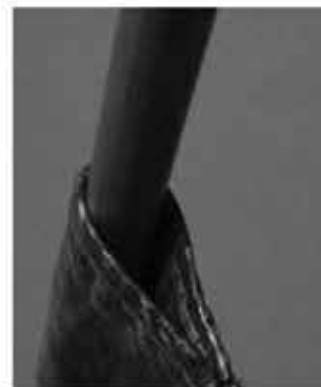
Figure 28

TRAP POSITION

SMALL NON-VOLANT MAMMALS

SMALL NON-VOLANT MAMMALS

Burton K. Lim[1] and Victor Pacheco[2]

Definition

Small non-volant mammals are defined as species that are approximately less than 1 kg in weight in groups such as rodents, marsupials, shrews, and tree shrews. However, there is overlap in size with some opossums being larger and species may overlap with more than one survey methodology such as squirrels being documented by live trapping of small animals, during walking line transects of large arboreal species, and during camera-trap surveys. Notwithstanding a few exceptions, small non-volant mammals are nocturnal and difficult to identify without live-trapping methods.

Suitability

This group is suitable for rapid biological surveys because small non-volant mammals represent over half of the mammalian species diversity in the world. Many species are also relatively abundant and can be documented by live trapping which makes them amenable to standardized analytical methods of study. Some species, such as rats and mice, can also be used as indicator taxa of disturbed habitats. They usually have small geographic ranges and species turnover can be high between sites, which makes small non-volant mammals suitable for identifying unique regions of endemism. Although represented by only a small proportion (<10%), a few in this group are endemic to specific habitats or elevations and have restricted distributions that make them of conservation concern. Small non-volant mammals are also the primary prey species for many vertebrate predators, which in turn may belong to the most threatened or endangered species in the world. In addition, several species of rats and mice are implicated as reservoirs of important emergent diseases.

[1] Department of Natural History
Royal Ontario Museum
100 Queen's Park
Toronto, ON M5S 2C6 Canada

[2] Departamento de Mastozoología,
Museo de Historia Natural,
Universidad Nacional Mayor de San Marcos, Av.
Arenales 1256, Lima 14, Lima, Perú

Core Methods

Live-trap transects

Although there are many sampling protocols, such as those discussed later in supplementary techniques, the core method for standardized rapid surveys is defined as a line transect of large (9" x 3" x 3.5") aluminum box-style live traps (e.g., Sherman LFA Folding Trap, Longworth). An outline of a trapping procedure is given below and ideally suited for 2 experienced biologists. If personnel and budget is not constrained, the core method can be increased at a percentage that facilitates scaling for standardization purposes and supplementary techniques are highly recommended.

• Along each transect, 2 traps set every 10 meters (e.g., 1 trap every 5 m or 2 traps at a station every 10 m to standardize for different methods of trap setting).

• 3 transects of 200 m or 2 transects of 300 m with transects radiating out from camp to maximize coverage; unlike relatively intact habitats, for areas that are more disturbed and to avoid noise disturbance, transects may be placed at a considerable distance from camp.

• Minimum of 120 traps per night of sampling effort, but whenever logistically possible should be increased by a convenient factor (e.g., 50% or 100%) for standardization purposes among studies. For rapid surveys, each locality should be sampled for at least 5 nights or ideally for 1 week. Some preliminary inventories of a habitat recommend more trap-nights of effort (Fraser *et al*. 2003, Jones *et al*. 1996), but this may not be feasible for monetary reasons (see Box 1 for cost of traps).

• Whenever possible, traps should be set off the ground to sample arboreal species traveling on vines, tree trunks, or low branches (flagging tape or twine can be used to secure the traps to the branch or vine) because this micro-habitat will not always be available at every potential trap site; for example, if 2 traps are set every 10 metres then one can be set on and the other off the ground, or alternate traps on and off the ground every 5 metres when possible.

• Traps on the ground should be set in areas where animals may be foraging, such as at the base of large trees, or along likely corridors of movement, such as along tree falls, and on top of logs.

• In closed forested habitats, animals will not be typically foraging in open areas, and will be wary of predators and keep closer to trees or logs for cover. Consequently, traps should be set with the open door facing trees or logs at about the same distance as the width of the door (e.g., 3" for large Sherman traps)

• In open grassland habitats, traps should be placed along possible foraging runways on the ground or near potential cover such as shrubs or solitary trees

• Check that the trigger is properly set on each trap by testing the treadle sensitivity with your hand and adjusting the trigger accordingly

• Every trap is marked by a piece of 8" flagging tape numbered sequentially with a waterproof marker and tied to nearby vegetation.

- Traps should all be baited, for example with raw unsalted unshelled seeds such as sunflower or a mixture of rolled oats, peanut butter, honey, bacon fat, etc.; if seeds are used, scatter a few (not many, ~12) in front of the trap and leading into the back of the trap; if a mixture bait is used, it should be placed on a piece of paper at the back of the trap to make removal and cleaning easier; seeds are recommended as the default bait as this is usually readily available in food markets; a secondary bait is dried corn kernels or rice. In the tropics the oily, shaved-off pericarp of oilpalm nuts (*Elaeis guineensis*) and bananas are also good bait, either alone or in the mixture with rolled oats, especially for marsupials.

- Trap lines should be checked in the early morning before it gets too hot or before ants discover the animals in the traps. In addition it is suggested to check traps, once or twice later in the day, if they are left open for diurnal species.

- In temperate or cooler regions, bedding material such as cotton should be put in the trap

- Traps should be rebaited if heavy rain has spoiled the bait or bait has been eaten by ants, which may be a daily occurrence in some areas; if this becomes a problem, traps can be baited in the late afternoon. Alternatively, bait could be wrapped with cheesecloth to retard ant activity.

- Normally traps can be left open during the day for possible diurnal mammals, but if by-capture of non-target groups such as reptiles is detrimental then traps can be opened and baited in the late afternoon

- If a trap has caught an animal, it should ideally be processed (examined, marked and released) on the spot. Alternatively, the trap should be replaced by a spare trap or by a small piece of flagging and the trap (with the animal inside) brought back to camp for specimen processing and data recording (the flagging acts as a reminder of where the trap should be reset the next day). The removed trap can also be identified by writing the station number and transect on the trap, but this may be difficult in wet or cold conditions.

Sampling Considerations

Sampling sites should be selected based on the objectives of the study including if comparisons between sites or habitats are important. Traps are set within walking distance of camp (e.g., 1-2 km) on transect lines that should in general include different habitat types such as well-drained forest, swamps, and creeks, if spatial heterogeneity is of interest. General comparisons of sites are also useful for assessing environmental disturbances or change against pristine or baseline habitats. However, specific habitat comparisons may be of primary importance, so transects should usually be confined to particular, distinct habitat types. Transects may need to be shorter but replicated across smaller, patchy habitat mosaics where space is limited. Environmental disturbance may also result in fragmented habitats that are too small to accommodate a complete transect. If the intervening disturbed areas are not important to the survey, then this will also necessitate the partitioning of transects.

This small non-volant mammal sampling protocol could be implementable in all biogeographic regions of the tropical or temperate climatic zones. An exception in temperate areas is when there may be a greater need to take into account seasonality and the possibility of hibernation or topor in animals, depending on latitudinal conditions. In tropical areas, seasonal considerations may include the effects of the rainy season, especially in low-lying or semi-inundated areas where trapping effort may be adversely affected because transects are flooded or bait washed away. This may negatively bias the calculation of relative abundance and species diversity. Also, logistically it is in general more difficult to do fieldwork in the rainy season and many species of mammals may be reproductively synchronized at this time of year if the trapping of pregnant females is of possible concern. However, an objective of the study may be to ascertain seasonal variation or regional differences.

Trap-nights, defined as the number of traps times the number of nights the traps were set, form the measure of effort for small non-volant mammals. This standardized unit allows the comparison of different sites and different studies. In addition, relative abundance and species indices can also be calculated. However, trap success, the number of traps that catch an animal divided by the number of total traps set, can be quite variable throughout the world. For example, rat trapping in the Yucatan Peninsula can easily yield 50% trap success on any given night.
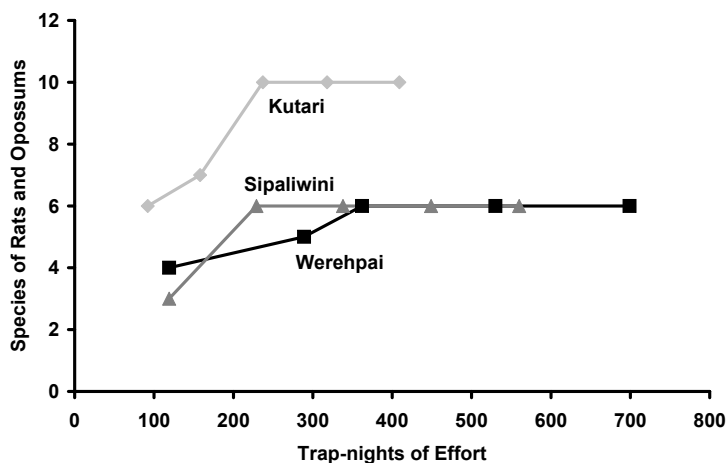

Figure 1
Species accumulation curves of small non-volant mammals at 3 sites in southern Suriname (Lim and Joemratie, 2011).

By contrast, success rates in the Guiana Shield region are notoriously poor in terms of both relative abundance and species diversity. One exceptional survey at Kawamalasamutu in Suriname caught 152 small non-volant mammals that documented 12 of 28 species expected in the area (Lim and Joemratie, 2011). Similar surveys in other parts of the Guianas have had a fraction of this trapping success with 11 individuals of 5 species in the Eastern Kanuku Mountains of Guyana (Lim and Norman, 2002) and 20 individuals of 11 species in the Upper Palumeu River of Suriname (Lim and Banda, 2012). However, the species accumulation curve for Kawamalasamutu had reached an asymptote after the 3rd night of survey at each of the 3 surveyed sites (Fig. 1). These site surveys were of 5-nights duration, which was a minimum to confirm the leveling off of the curve with the standardized field methods used. If more time is available at each site, then it may be worth investing effort into implementing specialized secondary methods to more completely inventory species diversity. But usually time is the limiting factor in rapid assessment surveys so comparisons are done using extrapolation methods.

Supplemental Methods

When possible, pitfall traps should be complementary to live traps that target the more typical terrestrial fauna. Pitfalls over longer periods (so possibly not ideal for surveys of shorter duration) are good at documenting scansorial species such as mouse opossums and arboreal rats, shrews and short-tailed opossums that are foraging on the ground. However, pitfalls require quite a bit of work to set up, requiring the digging of large holes for 20-liter buckets. Pitfall traps need to be set along their own dedicated transect and separated from the trapping lines. The traps should be checked in the early morning. Sampling effort is measured as Bucket-nights, defined as the number of buckets times the number of nights. This method may be efficiently used in conjunction with a herpetological or entomological survey because pitfalls also catch amphibians, reptiles and beetles etc. Pitfall traps are especially effective at sampling shrews and terrestrial opossums.

Snap traps complement or may be a more efficient method than live traps, which may be hindered by "trap shyness" whereby animals may be skittish to enter a foreign (aluminum) enclosure or may require a longer period of time to become familiarized with or accustomed to a trap in its environment. Snap traps can be used as the second trap at a station every 10 meters or used as the sampling unit in its own dedicated transect line.

Extra-large aluminum box-style live traps (e.g., XLK or XLF15 Sherman, 201 - Collapsible Trap - Chipmunk/Gopher/Rat Size or equivalent Longworth traps) may more easily catch larger-sized animals such as spiny rats (*Proechimys*) in the Neotropics or giant rats (*Leopoldamys*) in the Paleotropics, although some can squeeze themselves into the large-sized traps. Extra-large Sherman traps typically come in a box of 20 traps so these can be set as a unit within a transect depending on the expectation of catching larger rats.

Larger wire box-style live traps (e.g., Tomahawk 203 or National) target larger animals such as opossums that are not typically caught in smaller traps in the Neotropics (Voss *et al.* 2001). In the Paleotropics, they are needed for Giant pouched rats (*Cricetomys* sp.) or smaller porcupines (e.g. *Atherurus africanus*). If this method is used, a standard protocol is to set 4-6 traps evenly among the 2 or 3 trap lines, or more opportunistically on runways and at burrow entrances detected with help of local hunters/guides.

Traps should be set ideally at the base of large trees with vines reaching up to the canopy. Sardines or other suitably odoriferous bait such as bananas can be used to attract the omnivorous scavengers. Line the bottom of the trap with leaves to minimize exposure of the wire bottom. Mark the trap with a long piece (3') of flagging tape tied to nearby vegetation to make it obvious and to differentiate it from a standard live-trap transect line.

Voss and Emmons (1996) and Kirkland and Sheppard (1994) described the pitfall methodology. The only suggested modification is that buckets are dug into the ground every 10 m for 100 metres and a plastic sheeting driftfence is used to direct animals into the buckets. Holes should be punched into the bottom of the buckets to prevent a rising water table to displace the buckets and to allow drainage to minimize the amount of standing water on the bottom. Ideally, buckets with lids should be purchased to allow the closing/covering of the pitfalls or rain covers can be constructed from small boards and rocks as spacers that keep rain out but allow small animals to still enter the buckets.

Data Management

Identification of Species

Species identification for small mammals usually requires that vouchers are obtained in the field as a representative collection of species diversity, unless a well-trained researcher and comprehensive regional faunal key are available to confidently identify species. However, species identification in the field of small non-volant mammals is not trivial, and almost impossible, for example for African shrews, and the percentage of misidentification could be high even among experienced biologists. Capturing and releasing specimens should be considered carefully and identification must rely on expert opinion, access to keys of external traits and illustrated books. Good photos of the specimens showing diagnostic characteristics are an essential complement to confirm the identification. Standard measurements (total, tail, foot, and ear lengths) and weight should be obtained and recorded. Released specimens could be marked by toe/ear clipping if subsequent assessment or monitoring is considered. Preservation of tissues samples could be considered as well for DNA identification (such as the International Barcode of Life genetic-based identification system for all species of animals); toe/ear/wing clips are good sources for DNA.

Specimen Preparation

If voucher specimens are to be preserved (Hall 1962, Nagorsen & Peterson 1980), this request must be included in the research application and animal use protocols must be obtained ahead of time. Specimens must be euthanized in the fastest and least distressful way possible to animals (Sikes *et al*., 2011). Several inhalants (e.g., isoflurane, Halothane) are commercially available. In addition, non-inhalant products in the veterinary field are also available, e.g., Halatal (Ketamin 10%), and are injected directly into the heart that euthanizes the animal almost immediately (AVMA, 2013). In several countries, these products are restricted, however, the American Society of Mammalogists considers thoracic compression and cervical dislocation as acceptable options for humane euthanasia for small mammals (Sikes *et al*., 2011).

Standard measurements, photos (before euthanasia), and other data are taken before specimen preparation (e.g., Yates *et al.*, 1996). The simplest specimen preparation method is preserving in formalin (10%) to stop enzymatic breakdown. Specimens can also be prepared as dried skins with associated skull and skeletons, which are important identification materials. However, this preparation type requires experience and a dermestid beetle colony facility to clean the skinned carcass down to the skeleton and technical lab assistance to complete this labour intensive collection management procedure. Preservation in only ethanol is not recommended because specimens will deteriorate over time with the potential loss of valuable material without fixation in formalin. In all cases, it is now standard procedure to preserve a pea-sized piece of soft tissue sample in a 2-ml vial of 95% ethanol (or hypotonic buffer, but this preservation may be of shorter term) for DNA analysis. Ideally, and if possible, tissue samples for genetic study should be frozen in liquid nitrogen as new methodologies such as transcriptome analyses require higher quality molecular preservation, and this is the best long-term storage method. In the field, vouchers should be kept safe from ants, domestic dogs and cats, and other potential pest species that can damage them.

Handling and shipping of specimens are as important as other parts of the study. Collecting, exporting, and importing permits must be obtained and accompany the specimens as an attachment both outside and inside the boxes for transportation, which must be marked as fragile and with a tag of NON-COMMERCIAL VALUE. Excess formalin and alcohol should be removed and specimens kept moist with cotton and gauze in heat-sealed (if possible) plastic bags that can retain the moisture for several days before arrival at its final destination of deposition. Small mammals from certain areas (African rodents, Malagasy tenrecs) are of special interest to the CDC and require separate permits for importation to the USA.

Data Recording

In the field, general survey locality habitat (e.g., shrub, grassland, secondary forest) and/or microhabitat of collection (e.g., inside burrows, at the base of a tree, in a branch) should be documented. Standard measurements, age estimation, and reproductive condition (e.g., lactating, pregnant, scrotal testicles, etc.) are recorded in a catalogue or journal. Data should be written in water-resistant notebooks with pencil or permanent ink and later input to a database or spreadsheet. Photos of the specimen, ectoparasites, feces and other ancillary data are to be collected if possible.

Trapping sites or transects may require more detailed habitat description, with emphasis on the kind of vegetation, ecotone, and type of soil; and a qualitative estimation of disturbance. Elevation and geographic coordinates are also standard information, and must be taken for each transect. Some also take them for each trap station or have transects tracked with a GPS unit. Photos of the transects are also recommended to document the habitat description. A journal to record daily trapping effort, including types of bait, number and kind of traps, and success rate is strongly suggested (see Hall 1963 for recording standards used by the American Society of Mammalogists).

Conservation Implications and Limitations

Small non-volant mammals are ecologically important in that most are prey species for many different kinds of predators ranging from wild cats to snakes. Changes in their species diversity and relative abundance can have a profound effect on large predators that may be of conservation concern. They may also be indicators of human disturbance, as invasive species such as house rats (*Rattus rattus*) and house mice (*Mus musculus*) typically out-compete the indigenous fauna and can become established in secondary modified habitats. In addition, some species are endemic to specific habitats or elevations, making them a high conservation priority.

One limitation is that overall species diversity of small non-volant mammals is high, but trap success may be low in some parts of the world due to "trap shyness" so many species may not be detected in a rapid survey. This can be partially offset by incorporating snap traps, but is obviously not a suitable method if a representative collection is not being made, or using other supplemental techniques such as pitfall traps that target other groups such as scansorial species. The investigator needs to do a preliminary assessment of the objectives of the study and the appropriate field methodology to employ based on the amount of time and personnel available.

BOX 1: Examples of Equipment and Supplies

Core Methods:
- Sherman large LFATDG Folding Live Trap (120 @ $19.04 = $2,284.80)
- Alternatively for tropical environments with long-tailed rodents such as kangaroo rats: Sherman extra-large XLK Folding Live Trap (120 @ $25.59 = $3,070.80)
- Bait (raw, unsalted, unshelled seeds; e.g., sunflower seeds from local market; oil palm nuts in the tropics)
- Flagging tape (1 roll)
- Waterproof marker

Secondary Methods:
- Forestry Suppliers Museum Snap Trap (30 @ $6.95 = $208.50)
- Sherman XLF15 Folding Live Trap (40 @ $33.71 = $1,348.40)
- Tomahawk 204 Single Door Collapsible Live Trap (6 @ $45.48 = $272.88)
- 201 - Collapsible Trap - Chipmunk/Gopher/Rat Size (6 @ $ 37.86 = $ 227.16) or Tomahawk 203 Double Door Collapsible Live Trap (6 @ $49.26 = $295.56)
- Pitfalls – 11 20-liter buckets, plastic sheeting (100 m X 0.5 m), shovel, machete, staple gun or hammer and nails for securing plastic to wooden stakes that can be cut from staplings

Specimen Preparation:
- Field notebooks and catalogue sheets (water resistant paper)
- Indelible ink pens (e.g. Pigma Micron or Rotring Tikky Graphic) and pencils
- 12" ruler and/or tape measure for larger species
- Pesola scales (30g, 100 g, 500 g and 1000g)
- 2-ml tissue vials
- 95% ethanol
- Formaldehyde (dilute to 10%)
- Anesthetic (Isoflurane, Halothane etc.)
- Forceps (fine tipped)
- Scissors (fine tipped)
- Cotton tags for alcohol and dry specimens   See Wilson *et al*. (1997) for more complete list of field equipment and supplies.

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BIRDS

TOWARD A STANDARDIZED PROTOCOL FOR RAPID SURVEYS OF TERRESTRIAL BIRD COMMUNITIES

Sebastian K. Herzog[1], Brian J. O'Shea[2] and Tatiana Pequeño[3]

Introduction

Birds are found on all continents, throughout the oceans, and in virtually all terrestrial and aquatic habitats – there are few places on Earth where birds do not regularly occur. They play important roles in many ecosystems and contribute to ecological processes such as pollination, seed dispersal, and biological control (e.g. Şekercioğlu 2006). Some species are important to local indigenous communities as sources of protein, contributing to food security. Recreational bird watching is a rapidly growing sector of the international tourism industry, and tourism revenue can provide an important contribution to local and regional economies. Tens of thousands of recreational birdwatchers have also become citizen scientists by uploading their observational records to global (e.g. eBird) and national (e.g. WikiAves) open access online portals and data sharing networks.

Birds are ideal subjects for rapid biodiversity surveys. They are perhaps the best known group of organisms in terms of their taxonomy, biology, ecology, biogeography, and conservation status. Most species are diurnal and easy to identify under field conditions relative to other taxonomic groups, and nearly complete species lists can be produced during a rapid survey. The limited time and effort required for post-survey data processing allows for rapid data analysis. Birds are amenable to a variety of standardized survey methods (Bibby *et al*. 2000) and are highly cost-effective to sample (Gardner *et al*. 2008, Kessler *et al*. 2011). They are widely recognized as indicators of ecological integrity due to their habitat specificity and rapid responses to human impacts from local to regional scales (e.g. Furness and Greenwood 1993, Niemi and McDonald 2004). The global conservation status of all species is updated at regular intervals and many represent species of conservation concern (13% of extant species; BirdLife International 2013). Consequently, priority areas for biodiversity conservation have often been identified largely based on birds (e.g. the Endemic Bird Area – EBA – and Important Bird Area – IBA – frameworks of BirdLife International).

[1] Dr. Sebastian K. Herzog
Scientific Director
Asociación Armonía
Ave. Lomas de Arena 400, Casilla 3566
Santa Cruz de la Sierra - Bolivia
Tel/Fax: +591-(0)3-3568808
Email: skherzog@armonia-bo.org
www.armoniabolivia.org

[2] Brian J. O'Shea
Research and Collections
North Carolina Museum of Natural Sciences
11 W. Jones St.
Raleigh, NC 27601
Email: brian.oshea@naturalsciences.org

[3] Tatiana Z. Pequeño Saco
Directora de Desarrollo Institucional
Centro de Conservación, Investigación y Manejo
de Áreas Naturales - Cordillera Azul
(CIMA-Cordillera Azul)
Av. A. Benavides 1238 Of. 601 Miraflores, Lima 18
Tel: (511) 241-2291; 241-2295
www.cima.org.pe

A variety of resources exist to facilitate the identification of birds by both sight and sound. A similarly wide range of methodological approaches have been developed to count birds and quantify abundance and community composition (Bibby *et al*. 2000). However, not all of these approaches are suitable for rapid surveys because they are too time- or labor-intensive. In addition, different rapid assessment surveys have used different methods, limiting the comparability of results across surveys. The scope of this chapter therefore is to recommend a core set of standardized sampling protocols for rapid assessments of terrestrial bird communities that can be applied under most conditions worldwide.

General Approaches to Surveying Birds

Established methods can be broken down into four general categories: (1) audiovisual methods; (2) sound recording (acoustic documentation); (3) mist netting; and (4) specimen collecting. Strengths and caveats of these methods are summarized in Table 1. The most commonly used audiovisual methods in the tropics are point counts, line transects, and the species-list method (e.g. Poulsen *et al*. 1997, Fjeldså 1999, Haselmayer and Quinn 2000, Herzog *et al*. 2002, 2005, Söderström *et al*. 2003, O'Dea *et al*. 2004, Abrahamczyk *et al*. 2008, Clough *et al*. 2009, MacLeod *et al*. 2011), owing their popularity to time- and cost-effectiveness. Sound recording is an integral part of these methods, as it provides documentation of a large number of species, and enables analysis and identification of unknown vocalizations after surveys are completed. In many tropical environments, especially forest habitats, most bird species are much more often heard than seen. The recent development of automated sound recording technology (Brandes 2008) is likely to lead to an increased use of autonomous recording stations as a stand-alone method for documenting and monitoring tropical bird communities.

Mist nets were widely used during past decades for both bird surveys and specimen collecting, especially before the advent of modern field guides, affordable, portable sound recording equipment, and online audio reference libraries. Their time- and cost-effectiveness is considerably lower than that of audiovisual methods, and they are subject to a variety of biases, such as net avoidance, weather, habitat structure, and behavioral differences between species and among individuals of the same species (e.g. Jenni *et al*. 1996, Remsen and Good 1996). Nonetheless, under certain circumstances they should be considered as a supplemental method (see below). Specimen collecting is even more labor intensive than mist net surveys and therefore rarely suitable for rapid assessments. We assert, however, that there is a continued need for specimen collecting, especially in the tropics, where new species to science are still being discovered regularly. In regions poorly explored by ornithologists, specimen collecting is an essential tool to reveal cryptic biodiversity and document newly discovered taxa.

Core Standardized Methods

The most cost-effective way to survey the greatest proportion of bird species in a short period of time is to use a combination of audiovisual methods and sound recording. This combination of methods must also be sufficiently rigorous for comparative analysis and flexible enough to be adapted to specific conditions of different regions and ecosystems. Here we propose a set of efficient survey protocols that comply with these general prerequisites. We do not provide in-depth descriptions of particular methods and expect readers to have prior experience with these techniques (see Bibby *et al*. 2000 for detailed treatments).

Among audiovisual methods, 50-m radius point counts probably are the most widely used approach; points can be placed flexibly in any habitat type, are easily and precisely georeferenced, and their results can be analyzed quantitatively with a variety of robust parametric statistical techniques.

The 10-species or MacKinnon list technique is a rather new audiovisual method first proposed by MacKinnon and Phillipps (1993) that has been further developed since (e.g. Poulsen *et al*. 1997, Herzog *et al*. 2002, MacLeod *et al*. 2011). It is the logistically most flexible method but has limitations with respect to statistical analyses. Unlike point counts, consecutive 10-species lists are not necessarily spatially independent, and there is a greater probability of counting the same individual more than once. This method does allow, however, for estimation of relative abundances of species (and it is particularly suited for comparing abundances of the same species across sites; Herzog *et al*. 2002, Herzog 2008), and it is relatively robust with respect to potential observer biases and differences in experience (Fjeldså 1999, MacLeod *et al*. 2011). It also explicitly encourages the extensive use of sound recording (Herzog *et al*. 2002). Statistical analyses essentially are limited to construction of species accumulation (rarefaction) curves, both individual- and sample based, and curve extrapolation with confidence intervals (see Colwell *et al*. 2012), as well as the use of species richness estimators and similarity indices (Colwell 2013). Nevertheless, given that the main goal of rapid assessments often is a provisional estimate of overall species richness, relative abundances, and community composition, 10-species lists are an appropriate core survey method.

The importance of sound recording for documentation purposes and later identification of unknown vocalizations has already been mentioned. In addition to opportunistic sound recordings, standardized use of this method is crucial, particularly during the dawn chorus, at which time the greatest number of species vocalize almost simultaneously. In particularly species-rich habitats, the sheer diversity and abundance of sounds at dawn is likely to overwhelm even the most experienced observers. Obtaining clear recordings of the dawn chorus is the most efficient way to document the greatest possible number of species in most habitats.

Combining Core Methods into a Coherent Survey Protocol

Because each method has its own strengths, shortcomings, and biases, we recommend a combination of all three to generate robust, comparable data sets given the inherent constraints of rapid surveys. Note that the three core methods vary substantially in the required minimum level of observer expertise and experience with a given avifauna. Dawn chorus sound recording per se requires the lowest level of expertise and experience, and, although not ideal, vocalizations can be identified entirely by an expert after the survey is completed. 10-species lists are relatively robust with respect to potential observer biases and differences in experience, but do require at least intermediate knowledge of a given avifauna. Point counts, on the other hand, require high levels of expertise and experience and clearly is the most demanding of the three methods. In addition, in highly diverse tropical habitats they generally detect the lowest proportion of species and are the least amenable to comparisons of results among observers.

Dawn chorus recordings. We recommend conducting two to three 15-min stationary dawn chorus recordings each morning starting with the first vocalizations of diurnal bird species. Minimum distance between recording stations should be 200-250 m. Different stations should be sampled each morning. All stations must be georeferenced using GPS units. To standardize the recording procedure, we suggest the following protocol. Recordings should be made using a directional shotgun microphone (such as the Sennheiser ME-66) held at an angle of 20° above the horizontal or ground level in forest habitats (Haselmayer and Quinn 2000) and 0-10° in low-stature habitats such as grass- and scrubland. At the beginning of each recording the microphone should be pointed in the direction of greatest vocal activity; microphone direction is then rotated by 90° every 60 seconds until two full circles are completed after eight minutes. For the remaining seven minutes, microphone direction and angle may be changed at will to record newly vocalizing species, or to obtain clearer, louder documentation of species whose vocalizations may have been captured poorly during the first eight minutes of the recording.

Point counts. Following dawn chorus recordings, we recommend conducting 10-min (Fig. 1), 50-m radius point counts in early to mid-morning; stopping time will depend on bird activity, which varies with weather, season, and habitat. Minimum distance between point count stations should be 200-250 m; this is the maximum distance at which most forest bird species can be detected acoustically, and ensures spatial independence between points when a 50-m count radius is used. The same stations used for dawn chorus recordings may also be selected for point counts, provided they meet the minimum distance criterion. All birds heard and seen within 50 m of each point should be counted, and sex and age class noted if possible. If time permits, each station should be visited twice on different days at different times of the morning. We do not recommend estimating distances to unseen birds in tropical forest, due to the known incidence of high observer bias in distance estimates and the many variables affecting sound transmission through forest, which often make birds appear much closer or farther away than they actually are.
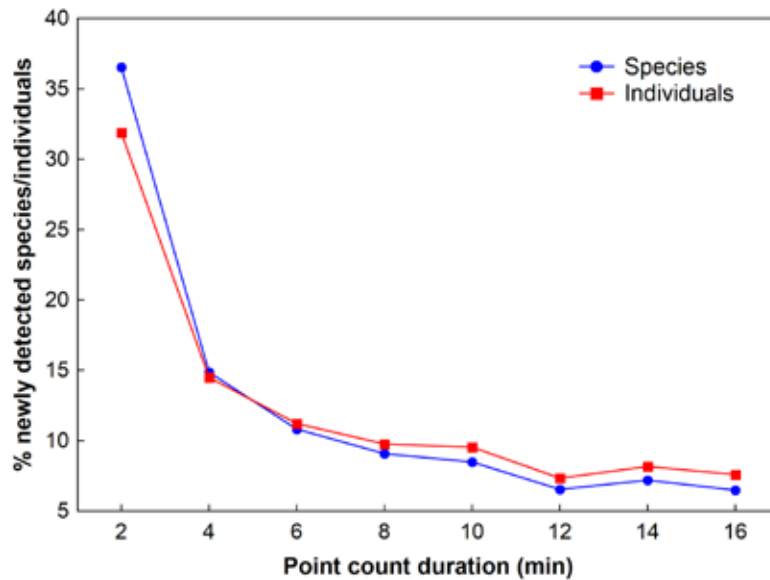
Figure 1
Percentages of newly detected species and individuals with increasing point count duration for eight 2-min intervals in semi-deciduous foothill forest of the central Bolivian Andes (dpto. Santa Cruz, Refugio Los Volcanes: 18°06'S, 63°36'W, 1000-1200 m; S.K. Herzog unpubl. data). Values are means of 172 counts (50-m radius) conducted at 12 stations between March 2003 and January 2004. The total number of detections per 16-min count ranged from 4 to 25 species (mean ± SD = 15.6 ± 4.6) and 7 to 48 individuals (mean ± SD = 24.7 ± 7.7). New species and individuals still were detected even during the last 2-min interval, but detection rates leveled off after 10 minutes for both species and individuals. On average, 80% of all species and 77% of all individuals detected during the entire 16-min point count were observed within the first 10 minutes.

10-species or MacKinnon lists. All individuals heard and seen between dawn chorus and point count stations as well as afterwards should be noted in consecutive order. A digital voice recorder (dictaphone) should be used during surveys and observations transcribed daily to a field note book outside survey hours. Species-list surveys should be carried out until at least mid-day to include peak hours of mixed flock activity. Stopping time will depend on bird activity, which varies with weather, season, and habitat. Surveys should be resumed in mid- to late afternoon until dusk. On at least 2-3 days per site, species-list surveys should also be conducted 1-2 hours prior to dawn and after dusk to detect nocturnal species. Detailed instructions for species-list surveys are given in Herzog *et al*. (2002), but some key considerations should explicitly be covered here. As with point counts, observations at distances of >50 m should be noted but excluded from analysis. It is crucial that provisional names be assigned to species not confidently identified by sight or sound at first (and later replaced with definite identifications). Ten-species lists should not actually be compiled in the field, but only later during data analysis and after all sound recordings have been reviewed and identified, so that birds recorded can be incorporated into the species lists. When longer time periods are spent in one spot or when resampling a given section of the study area, repeated counts of known territorial individuals should be avoided.

Obviously, it will occasionally be difficult to determine whether a bird has already been counted; when in doubt, it is best to adopt a conservative approach and omit a given observation from the analysis.

Data obtained during dawn chorus recordings and point counts can be combined with those produced by the species-list surveys per se to construct 10-species lists for the entire data set procured during a rapid assessment (see Fig. 2). All individuals detected by the three methods are simply listed in consecutive order and then broken down into 10-species lists, followed by the construction of species accumulation curves (sample-based rarefaction). These curves may also be constructed without dividing the total list of accumulated individuals into sub-lists (individual-based rarefaction). For dawn chorus recordings, in some cases it might be difficult to determine the total number of individuals when multiple individuals of the same species are vocalizing. In such cases, only the incidence (presence or absence) of each species on each 10-species list may be used for analysis (sample-based incidence data). Data can further be analyzed separately for each method, including both rarefaction curves (Fig. 3) and, in the case of dawn chorus recordings and point counts, parametric statistical comparisons between habitats or different rapid assessment localities.

The free software EstimateS (Colwell 2013) is readily available for rarefaction, curve extrapolation, and the computation of species richness estimators and similarity indices. Relative abundances of species of conservation concern and other key or indicator species can also be compared between habitats and localities for each method individually or the combined species-list data set (e.g. Herzog 2008, MacLeod et al. 2011). Sound recordings should be archived in a publically accessible repository or sound library (e.g. xeno-canto, Macaulay Library, British Library of Wildlife Sounds) so they are available for comparison and verification, contributing to an ever-increasing volume of available reference material.
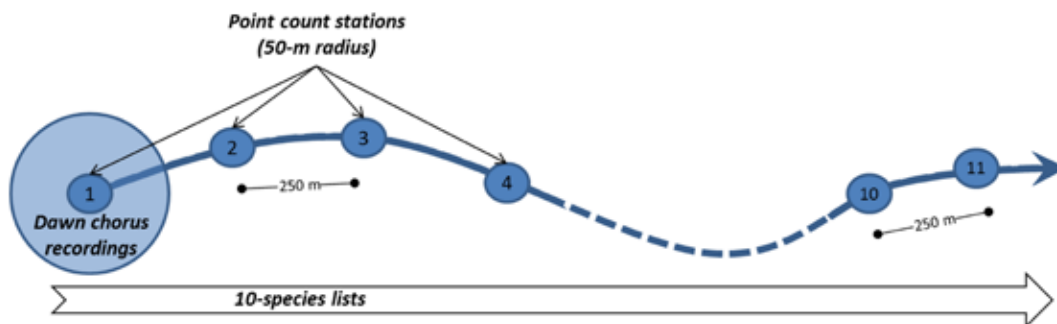
Figure 2
Schematic illustration of the combination of the three core methods into a coherent survey protocol. Daily surveys start with two to three 15-min stationary dawn chorus recordings followed by 10-min point counts until about mid-morning. All individual birds heard and seen between dawn chorus and point count stations as well as afterwards are noted in consecutive order, using opportunistic sound recording as deemed necessary. Data obtained by all three methods can then be combined to construct 10-species lists for the entire data set: all individuals detected are simply listed in consecutive order and then broken down into 10-species lists.
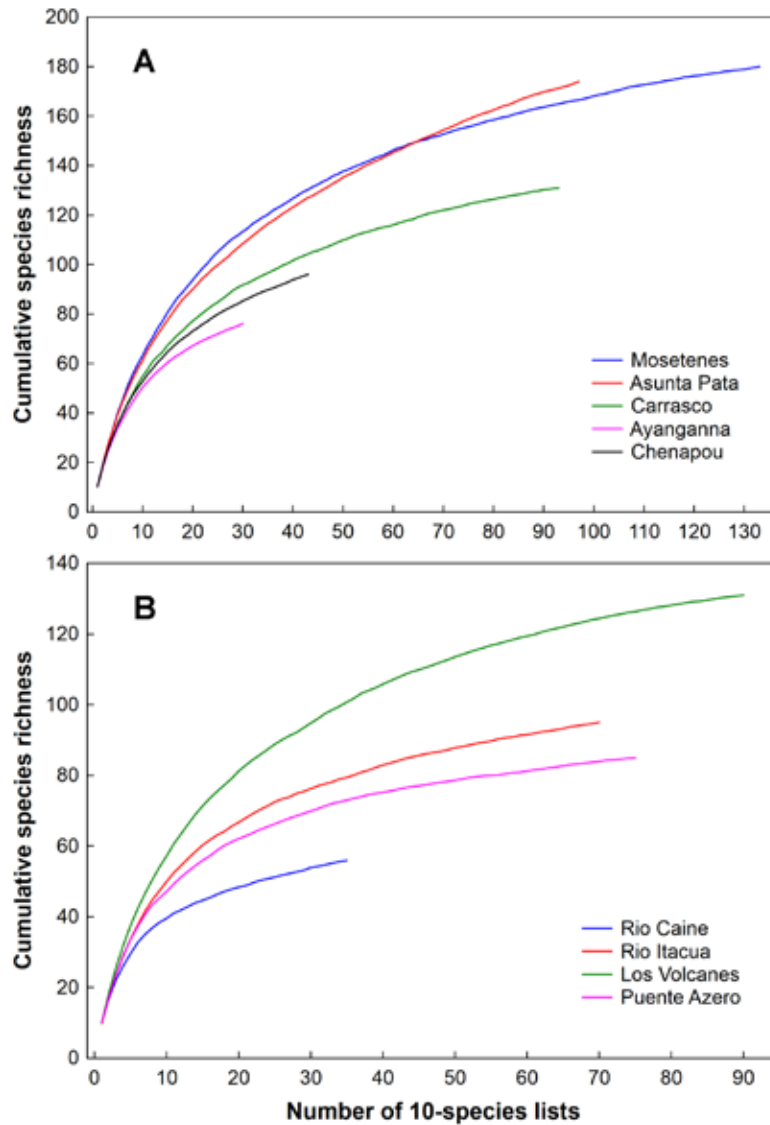
Figure 3
Examples of species accumulation (rarefaction) curves based on samples obtained by the 10-species or MacKinnon list technique. A: humid tropical forest localities in the northern Bolivian Andes (Mosetenes: 16°14'S, 66°25'W, 1180-1600 m; Asunta Pata: 15°03'S, 68°29'W, 1150-1500 m; Carrasco: 17°08'S, 65°35'W, 1180-1600 m; Herzog 2008) and on the Potaro Plateau in Guyana (Ayanganna: 05°18'N, 59°50'W, 700 m; Chenapou: 05°01'N, 59°38'W, 480 m; B.J. O'Shea unpubl. data). B: tropical drought-deciduous forest localities in the southern Bolivian Andes (Río Caine: 17°58'S, 66°51'W, 2100-2600 m; Río Itacua: 19°54'S, 63°31'W, 850-1000 m; Refugio Los Volcanes: 18°06'S, 63°36'W, 1000-1350 m; Puente Azero: 19°39'S, 64°03'W, 1100-1400 m; Herzog and Kessler 2002).

Sampling Effort and Site Selection

Before starting to survey birds, it is very important to consider habitat heterogeneity (spatial variations in topography, vegetation structure, and microhabitats within the same general habitat type) and diversity (total number of general habitat types) in the overall survey area. If possible, every general habitat type should be surveyed and analyzed separately to ensure comparability between different assessment sites with different degrees of habitat diversity. Ideally, habitat diversity along with the extent and spatial distribution of different habitat types should be determined beforehand using satellite images.
A single well-trained and experienced surveyor will often be sufficient to conduct rapid assessments with the combination of core methods outlined above. For particularly species-rich regions and habitats, however, a total of two surveyors are recommended.

Minimum survey effort will vary substantially between regions and habitats depending on overall species richness. We recommend a minimum number of ten point count stations in any type of habitat to account for natural and stochastic variability in the environment, and this sample size will usually be sufficient in areas with relatively few species, such as high elevation environments. In exceptionally species-rich environments, such as Amazonia and the eastern Andean foothills where several hundred species can be crowded into an area as small as 100 ha, ten point count stations will be insufficient. For such environments we recommend the use of at least 30 point count stations per habitat type. Elsewhere, 20 point count stations will probably suffice for most habitat types. Such numbers of point count stations require a fairly extensive trail system to ensure spatial independence between points. These considerations need to be taken into account when makeshift trail systems are established specifically for rapid surveys.

If available trail systems are inadequate, topography is highly complex, and/or the distribution of different habitat types is patchy at a fine spatial scale (e.g. patches of less than 300-400 m in diameter), point counts may not be an appropriate survey method. In these cases we recommend applying only sound recording and 10-species lists.

Establishing minimum recommended sampling effort for 10-species lists is less straightforward than for point counts. A 10-species list is defined neither by time nor space. Detecting ten different species may be accomplished in as little as 30 seconds, or it may take 30 minutes or even longer, depending on overall species richness at a site, season, weather, time of day, and observer skill, among other factors. As the main method for analyzing 10-species list is the construction of species accumulation curves, the number of newly detected species each day should be determined in the field as an approximation of sampling completeness. Computationally simple species richness estimators such as Chao 1 can also be used in the field for this purpose (Herzog *et al*. 2002). Overall, based on our experience in the Neotropics, three days of intensive species-list surveys by a single observer should be sufficient in areas with relatively few species such as high-elevation environments, whereas exceptionally species-rich environments may require as many as 8-10 days to record about 80% of the resident species.

A final consideration in mountainous areas is the elevational range covered by a survey. The greater the range, the stronger the influence of elevational species turnover on the number and proportion of species detected. Analogous to surveying different habitat types separately, different elevational zones in areas with wide gradients should be treated as separate habitats. We consider a range of 200-300 meters as a reasonable maximum for separating habitats by elevation.

Supplemental Methods

Line transects, in the strict sense, are straight lines along which the observer moves at a constant speed. In many tropical habitats, placing straight lines is logistically challenging, especially in mountainous regions. Therefore, the number of line transects surveyed will be lower than the number of point counts that can be covered in the same amount of time, leading to lower statistical power in quantitative analyses. Another issue with line transects is the requirement of constant observer speed, which is unrealistic, particularly in dense forest habitats, where birds often are partly hidden by foliage and mixed-species foraging flocks are common. This quite simply requires the observer to stop frequently for variable periods of time. Therefore, line transects rarely are a suitable core method for rapid surveys but may be used in open and/or species-poor habitats when logistically feasible.

Although mist nets are time- and labor-intensive, they do tend to detect a small proportion of species that might be overlooked using audiovisual methods (including rare, skulking, and quiet species). They also allow for photographic documentation of species and may provide additional information on age, sex, reproductive condition, molt patterns, and parasites that are not obtained by other methods. Therefore, they should be used as a supplemental method when time and human resources permit. At least two additional surveyors exclusively managing mist nets will be required to run a sufficient number of nets (10-15). All mist nets should be moved to new locations at least every other day to maximize capture rates.

The deployment of automated recording stations should be considered whenever funding permits, especially when only one surveyor is available. Within a survey site, this method provides documentation of vocally active birds simultaneously at several independent locations and at any time of day specified, enabling the surveyor to focus on point counts, 10-species lists, and/or opportunistic recording. Because automated recording can be conducted continuously, it can provide a vast amount of data with no observer bias, but also requires time-consuming analysis to identify individual sounds post-fieldwork. The recordings can act as a permanent repository of species present during the sampling period even if they are not analyzed until much later, and increasingly sophisticated analytical software may be able to automatically identify individual species in the future.

Drawbacks of automated recording stations currently include their relatively high cost and weight, and their recording quality is still inferior to that of standard hand-held digital recording equipment. They also suffer from problems associated with any electronic equipment in warm or humid locations — malfunctions can be common but may not be recognized immediately. Thus, recordings should be

spot-checked daily to ensure that recorders are functioning properly. Animals such as ants and monkeys may also damage recording stations. Nonetheless, technological improvements and more affordable prices in the near future are likely to make the use of automated recording stations increasingly suitable for rapid assessment surveys.

Context-Dependent Sampling Considerations and Limitations

Point counts, 10-species lists, and sound recording are amenable to virtually any terrestrial habitat in the tropics and subtropics. However, the detectability of certain bird species can vary seasonally and bias results obtained by audiovisual methods at different times of the year. This needs to be taken into account when comparing data between sites. Ideally, rapid assessment surveys should be carried out in the season with the greatest overall activity and detectability of birds.

Standardized dawn chorus sound recordings are not always necessary. In comparatively species-poor habitats and those without a pronounced vocal activity peak at dawn (e.g. Amazonian white sand forest, certain grasslands, high-elevation forest), opportunistic sound recordings as part of the 10-species list technique are sufficient and often a better investment of time and effort than standardized dawn chorus sound recordings.

Rapid assessments are snapshot biodiversity surveys. They do not capture inherent natural variations in species richness and composition over time. Communities of highly mobile species such as birds are subject to not only seasonal, but also interannual and longer term variation, both naturally and as a result of anthropogenic pressures. Longer term variation in tropical bird community composition and richness is extremely poorly known due to the scarcity of long-term monitoring sites. Interannual and seasonal variation can be pronounced (e.g. Herzog *et al.* 2003, Latta *et al.* 2011), especially in areas with seasonal differences in climatic variables such as precipitation. The potential magnitude of seasonal variation in tropical bird communities must be taken into account when comparing results of rapid surveys from different areas.

Conservation Implications

As traditionally practiced, rapid assessments had the primary objective of identifying areas of exceptional biodiversity without regard to underlying processes and temporal change. Considering current rates of land conversion, rapid global climate change, and their synergistic, potentially detrimental effects on biodiversity (Travis 2003), this is an outdated approach. Today, rapid surveys should establish georeferenced baseline data using replicable sampling protocols that can contribute to long-term monitoring of both naturally and anthropogenically induced changes in particular areas at both the community and species level. This is especially important for a relatively large number of species of conservation concern as well as ecologically important functional groups such as seed dispersers and pollinators: using standardized sampling protocols during rapid surveys will help determine population trends and facilitate comparisons of abundance between sites, aiding in the identification of high extinction-risk species and priority areas for conservation.

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BOX 1: Equipment and supplies needed for rapid assessment bird surveys

Core Methods:

- Waterproof 8x or 10x binoculars
- Telescope (optional)
- Portable digital sound recorder (make sure the latest firmware version is installed) with corresponding memory cards and, if necessary, external speaker for playback
- Directional microphone with foam windshield and cables (at least 2-3 spare cables)
- Digital voice recorder (dictaphone)
- Field notebooks and pencils or pens with waterproof ink
- Field guides for bird identification
- Bird sound reference recordings in digital format
- GPS
- High-precision/professional altimeter
- Digital camera, 300-400+ mm telephoto lens

Secondary Methods:

- Mist nets and supplies used for bird banding (bird bags, spring scales, calipers, rulers, data sheets)
- Automated recording stations

TABLE 1. Overview of common ornithological survey methods, their strengths and caveats.

AUDIOVISUAL METHODS

Survey method	Strengths	Caveats
Point counts	• Thoroughly documented and tested method • High time- and cost-effectiveness • Points can be placed flexibly in any habitat type, are easily and precisely georeferenced • Results can be analyzed quantitatively with robust parametric statistics • Allows for relative abundance and density estimates of species	• Requires high to expert levels of observer expertise and experience • Tends to detect a lower proportion of resident species than other audiovisual methods
Line transects	• Thoroughly documented and tested method • Results can be analyzed quantitatively with robust parametric statistics • Allows for relative abundance and density estimates of species	• Transect placement is logistically challenging and time-consuming in many tropical habitats, especially in mountains • Lower time-effectiveness than other audiovisual methods, resulting in lower sample size and statistical power in quantitative analyses • Requirement of constant observer speed is unrealistic in many tropical habitats, especially forests, where most birds are hidden by foliage and mixed-species foraging flocks are common • Requires high to expert levels of observer expertise and experience
Species-list method	• Very high time- and cost-effectiveness • Logistically highly flexible • Generates data at all times during survey hours (no idle time while moving between survey stations) • Extensive sound recording is an integral component of the method (unlike point counts or line transects) • More robust with respect to observer bias and expertise than point counts or line transects • Allows for of relative abundance estimates	• Fairly recently developed method, less thoroughly tested than other audiovisual methods • Sampling units lack spatial independence, prohibiting use of robust parametric statistics • Statistical analyses limited to construction of species accumulation curves, curve extrapolation, species richness estimators, similarity indices

SOUND RECORDING (ACOUSTIC DOCUMENTATION)

Survey method	Strengths	Caveats
Manual recording	• Very high time- and cost-effectiveness in the field • Easy documentation of a large proportion of resident species • Recordings serve as acoustical voucher specimens, permits post-survey expert review of identifications • Dawn chorus recordings: most efficient way to document the greatest possible number of species in most tropical habitats; may be analyzed with robust parametric statistics	• Often requires time-consuming post-survey review of recordings to identify all vocalizing species • Equipment malfunctions may occur, especially in warm and wet environments
Automated recording stations	• Same strengths as manual recording, but less cost-effective (but equipment prices may drop) • Documentation of vocally active birds simultaneously at several independent locations and at any time of day specified • Provide vast amounts of data with no observer bias • Increasingly sophisticated analytical software may enable automatic species identification in the future	• Relatively high cost and weight of equipment • Recording quality is still inferior to that of standard hand-held digital recording equipment • Equipment malfunctions may occur, especially in warm and wet environments; animals (e.g. ants, monkeys) may also damage recording stations
Mist netting	• Tends to detect a small proportion of species that might be overlooked using audiovisual methods • Allows for photographic documentation of species • Provides additional information on life histories (e.g. molt, reproduction) not obtained by audiovisual methods • Requires only moderate bird identification expertise	• Very time- and labor-intensive • Requires substantial expertise extracting and handling birds • Detects only a small proportion of resident species in most tropical habitats • Subject to a variety of biases (e.g. net avoidance, habitat structure, interspecific behavioral differences) — unsuitable for statistical comparisons across survey sites
Specimen collecting	• Can be an essential tool to reveal cryptic biodiversity and document newly discovered taxa in poorly explored regions • Provides voucher specimens that can be subjected to post-survey expert review of identifications and made available for future research • Provides additional information on life histories (e.g. molt, reproduction) not obtained by audiovisual methods	• Very time- and labor-intensive • Requires special training in taxidermy, museum science, safe firearm handling • Requires additional research or collecting permits that are often more difficult to obtain • Weight and bulk of specimen collecting and preparation equipment and supplies, bulk of prepared specimens

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HERPETOFAUNA

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Alessandro Catenazzi[1], Stephen Richards[2] and Julian Glos[3]

Introduction

Herpetofauna includes two groups of tetrapod vertebrates: Amphibians are ectothermic animals characterised by permeable skin, eggs without shells, and complex life cycles often but not always including an aquatic larval stage. They have a high diversity of reproductive modes. There are ~7,528 species comprising: frogs (~6,640 species), salamanders (~683 species) and caecilians (205 species).

Reptiles are predominantly oviparous ectotherms characterised by scaly skin and eggs with shells. There are ~10,272 species comprising: lizards (~6,145 species), snakes (~3,567 species), turtles (~341 species), amphisbaenians (~193 species), crocodiles (~25 species) and tuataras (1 species).

We restrict our discussion to tropical regions because the tropics have exceptionally high herpetofauna diversity, remain under-sampled in most regions, and so are commonly targeted by rapid biological inventory surveys. Many techniques used for species inventory in temperate regions are unsuitable for use in tropical environments, and particularly for rainforest habitats. In particular, our discussion will emphasize methods to survey groups of herpetofauna that are most species-rich in these environments.

Suitability of amphibians for rapid inventory surveys and for guiding conservation decisions

Amphibians, particularly anurans, are a moderately but not overwhelmingly diverse group with a well-established taxonomy. A comparatively high proportion of a species pool can be assessed in a short time using cost-effective techniques, because amphibians are typically abundant. Heyer *et al*. (1994) and Dodd (2010) describe a variety of standard monitoring techniques for amphibian populations.

Furthermore, amphibian populations are known to respond rapidly to environmental perturbations in both aquatic and terrestrial environments (anthropogenic disturbances, climate change, etc.) so they are a suitable group for making historical comparisons. As of 2015 nearly a third of described amphibian species that have been assessed by the IUCN are classified as threatened and are therefore important in guiding conservation decisions. Nearly a quarter of all amphibian species have been described over the past 10 years and there is a great potential for further discoveries in tropical regions during rapid biological inventories. New species discoveries often generate considerable media interest and catalyse conservation actions.

[1] Department of Zoology, Southern Illinois University, 1125 Lincoln Dr., Carbondale, IL 62901, USA.

[2] Herpetology Department, South Australian Museum, North Terrace, Adelaide, South Australia 5000, Australia.

[3] Department of Animal Ecology and Conservation, University of Hamburg, Martin-Luther-King Platz 3, 20146 Hamburg, Germany

Suitability of reptiles for rapid inventory surveys and for guiding conservation decisions

Among reptiles, terrestrial lizards are the most amenable to rapid biological surveys, and we therefore focus our survey techniques predominantly on them. Lizards are abundant in many tropical environments, are generally readily identified with existing resources, and can be sampled efficiently, especially in habitats such as tropical woodlands and savannas. Some groups of lizards, such as skinks, are also amenable to rapid quantitative sampling in tropical rainforest habitats.

Freshwater turtles, although not very abundant or diverse, are highly threatened (e.g. when used as food), good survey techniques exists, and chelonians are a good tool to raise public awareness; they should therefore be included whenever possible. Often, many data regarding the presence and distribution of turtles will be obtained by interacting with local people and with researchers sampling large mammals (who typically cover larger areas on foot, and thus have higher chances of encountering terrestrial turles) and with researchers sampling fish communities (who often see, net or trap freshwater turtles during their work).

Due to their low abundance and often specialised habitat requirements, most other reptiles (e.g., snakes) are difficult to sample quantitatively during rapid surveys in tropical rainforest and aquatic habitats. Moreover, visual detectability of snakes can be heavily biased by observer's abilities and experience, making comparisons among sites surveyed by different teams difficult to interpret. McDiarmid *et al* (2012) provide a detailed discussion of these issues, along with descriptions of standard monitoring techniques for reptiles.

Nineteen percent of reptile species, and 21% of lizards, that have been assessed by IUCN are globally threatened (Böhm *et al*. 2013) and the percentage is exceptionally high among turtles, crocodiles and tuataras. Furthermore, several species of reptiles are listed in CITES appendices regulating trade of wildlife. Threatened species and species listed in international agreements are important to guide conservation decisions.

Given the predominantly diurnal activity patterns of most lizards (geckos are an exception), survey regimes that complement those of nocturnal frogs can be undertaken in a time- and cost-efficient manner by herpetologists having familiarity with both groups.

Importance of molecular data for amphibian and reptilian identification

The diversity in many groups of amphibians and reptiles is still poorly known. Reptiles are the largest class of vertebrates and yet many new species are discovered every year; and among amphibians hundreds of new species have been discovered and described over the last 10 years (Catenazzi 2015). Furthermore, groups with large radiations typically contain many cryptic species, which require the integration of morphological, behavioral and molecular data to be identified. Therefore, it is important to collect tissues from voucher specimens obtained during rapid inventory surveys. Collected tissues should be suitable for DNA extraction while minimizing damage to morphological features (e.g., liver, thigh muscle or tongue for amphibians; scales or tail tip for reptiles).

Core Methods

Time-constrained transects (frogs, salamanders and lizards)

Transect surveys are the technique most frequently used to document herpetofauna species diversity during rapid species inventories. They are cost-effective, applicable in almost all terrestrial habitats, and suitable for sampling both terrestrial and arboreal species. They also permit the collection of relative abundance data. Transect surveys have traditionally been either fixed-length or time-constrained; we recommend the latter because in the difficult terrain frequently encountered during rapid biodiversity assessments it is often difficult or impossible to replicate transects of the same length both within and across sites. In contrast, time-constrained samples allow calculation of search effort in person-hours even if time spent on each survey varies due to unavoidable environmental or other factors.

Although transect surveys are commonly divided into Visual Encounter Surveys (VES) and Acoustic or Audio Transects, we recommend combining the two techniques to maximise the efficiency of transect surveys (i.e., the ability to detect the greatest number of species) during rapid inventories. It should be noted that Audio Transects only detect calling males so this technique cannot be used to estimate relative abundance of non-calling males, females and juveniles. Also, it should be noted that the combination of both methods might lead to double counts of some individuals, depending on habitat structure and calling habitats of frogs.

Time-constrained transect surveys are normally conducted in the following manner at each site.

1. Identify all habitats present within the survey area.

2. Identify and clearly mark all hazards that may present a danger during field surveys, particularly at night.

3. Establish (if not already done) a network of trails that provides access to each of the habitats identified. Keep cutting of bush to a minimum, trails should be large enough only to allow safe passage.

4. Flag trails with biodegradable flagging tape.

5. The number of transects established at each site will depend on a range of factors including terrain, but we recommend a minimum of 5 transects each at least 100 m long. Single transects can traverse a range of habitats, or transects can sample predominantly within a particular habitat type so that all habitat types are not adequately sampled until all transects have been completed. The diversity of habitats within a transect will be determined by extent of habitat diversity within the broader landscape, and by accessibility of the local terrain at each survey location. The sampling design should maximise the diversity of habitats sampled within the limited time available during rapid surveys.

6. Each transect should be surveyed at least once each day and each night. Crepuscular species should be sampled 1 hour before and after sunrise and sunset. The order in which transects are surveyed at each site should ideally be randomised, but if conditions make this challenging, they can also be searched in turn until all transects have been sampled at least once. Day surveys for reptiles should be conducted from about mid-morning to sunset, while night surveys should start between 30 and 60 minutes after dark.

7. The start and end point of each transect should be GPS-marked. Additional GPS points should be recorded on longer transects to ensure that voucher specimens are adequately georeferenced.

8. All transects should preferably involve a minimum of 2 (for safety) and a maximum of 3 (to reduce disturbance) persons.

9. At the start of each transect survey the following data should be recorded in a waterproof field notebook: location, transect number, date, time, numbers and names of investigators, habitat and weather information and any other site-specific data required. The time when the sample is finished should always be recorded.

10. The survey technique involves the investigators walking along the transect and recording each individual frog, lizard and salamander observed within a strip approximately 2.5 m wide on either side of the trail (maximum detectable distance in rainforest, pers. obs.). The numbers of calling frogs within the ~5 m wide transect are also recorded during the survey, and care should be taken not to double-count frogs that are both seen and heard. Frogs producing calls that are unknown should be searched for to allow positive identification. During day samples for lizards the investigators should turn logs, rocks and search other potential refuges as they move along the transect.

11. To generate the most robust and comparable species accumulation curves at each site the time that each animal is seen or heard should also be recorded.

12. An additional, optional technique that permits estimation of abundance of calling male frogs along transects is described under Supplemental Methods.

Acoustic surveys with automated recording devices (frogs)

During rapid surveys time limitations often prevent researchers from sampling all habitats or from conducting surveys at specific times. Automatic recording devices (Fig. 1) offer the opportunity to sample frog species that emit vocalizations without the need for researchers to be present. Thus researchers can maximize their time in the field by extending sampling periods and number of habitats that can be surveyed simultaneously. Furthermore, the use of automatic recording devices is amenable to a variety of sampling designs, from focused surveys of threatened taxa to documentation of the distribution and relative abundance of common species across a larger landscape. Automatic recording devices also remove any sampling bias, and can be deployed without experts present in the field once target taxa and habitats have been identified.

This technique requires that there is a library of calls available for the site or for the region more generally, and that there is sufficient expertise available to identify the presence of new species and species for which the calls were not previously known.

There are logistical constraints to the number of automatic recording devices that can be deployed during a rapid biological inventory but it is recommended that a small number of recorders (i.e., 5-10 recorders depending on the size and battery requirements) be used within each broad habitat type for sampling of focal microhabitats that are most likely to be used by calling amphibian species. Appropriate placement targets will depend on the composition of frog communities at each site, but they are likely to include wetlands and ponds, oxbow lakes, riparian habitats, forests rich in epiphytes with water-filled leaf axils, wet or moist grasslands, etc.

Programming of recording devices should take into account technical limitations (i.e., battery life and memory size), optimal recording periods and time needed to inspect recordings. Although several software programs are available to assist with call recognition (e.g. Arbimon, XBAT, SongScope), careful visual and auditory inspection of each recording is often required during surveys of areas that are poorly known or likely to contain species whose calls are unknown. Sound analysis software visualizing the spectrogram (e.g., Raven, SongScope, Avisoft) greatly facilitate the rapid visual inspection of recordings. However, most frogs vocalize at frequencies that overlap with those of many other organisms, and often display limited frequency modulation so visual inspection should complement, rather than replace, listening to recordings.

Recording times should target periods of greatest calling activity. Most amphibian species call at dusk and during the first hours of the night, and short and frequent recordings (e.g., 5 minutes every 30 mins) during these hours will allow detection of a large proportion of species in lowland tropical rainforest. Depending on the region and elevation, acoustic recording during dawn hours may also be necessary (e.g., dendrobatid species in the Neotropics), as well as recording during daytime (e.g., grassland species in high-elevation tropical grasslands).

Unfortunately no centralized database is available to compare recordings with calls of described species and voucher-associated recordings, so the use of recordings relies on the researcher's previous knowledge and experience. However this knowledge can be supplemented, for some species, with recordings available from electronic databases (i.e., Fonozoo, Amphibia Web) or call descriptions from the literature.

Dip netting (larval and aquatic amphibians)

Activity levels of adult amphibians are highly dependent on environmental factors such as rainfall and air temperature so differences in anuran activity between seasons, within seasons, and within a day can be dramatic. In contrast larval amphibians are confined to aquatic habitats where dip-netting provides an easy-to-apply method that is efficient irrespective of these environmental factors.

It provides presence/absence data (i.e., no abundance data) and potentially expands species lists by documenting both aquatic species that are unlikely to be detected during transect or acoustic samples, and those species with adults that are not active at the time of the survey due to environmental conditions.

Aquatic and larval amphibians are dip-netted in both lotic and lentic waters. A sampling unit is one body of water (stream, pond) or a discrete habitat within a larger body of water. Sampling effort will vary depending on the size and structure of the aquatic habitat but should be quantified as much as is possible. Dip-net strokes should be performed in all microhabitats (i.e., at different water depths, in differently vegetated parts, in fast and slow flowing parts, on different substrates such as rocks, mud or sand and within substrates such as pebbles and leaves). If the water is difficult to sample due to structures (e.g., branches) within the water, these structures should be removed a minimum of one day before the sampling whenever possible. The number of dip-net strokes might differ between sampling units (e.g., depending on habitat characteristics, individual abundance, etc.) but if possible a minimum of five dip-net strokes should be made and the sampling units should be recorded following each sample. Dip-netting is performed with minimum perturbation of the water to maximize catching efficiency, i.e., in lotic systems by moving from downstream to upstream pools, and in lentic waters by sampling at the water edge. All larval amphibians are collected from the dip-net (using a teaspoon will minimise the chances of harming larvae) and kept alive temporarily. After sampling vouchers of each morphotype encountered are retained and identified to species level, where possible, based on (a) their morphology (if possible) or (b) via DNA-barcoding using the techniques outlined below.

Species identification via DNA-barcoding is done applying a five-step procedure: (1) sorting all individuals into morphotypes, (2) euthanizing at least one individual of each morphotype using e.g., MS-222 or Chlorobutanol, (3) taking a tissue sample from the tail and storing it in 99% ethanol, (4) storing the tadpole voucher in 5% formalin, (5) sequencing appropriate genes (e.g., 16S) and blasting using appropriate gene databases (e.g., GenBank). If time is short, whole tadpole vouchers can be stored in ethanol so that tissue samples for DNA-analysis can be taken later. However it should be noted that storage directly into ethanol will severely distort the voucher tadpole reducing its future value for morphological identification, so completion of step 4 is preferred.

Data to record should include (a) collector name, (b) date and time, (c) GPS-coordinate, (d) lentic or lotic habitat, (e) surface area/stream width, (f) maximum depth, (g) presence of other fauna, e.g., predatory fish, (h) number of tadpole individuals, and (i) brief description of vegetation structure and substrate of the water.

This field method is relatively easy to apply without extensive training or experience, and has the advantage of detecting species irrespective of the simultaneous activity levels of adults. It can also be done at any time of the day. However the technique produces only presence/absence data, is applicable only for amphibians with aquatic life stages, and identification of species can be relatively time-consuming and costly (DNA-barcoding) when morphological ID is not possible. Because of concerns about moving the amphibian chytrid fungus around nets should be thoroughly cleaned before changing sampling sites.

Figure 1
Automatic digital sound recorder used to monitor populations of marsupial frogs (*Gastrotheca excubitor* in the image) and terrestrial breeding frogs (*Noblella pygmaea* and *Psychrophrynella usurpator*) at the Abra Acjanaco mountain pass in the Peruvian Andes (photo by A. Catenazzi).

Supplemental Methods

Audio strip transects (frogs)

Audio strip transect is a method to assess densities of calling individuals (i.e., ind/ha; Zimmerman 1994) and is most commonly used in conjunction with transect surveys (see Time-Constrained Transects under Core Methods). It is a useful tool to detect changes in population densities of calling individuals and to compare densities of calling individuals between sites. However, it is time-consuming and applicable only to species that are evenly distributed at the site, i.e. not aggregated. Species that are encountered during breeding aggregations around ponds or streams are not suitable for this method. Accordingly, suitable species for distance sampling are often those that breed independent from larger waters (e.g., direct developing frogs, some phytotelmata and terrestrial nest breeders).

Audio strip transect protocol:

(1)   Identification of suitable species.

(2)   Preparation of line transects (points 1 to 10 in 'Time Constrained Transects' section above).

(3)   Walking slowly along the transect and counting calling individuals of focal species.

(4)   Visual searching to detect the location of calling individuals.

(5)   Measuring perpendicular distance (m) of individual to transect.

(6)   Repeating (4) and (5) for the first ten individuals.

(7)   Average the perpendicular distance of the first ten detected individuals (m).

(8)   Continue (3) for the rest of the transect and count number of calling males of each species.

(9)   Density of each species along the transect is determined by dividing the number of calling males encountered by the species-specific area of the transect (mean detectability distance X transect length).

Digging (caecilians)

Given the evolutionary distinctiveness and poor state of knowledge of the group, attempts to document caecilians should be made in those parts of the world where the group occurs (circumtropical). Due to their fossorial lifestyle caecilians are not readily detected with the range of methods described above. Caecilians can be found in pitfall traps after rain, and detected by turning over the soil in moist habitats that are likely to harbour them. Soil should be removed to a depth of up to 40 cm (depending on substrate properties) and the removed soil should be searched for caecilians. The best results are obtained when teams work in groups of two, with one person digging and the other pouncing when an animal is spotted, before it can disappear back into the soil. Showing images of caecilians to locals and asking for information about their presence may greatly facilitate the success rate of encountering these secretive species. In regions where new roads are being built thought the rainforest, examination of recently moved soil near creeks and interviews with road workers may increase detection of caecilians. Given the difficulty of detection and requirement to use non-quantitative techniques to maximise encounter rates, only presence/absence data can be generated for this group during rapid inventory surveys.

Environmental DNA (aquatic amphibians)

Amphibians that use aquatic environments can be detected by filtering water and amplifying genetic markers that allow identification of taxa, particularly for endangered and elusive species. This approach can be adapted to suit a variety of needs, and is amenable to rough estimates of population abundances. The main benefit of this approach is that it requires no previous knowledge of which species are present at a given site, and is less sensitive to detection biases due to differences in observer's abilities. However, several potential impediments make this technique less suitable under typical conditions of rapid biological inventories. For example collecting samples requires filtering of considerable volumes of water, and proper storage of filters and processing of samples requires good knowledge of molecular biology techniques, access to thermal cyclers, primers and reagents, and time to analyse the samples.

The general sampling and analytical procedure to generate a species list where no previous knowledge or expectation of finding specific species exists includes the following steps: collection and filtering of water samples (or other samples that may contain amphibian DNA), storage of filters and transfer to a molecular biology lab, extraction of DNA from filters, use of amphibian-specific primers and/or blockers to selectively amplify 16S and other frequently used genes, sequencing, and comparison with sequences in GenBank by using BLAST.

Pitfall traps

Pitfall traps with associated drift fences are commonly used to document small, terrestrial herpetofauna in a wide variety of temperate and arid zone habitats. Pitfall trapping is a useful technique for documenting small, rare, often fossorial species that are difficult to detect using other techniques. However it is time-consuming and few additional species are added to a typical inventory using this technique.

Pitfall traps (with drift fences) should be established in 'straight' lines of 10 m length with buckets placed at equal distances along the fence-lines (Fig. 2), or in arrays with prongs of the same length that are designed to 'funnel' animals that are not caught in one set of buckets towards another fence line, such as 3-pronged arrays.

Comprehensive instructions for the construction of pitfall lines and arrays, and examples of survey design, have been provided elsewhere including Corn (1994) and Fisher and Rochester (2012). The survey design used during rapid biodiversity inventories will be determined largely by the terrain but we recommend that at each site a minimum of six pitfall lines, each 10 m long and containing three 20 L buckets should be established and that they be placed to sample the broadest range of habitats possible. If logistically feasible we recommend that three straight-line and three 'funnel design' arrays are used at each site.

Although this technique is useful in tropical savannas and woodlands, establishment of pitfall arrays in remote rainforest habitats is logistically challenging, time consuming, and when pits fill with water after heavy rain can potentially lead to high mortality of trapped animals. Furthermore Rödel and Ernst (2004) found that pitfall trapping was the least effective method that they tested for producing a comprehensive herpetofaunal inventory in tropical rainforest. We therefore do not recommend the use of this technique in rainforest environments.

Supplies/equipment needed in the field and after field work

For all methods:

- GPS, rubber boots, waterproof notebook or data sheets, thermometer, dissecting kit, tissue vials, chemicals (ethanol, 99% Laboratory Grade for DNA samples, 70-75%% 'standard grade' [can be diluted from higher concentration]; formalin, 5-10%, can be diluted from absolute to minimise volumes requiring transport [note that 37% formaldehyde = 100% formalin, so formaldehyde labelled as 37 or 40% should be treated as 100% formalin), calipers, single-use small plastic bags to house frogs, permanent markers.

For transects:

- Headlamps, measuring tape, portable recording device, camera with macro lens and flash, biodegradable flagging

For acoustic surveys with automated recording devices (frogs):

- Recording device (Wildlife Acoustics Songmeter, smartphone with microphone, Sieve Analytics Arbimon)

For dip-netting:

- Dip-nets of different sizes, teaspoon, plastic sieves, waders, plastic jars, magnifying glass, sorting tray.

For pitfall trapping:

- 20L buckets, drift fence (e.g., plastic), staple gun, poles, shovels, posthole diggers.

For audio strip transects:

- Headlamps, measuring tape, portable recording device.

For digging:

- Spades.

For environmental DNA:

- 0.45 µm filters, funnel holders, hand-held or portable electric pump, vials.

Additional equipment required post-survey:

- Sound analysis program, microscope and/or stereoscope, access to DNA laboratory with appropriate facilities, and chemicals for long-term storage of vouchers.

Figure 2
Arrays of drift fences and pitfall traps for surveying lizards in the Florida scrub, USA (left) and in the tropical dry forest of north-western Peru (photos by A. Catenazzi).

Selecting sampling sites

It is important to select sample sites to encompass the environmental heterogeneity present in each survey area. This process should start prior to mobilisation by examination of topographic maps, GIS layers, satellite imagery, etc. to determine the major topographic features present within the broader study area. Aerial reconnaissance en route to site and interviews with local landowners can provide valuable additional information about habitat features within the study area.

Ground-based reconnaissance is particularly important when sampling amphibians, because they exhibit a high diversity of reproductive modes and therefore occupy a wide range of aquatic and adjacent terrestrial habitats. Investigation of habitats known to be occupied by threatened or otherwise significant taxa should also be a priority.

Typical sampling effort required

Information about the comprehensiveness of herpetofauna inventory data from tropical sites is lacking. However, based on many years' experience conducting surveys in both the new and old-world tropics we expect that the percentage of frogs documented at a given site during a seven day rapid inventory using only core inventory techniques can be as low as 30% in the hyper-diverse lowlands and foothills of the Neotropics but may be as high as 70% in other tropical regions. (Fig. 3 and Fig. 4)

By adding opportunistic methods this percentage may be further increased. The percentage of lizards and snakes documented using the core techniques is lower than it is for frogs, but can be increased by a substantially larger amount in tropical woodland habitats when intensive opportunistic sampling techniques are employed.

Context-dependent sampling considerations

There are important geographical and historical differences in herpetofaunal composition that will affect the effectiveness of survey techniques. Differences in reproductive modes, diel activity patterns (diurnal vs. nocturnal), spatial distribution, habitat use, body sizes and calling activities influence detectability of individuals and affect the likelihood that certain species or groups can be found during rapid surveys. For example terrestrial-breeding species, which dominate some tropical amphibian faunas, disperse more homogeneously over space than aquatic breeding amphibians, and thus are best surveyed using techniques such as time-constrained surveys and auditory transects. There also are geographic differences in activity times of amphibian and reptiles, which will determine whether there is a need to include diurnal and/or nocturnal transects. For example while diurnal frogs are a conspicuous component of some Neotropical forests, they are nearly completely absent in the Melanesian region. Similar considerations should guide selection of core methods along with a general knowledge of the main faunal groups expected to occur at a site.

Activity patterns, and hence detectability, of all groups of herpetofauna will be influenced to some extent by seasonal factors. However the effects of climate, and in particular of rainfall seasonality, are most dramatic on frogs. The protocols described in this chapter are generally resilient to seasonal changes and can be conducted in most conditions. Minor modifications may be required in the event of heavy rainfall, and it should be recognised that under dry conditions frog activity is usually greatly reduced so that a smaller percentage of the total fauna will be documented within the seven day survey period. For those sites with strong seasonality of rainfall surveys for frogs are best done during, and particularly early in, the wet season. However reptiles are best surveyed during drier periods, and when more hours of sunshine are likely. It is extremely difficult to schedule rapid surveys in order to maximise detection rates of all herpetofaunal groups.
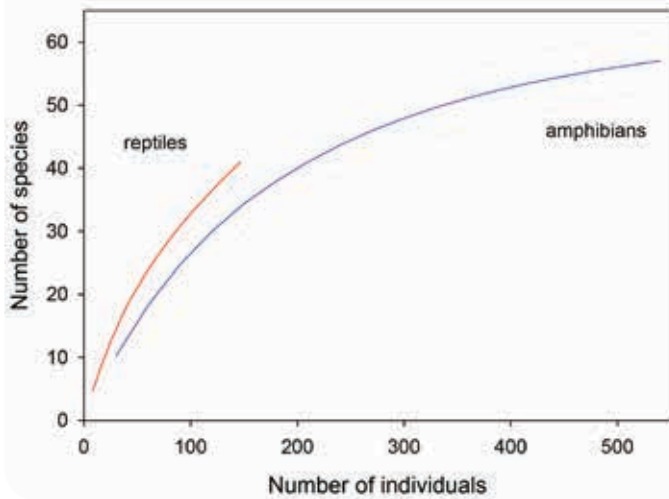
Figure 3
Individual-based species accumulation curve with 95% confidence intervals computed from time-constrained surveys conducted during 18 days of a rapid biological inventory of amphibians (blue line) and reptiles (red line) in submontane Amazonian forests (Catenazzi and Venegas 2012). The greatest majority of amphibians at this Neotropical site is composed of frogs, and that of reptiles is composed of snakes and lizards. See Veith *et al*. (2004) who tested necessary sampling effort in different regions of the world.

Figure 4
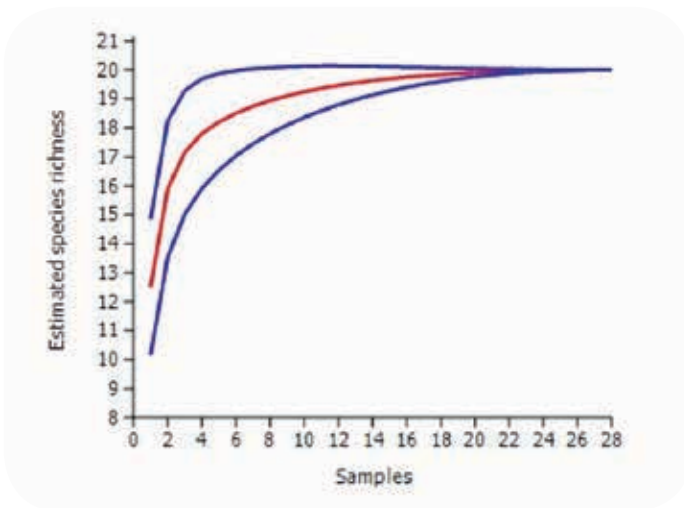Sample-based species accumulation curve during a rapid biological inventory for lizard species in a tropical deciduous dry forest during the rainy season (Menabe forest, Western Madagascar). After seven days, on average 94% of all lizard species were detected. Shown is estimated species number (red line) and 95% confidence intervals (blue lines).

Dendrobates tinctorius. Photo © Trond H. Larsen

The two core methods that will be most affected by heavy rainfall are dip-net sampling and pitfall trapping. The potential impact of heavy rain on dip-netting protocols is to turn slow-flowing or small streams into dangerous torrents, and to possibly sweep away tadpoles. These conditions might also compromise the ability to recover usable eDNA samples. Extreme care should be taken when sampling in fast-flowing streams and rivers and sampling should be suspended if there is a risk to personal safety.

Although pitfall trapping is an extremely useful method for documenting small terrestrial herpetofauna, animals quickly drown in pits that fill with water during heavy rain. If pitfall lines are to be established in areas and/or seasons prone to heavy rainfall events then solid lids for each pitfall bucket should be part of the survey's equipment inventory and these should be used to keep pits dry during rain. Holes drilled in the bottom of the buckets are insufficient to allow drainage if the soil is supersaturated and the buckets will pop out of the ground.

Data Management

Species identification, specimen processing and management

Species identification should be done in the field whenever possible, at least to genus level. However during rapid inventories some species may be unknown and possibly new to science, in which case one must photograph and describe coloration of live specimens before preservation. It is generally recommended to photograph as many specimens as possible, especially when visiting remote areas. Recommended photographs are dorsolateral, dorsal and ventral views for all groups, details of webbing and plantar and palmar surfaces in frogs, and details of head squamation in lizards and snakes.

Specimen processing, whether for collection or temporary examination followed by release, is greatly facilitated by assigning a unique identifier immediately upon capture. For example amphibians can be housed individually in disposable plastic bags, with the unique identifier written with a permanent marker on the bag. The identifier can then be associated with geographical, ecological, and behavioral data noted at the time of capture (which can also be written on the bag, or in a field notebook).

Collected individuals should be labeled with a second unique identifier code (i.e., the field series number), and information associated with each collected specimen should be recorded in a field catalogue. Although electronic tablets may seem attractive, we recommend the use of waterproof notebooks in the field. Electronic equipment often malfunctions in tropical environments, and its use is limited by the availability of electric power.

Types of data collected and data management

We recommend following guidelines of the Darwin Core biodiversity data standard, which has been adopted for example by VertNet (www.vertnet.org), as outlined by Wieczorek *et al*. (2012). The Darwin Core recognizes the following categories associated with biodiversity collection: record-level term (e.g., institutions and nature of data record), occurrence (type of observation), event (sampling protocol and associated data), location, taxon, and identification (linkage between taxon and occurrence; e.g., observer responsible for taxon identification).

Accordingly, biodiversity data during rapid surveys must include:

- Reference tag and/or field series number

- Species identification

- Date and time of collection

- Name of collector and of person identifying taxon (if different)

- Sampling protocol

- Location, typically in the sequence: Country, State or Department, County or Province, District, Town, Locality

- Geographic coordinates with system used

- Elevation

Additional data, such as notes on natural history, are of great help when characterizing the herpetofaunal communities or describing new species. Whenever possible, researchers should also record the following data:

- Name of digital photograph file or slide/negative number

- Name of digital audio recording file or tape number

- Body, substrate, air temperature (very important because call structure and frequency vary with temperature)

- Weather conditions

- Substrate type, height (for arboreal species)

Conservation Implications and Limitations

Detecting change over time and responses to disturbance/environmental change

Rapid biological inventory surveys assess the baseline biodiversity of an area. They provide preliminary species lists and identify areas of high species diversity. However, rapid inventory surveys do not provide complete species lists, either for amphibians or for reptiles. At the species level they provide information on distribution, on habitat utilization and on the ecological requirements of species. This information is of particular conservation relevance for endemic or restricted-range species, and for threatened species.

Assessing the impacts of environmental change and/or disturbance requires quantitative, replicable techniques and the core techniques described here (transect surveys, acoustic monitoring, dip-net sampling and pitfall trapping) and the optional audio-strip transects can provide data suitable for such assessments. As long as enough data are collected to produce accumulation curves, such curves can be compared among sites and across time to determine whether changes in species richness have occurred. Techniques such as auditory transects that measure density of calling males of the most common species can be prioritized to address specific questions regarding the effects of environmental disturbance on population size which is often more powerful than simply measuring presence.

However, it should be reiterated that obtaining quantitative and reliable abundance data for herpetofauna within the short time of a typical rapid inventory is difficult, and therefore comparisons of species abundances over time generally have limited value. However responses to disturbance and environmental change can be detected when a gradient of differently disturbed areas within the survey region is assessed for species richness; and the rapid inventory techniques described here are useful for detecting the presence of particular focal species of conservation concern.

Therefore rapid inventory surveys can be a starting point for assessing the conservation value of, and for guiding conservation actions in, the surveyed area; such actions might include assessing the status of threatened species, assessing the impact of anthropogenic disturbance, and guiding the design and creation of effective protected areas.

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FRESHWATER FISHES

STANDARDIZED RAPID BIODIVERSITY PROTOCOLS: FRESHWATER FISHES

José L. O. Birindelli[1], Vanessa Meza-Vargas[2], Leandro M. Sousa[3] and Max Hidalgo[2]

Introduction

Definition of taxon – With over 32 thousand species (Nelson, 2006), fishes are more diverse than all other vertebrates combined. Fishes comprise a variety of distinct lineages including lampreys and hagfishes, sharks, rays and chimaeras, ray-finned and lobe-finned fishes. All of these species live in or are associated with aquatic habitats and breathe through gills, even if only in a supplementary way. Fishes exhibit enormous diversity in their morphology, physiology, reproduction and genetics. Similarly, fishes occupy a wide range of habitats, from small streams at five thousand meters elevation in the mountains of Tibet to sea valleys deeper than seven thousand meters below sea level (Helfman *et al*., 2009). However, fishes are not homogeneously distributed on earth, as approximately half of all species occur in continental waters, representing no more than 3% of the water available on the planet.

What criteria make the taxon suitable for rapid baseline surveys and for guiding conservation decisions in general? Freshwater fishes are the most dominant group of vertebrates in inland aquatic ecosystems, including rivers, streams, lakes, floodplains, intermittent pools, and subterranean aquifers. Aquatic habitats occupied by fishes are extremely diverse in terms of their physicochemical characteristics (e.g., white, black and clear water), geological origins (e.g., uplift of mountains and plateaus, rifting of continents, and erosion of ancient crystaline shields), and vegetational associations (e.g., piedmont forest, floodplains, rainforest, savanna). All these attributes influence fish diversity and community structure.

Fish species and assemblages respond quickly to environmental disturbances, including anthropogenic activities that alter hydrology (e.g. construction of dams) and water quality (e.g. mining and agriculture activities). Due to recent human activities, many freshwater fish populations have undergone drastic reductions and many species are now considered to be threatened or extinct. Globally, 1,670 freshwater fish species (Actinopterygii) are considered threatened at some level, a number nearly four times greater than that of marine fishes. The most important threats to freshwater fishes are dams and soil erosion (sedimentation), which are respectively responsible for impacting 494 and 489 species on the IUCN red list (data available at http://iucnredlist.org).

[1] Museu de Zoologia, Departamento de Biologia Animal e Vegetal, Universidade Estadual de Londrina, Londrina, PR, Brazil.
Email: josebirindelli@yahoo.com

[2] Departamento de Ictiología, Museo de Historia Natural UNMSM, Av. Arenales 1256, Jesús María, Lima, Perú. meza.sv@gmail.com;
Email: mhidalgod@unmsm.edu.pe

[3] Laboratório de Ictiologia de Altamira, Faculdade de Ciências Biológicas Universidade Federal do Pará, Campus Altamira, Altamira, PA, Brazil.
Email: leandro.m.sousa@gmail.com

Fishes are often conspicuous, abundant and relatively easy to collect and store, either as living or preserved specimens. Distinct methodologies and efforts can be applied according to the taxon, habitat, and ultimate objectives of the study (e.g., diversity, ecology, genetic).

Because fishes occupy water that drains off or through the land, and are sensitive to changes in this water, they are excellent, integrative indicators of overall ecosystem health and can be a cost-effective means of conducting biomonitoring assays. Moreover, fishes often have high sociocultural and economic value as sources of food, recreation, and income (e.g., when collected and sold to the ornamental aquarium trade). Their sustainable management as a renewable resource can therefore provide a primary motivation for conserving natural ecosystems.

Core Method

Methods for collecting fishes vary greatly according to habitats, as all types of equipment cannot be applied in all situations. The following three main and most often-found habitat types are used as a guide:

1. small-scale lotic habitats (e.g., headwaters, forest streams),
2. medium and large-scale lotic habitats (e.g. rivers),
3. lentic and slow-water habitats (e.g., lakes, floodplains).

Small lotic habitats are those with flowing water and classified as first, second or third order streams according to the Horton method, modified by Strahler (1957). Medium and large-scale lotic habitats are those with flowing water and classified as fourth or larger order streams. Lentic and slow-water habitats consist of any still water body including lakes, swamps and floodplains. Distinct standardized protocols are suggested for surveying fishes in each of these habitat types. More specialized habitats, such as caves and temporary isolated ponds, for example, should utilize other protocols (see supplementary methods below).

Core standardized sampling protocols for rapid survey – Sampling methods are described below for each habitat type. Information regarding gear specifications, sample site selection, and sampling effort, are provided below.

Small-scale lotic habitats (headwaters and small streams):
Each sample should be carried out by at least three people working together in a 50 meter long stretch of waterway (i.e., reach) for one hour of effort using the following equipment: dip nets, sieves and seine nets. Prior to sampling, each end of the reach should be blocked by seine nets to prevent fish from escaping. If samples are made in the same stream, these should be separated by at least 500 meters to maintain independence among samples. Samples should always be made during daytime, for comparisons.

Medium and large-scale lotic habitats (large streams and rivers):

Each sample in a medium or large-scale lotic habitat should be made using combinations of seine nets, cast nets and gill nets. A seine net should be applied 10 times along the shore, preferably keeping a distance of approximately five meters between each sampling event. Cast nets should be applied 10 times along shallow stretches, usually found near the shore, preferably keeping a distance of at least five meters between each sampling event. Five gill nets should be placed in the water for at least 8 hours, preferably from sunset to sunrise, ideally at a safe distance from each other (e.g., at least 10 meters apart), to avoid influencing among them. Samples should be made during both day and night, emphasizing transitional periods between day and night when most fishes are more active.

Lentic and slow-water habitats (lakes, swamps and floodplains):

Each sample in a lentic or slow-water habitat should be made using gill nets and seine nets. A seine net should be applied 10 times along the shore, preferably keeping a distance of at least 5 meters between each sample. Five gill nets (net specifications below) should be placed in the water for at least 8 hours, preferably from sunset to sunrise, ideally at a safe distance from each other (e.g., at least 10 meters apart), to avoid influencing among them. Samples should always be made during day and night, emphasizing transitional periods between day and night when most fishes are more active.

Supplies/equipment needed in the field and after fieldwork

List of supplies and equipment:
Field work:

- 2 dip nets (mesh 1 to 3 mm, diameter of approximately 30 cm)
- 2 sieves (mesh 3 to 5 mm, diameter of approximately 60 cm)
- 2 seines (mesh 3 to 5 mm, approximately 5 meters long and 1.5 to 2 meters high)
- 2 cast nets (mesh 12 to 20 mm and 2 to 3 meters high)
- 5 gill nets (nylon mesh size ranging from 12 to 70 mm bar (vs. stretched), approximately 10 meters long and 2 meters high)
- 40% formalin solution (amount depending on the estimated number of samples and target place)
- 96% alcohol solution (amount depending on the estimated number of samples)
- Large gauge syringe and needles, and gloves
- Vials of 5 mL, dissection kit with scalpel, razor blades, and microscissors
- Field book and waterproof and alcohol-proof pen and paper
- Photographic camera and batteries
- Aquarium for photographs (including a supply of clear, bottled water specifically for photo tank)
- Anesthetic solution (e.g., clove oil)
- Plastic bags of different sizes (including rubber bands)
- Handheld GPS

Lab work:

- Trays and forceps
- 96° alcohol solution
- Jars of distinct sizes (ranging from 250 ml to 3 liters)
- Heavyweight label paper and printer
- Computer
- References for species identification (including internet access)

Selecting sampling sites – In order to apply each of the three suggested protocols for a selected site, it is necessary to previously determine if that water body is a small-scale lotic habitat, a medium or large-scale lotic habitat or a lentic or slow-water habitat. Thus, maps that allow determining the stream order according to the Horton method, modified by Strahler (1957), should be examined prior to going to the field, and also available during the field collection. Selection of sites should favor heterogeneity of habitat structure, in order to have the best opportunity to focus collecting and achieve the most complete species list possible.

Typical sampling effort required – For small-scale lotic habitats, the minimum effort needed for standardized sampling is a team of three people working together in the same site for one hour. For medium and large-scale lotic habitats and lentic and slow-water habitats, the minimum amount of effort needed for standardized sampling is a team of four people working together in the same site for one hour.

The minimum number of samples for a rapid assessment should be 30. Based on available data (Chernoff *et al.*, 1999a; Chernoff *et al.*, 1999b; Mol *et al.*, 2006; Anjos & Zuanon, 2007), it is likely that up to 90% of the species of fishes in small-scale lotic habitats are collected after approximately 30 samples in relatively small and homogeneous areas, using the protocols suggested herein (one sample corresponding to one collection event at one site).

However, if a more complete inventory is the objective of the survey, especially focusing on increasing the number of species sampled in larger areas, it is strongly suggested that more effort is made using additional sampling equipment and techniques, described below, as well as more samples.

As rapid assessments are intended to be fast and to last a short period of time, the sampling effort will depend ultimately on the amount of time available for field trips. Using the methods and protocols described herein, it is possible to make up to five samples in small-scale lotic habitats, or up to one or two samples in medium and large-scale lotic habitats or, lentic or slow-water habitats per day. In a field trip that lasts 15 days, it is possible to make up to 50 samples, usually less however due to difficulties in reaching each collection site.

Medium and large-scale habitats and lentic and slow-water habitats have usually more diverse fish fauna and more microhabitats to sample, which diminishes the percentage of species sampled during a rapid survey. Shotgun-style inventories based on qualitative surveys may detect more species, as more distinct sampling equipment is used and more effort is employed. However, shotgun approaches ignore standardized protocols, making the resulting data not suitable for later comparison in terms of species relative abundance. Species accumulation and rarefaction curves should be calculated based exclusively on data obtained through standardized sampling. Species accumulation and rarefaction curves can be obtained through distinct methods including Chao and Jacknife, for example, or a combination of these (Gotelli & Colwell, 2010; Ortega *et al.*, 2014).

Context Dependent Sampling Considerations

Sampling considerations for assessing different types of environmental change/disturbances —
If one of the goals of a rapid survey is to measure environmental disturbances, collections should be made in the same site prior to those changes, possibly more than one time in order to have more data that can be compared to the data obtained in the same site after disturbance. Alternatively, a reference site can be selected with similar overall characteristics (width, depth, water volume and velocity, type of substrate, abundance of vegetation, etc) but lacking the environmental disturbance. Data obtained in a reference site could replace those obtained in the studied area prior to disturbances, if the latter are not available. Some environmental disturbances change the type of the habitat, for example, from lotic to lentic, making comparisons of fish diversity much more complex, as the sampling methods applied in those habitats are distinct. In those cases, inventories should use a greater variety of equipment, techniques and more extensive effort.

Habitat considerations — The standard rapid assessment protocols herein suggested are not appropriate for every aquatic environment. There are several particular habitats that need special consideration. Details and suggestions on additional sampling methods are found below. Creeks and small streams with high elevation and steep slope (e.g., those found in the Andes of South America) cannot be efficiently sampled using only the protocols herein suggested for small lotic water bodies. Strong water flows prevent the suggested protocol from being implemented effectively (e.g., it may not be possible to block the stream with a net). For high gradient mountain streams, for example, electrofishing is often a requirement for efficient sampling (Ortega *et al.*, 2014).

Other unique and difficult to access habitats include the main channels of large rivers, most of which have highly specialized fishes occupying the bottoms of the main channel. Large predatory fishes of deep river channels can be collected by drifting large gill nets or through other local fishing techniques (e.g. fishtraps with certain kind of bait depending on the species), whereas the majority of small bottom fishes can only be collected using bottom trawl nets. This technique can yield many types of fishes that are not collected otherwise (Barthem & Goulding, 1997).

Temporary isolated ponds are generally shallow and ephemeral habitats. The rapid survey techniques proposed in this chapter do not apply to them, as it is impossible to use the same sampling equipment (e.g., gill nets in some cases). The most common method to inventory these habitats is to use sieves and dip nets. Similarly, caves and subterranean waters are complex and highly variable habitats. Sampling methods applied in these types of environments are mainly traps, sieves and dip nets (Bichuette & Trajano, 2003).

Biogeographic or regional considerations – Fishes are excellent subjects for biogeographic studies, as many fish species are often restricted to particular watersheds. It is common to find distinct species separated by relatively short distances, and distinct faunas in far away places (distinct continents, for example). Nevertheless, the basic methods proposed here for collecting fishes apply equally to sites globally.

Seasonality – The most significant change in the fish fauna in tropic environments is related to changes in the water level, turbidity, and chemistry. Those changes occur seasonally in many tropical environments, in which species composition vary in abundance and diversity of fishes tend to be greater in the rainy season, when food resources are more available as water floods into riparian forests (Matthews, 1998). Therefore, the minimum protocol must include collections in periods when the water is highest and also in periods when the water is lowest, to obtain a realistic representation of a region. On shorter timescales, rain events can often cause spates in which water levels rise rapidly. It is during these periods that many large fish move from their shelters, making such events ideal opportunities to sample using baited hook and line. On the other hand, it is easier to sample fishes, particularly in most water bodies in the dry season, when there are fewer places for fishes to hide and is easier for researchers to explore the water body and manage nets.

Supplemental Methods

Many other fishing methods could be used to maximize the number of species sampled during a rapid inventory. If the aim of the survey is to sample as many species as possible, the minimum protocol herein suggested should be supplemented by additional effort, using the methods and sampling equipment described above, and also other fishing methods detailed below.

Underwater observation – this method is only feasible in places where visibility is of 50 cm or (preferably) more. This method depends also on the target species, for example, to observe fast swimming fishes high visibility is needed, whereas slow benthic fishes can be observed in places with approximately 50 cm of visibility. There are many different techniques for collecting data based on underwater observation, most of them developed in studies of fishes in marine habitats where water clarity is much higher. In addition, some benthic fishes can be hand-caught (or caught with help of a dip net) once they are located underwater, a technique that works well for loricariid armored catfishes in South America, for example, and is known as hogging or noodling.

Electrofishing: this method is only feasible in water bodies with medium to high conductivity (i.e., 50 uS/cm or higher). This method is most effective in Andean rivers of South America, in addition to creeks and lakes (Ortega *et al.*, 2014). The standardized protocol for electrofishing includes the delimitation of a stretch of the water body (100 meters, for example) and time for determination of collecting effort.

Trawling: this method is only feasible in large rivers with bottoms that are free of large rocks and trunks (which can be determined by sonar or local fishermen). In addition, this technique requires the use of a motorboat and a fully equipped trawling net (with otter boards and cable). This technique is especially interesting for species that live deep in river channels (for example, catfishes and electric fishes in South America).

Hook and line (including trotline): methods including hook and line are among the oldest and most diverse fishing technique available. These methods can be used in a great variety of environments focusing especially on medium to large fishes, usually predatory species at low density in fast flowing rivers and deep lakes. However, even small fish can often be collected effectively using small hooks at the water's surface.

Trapping: most traps work on a "funnel" or "maze" principle, with fishes being attracted by bait, passing through an opening and being unable to find their way out. Traps are especially good in deep pools, lakes and places with difficult access such as caves.

Electric detectors: this method is used for detecting electric fishes. This method requires a small battery-powered audio amplifier and duplex cable with terminal on one end bare and is extremely effective at locating electric fishes (e.g., Gymnotiformes in South America, Mormyridae (Osteoglossiformes) in Africa). Once the fishes are located they can be caught by sieves and dip or seine nets.

Removal of structure: In medium to large size rivers, there are often zones near the bank where dead, partially rotten wood and/or lateritic rocks with many holes accumulate. In these habitats, it can be very effective to remove whole pieces of wood and/or lateritic boulders to the shore where they can be broken and their holes explored more thoroughly. Many types of fishes use these structures as hiding places and are often only collected by this method. Additionally, scooping submerged sand from the river bottom into a bucket and dumping it on dry land can sometimes yield specialized sand-dwelling fishes.

Data Management

Species identification, specimen processing and management – Rapid inventories must try to maximize the information collected in the field during a short time period. In fish inventories, fish specimens are traditionally collected and preserved in formaline solution in the field and kept in alcohol solution in the lab. For details of methods for processing and preserving specimens see Motomura & Ishikawa (2013). In more modern days, two additional sources of information collected in fish inventories, tissue samples and photograph of live specimens, should be encouraged in order to help species identification and to increase the current knowledge of fish diversity, evolution, and biogeography through future studies. Tissue should be taken primarily in the field and tagged by sequential numbers linked to individual specimens, which should be photographed for subsequent identification (more details in Motomura & Ishikawa, 2013). Other informative data include photographs of live specimens, which can be taken in the field with specimens anesthetized (details in Lucena *et al.*, 2013).

At least one specimen of each species per site should be photographed live, and possibly more than one when specimens of different sizes or dimorphically distinct genders are collected (details on methods in Sabaj Pérez, 2009; Motomura & Ishikawa, 2013). Photographs of each sampling site should also be taken and uniquely identified. Field forms should be filled for each site, including a field number associated to each collection event. Important information that should be included in the field forms are:

- locality,
- municipality/state,
- country,
- geographical coordinates (datum),
- collectors,
- date of collection, and
- general information on the site (e.g., type of substrate, water velocity, amount of underwater and marginal vegetation).

All fishes collected should be deposited in fish collections that are available to the scientific community. If possible, samples should be deposited in more than one fish collection, minimising the risk of losing all vouchers due to eventual disasters, and making data more easily available for the global scientific community. Many countries require, in order to issue permits, that at least 50% of the collected specimens are deposited in fish collections of that country.

Data collection – All data collected during a rapid survey through the herein suggested protocols consist of fish specimens. Additional collecting methods might include other types of data such as observations of fishes in the environment (using dive techniques, for example).

The data collected during a survey should include a species list per site, with number of specimens per species per site, and a species accumulation curve to demonstrate the effectiveness of the sampling. In addition, a survey should identify those species that are endangered species based on IUCN criteria, on national or regional red lists, endemic species, migratory species, potentially new species, invasive species, ornamental and commercial species, and new distribution records. The data obtained thorough standardized sampling should also be used to calculate Diversity and Richness of each site, indices that could be used to make comparisons among areas or in the same collection site at different times. Details on how to analyze data are summarized in Ortega *et al*. (2014).

Conservation Implications and Limitations

Conservation implications – When rapid surveys are effectively executed, the collected data provide information that can be used to recognize areas with high endemism, are well-preserved, or to identify target species for conservation actions. Also, data collected in this way facilitates the understanding of how ecosystems change due to disturbances, including those related to human activities. Diversity and abundance of fish communities can be used to understand the integrity of a studied area, as well as changes over time (Bozzetti & Schulz, 2004). In addition, the presence of indicator species, such as threatened, exotic, or species with particular needs (i.e., forested riparian zones) may help to evaluate the current status of a selected area (Cambray, 2003).

Rapid surveys can provide the chance to collect specimens of poorly known or new species, and improve the knowledge of a particular taxon or an area of interest. In addition, surveys are often performed by multi-institutional teams, strengthening local scientific communities.

Constraints and limitations for rapid survey – The most obvious limitation of rapid surveys is the short amount of time applied towards inventory efforts, which can result in incomplete species lists necessary to devise complete species richness and diversity. This is especially true for large and heterogeneous sampling areas. As a consequence, comparisons among distinct habitats and environments might be complicated by biased collections. However, the core standardized sampling methods proposed here strives to avoid these issues. Also of note, rapid surveys are more effective at sampling common and abundant species, whereas rare species are often missed. Rapid surveys can be supplemented by desktop searches via online fish collection databases (e.g., SpeciesLink, http://www.splink.org.br/index; VertNet: http://vertnet.org), and other regional studies in peer-reviewed published references.

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FRUIT-FEEDING BUTTERFLIES

A STANDARDIZED SAMPLING PROTOCOL FOR FRUIT-FEEDING BUTTERFLIES (NYMPHALIDAE)

Philip J. DeVries[1], Christopher A. Hamm[2] and James A. Fordyce[3]

Introduction

Butterflies are among the best-known insects in the world, and their great public appeal makes them a useful group for conservation inventories and monitoring. The Nymphalidae is the largest family of butterflies, and the feeding guild known as fruit-feeding nymphalids may comprise up to 50% of the nymphalid species richness in tropical forests (DeVries *et al.* 2012). One of the most salient characteristics of this group is that they can be sampled in a standardized manner to avoid human collector biases, thus facilitating comparisons of species richness, composition and abundance within and among habitat types. As such, standardized trap-sampling of fruit-feeding nymphalid butterflies has been shown to be an effective means for understanding tropical butterfly diversity in space and time, and for use in conservation efforts (DeVries and Walla 2001; Hill and Hamer 2004; Molleman *et al.* 2006; DeVries *et al.* 2012; Freitas *et al.* 2014). For these reasons, we propose focusing rapid, standardized sampling methods exclusively on fruit-feeding nymphalids, rather than on the entire butterfly community. There are many trap studies now being conducted, but most, however, are not directly comparable because they do not use consistent trap designs, sampling protocols or bait (see examples and citations in DeVries 1987, DeVries & Walla 2001, Batra 2006, Frietas *et al.* 2015). The sampling protocol provided here is based on more than 10 years of monthly sampling conducted in lowland Neotropical forests at Garza Cocha, Sucumbios Province, Ecuador and the Tirimbina Biological Reserve Heredia Province, Costa Rica that have been demonstrated to be directly comparable (DeVries & Walla 2001, DeVries *et al.* 2012).

Core Methods – The Trapping Protocol

Trap Construction – A completed trap is a cylinder 1 m tall and 37 cm in diameter with a closed top and open bottom (Fig 1). Two metal ring frames are sewn into the top and bottom, and the netting must completely close the top of the cylinder. A piece of transparent plastic sheeting can be placed on top of the cylinder to help keep rain out of the bait cup (optional, depending on sampling site, and rain frequency and intensity). The cylinder needs to be sewn such that the netting overlaps on the long axis by 2 cm leaving a 20 cm unsewn slit approximately 30 cm from the top to allow access to the trap interior. Suspended from the bottom ring of the cylinder is a 47-49 cm square trap base (3 mm durable

[1] Department of Biological Sciences
   University of New Orleans
   2000 Lakeshore Dr.
   New Orleans, Louisiana 70148 USA

[2] Department of Ecology and Evolutionary
    Biology
   University of Kansas
   1200 Sunnyside Ave – 5032
   Lawrence, Kansas 66045 USA

[3] Department of Ecology and Evolutionary
    Biology
   University of Tennessee
   569 Dabney Hall
   Knoxville Tennessee 37996 USA

plastic for wet habitats, 5 mm plywood for dry habitats) that hangs 6 cm below the opening of the cylinder (keeping this distance is important to minimize escapees). The diameter of the trap base needs to extend 5-6 cm beyond the cylinder diameter (this is important because it provides a landing platform). Holes are drilled on each side, and plastic cable ties or plastic cords can be used to attach the base to the trap. A small plastic bait cup is secured to the center of the base with a loop of thin, stiff wire that is passed through two holes drilled in the base. The wire is then pressed down into the mouth of the cup to keep the bait cup upright and centered on the base. The receptacle for the bait should have a volume of at least 200 ml (8 ounces), and just be tall enough to pass between the base and lower trap ring (6.5 cm maximum, not lower than 6 cm). Cheap, pliable plastic containers work well as they can be cut to size. A sufficient length of nylon cord needs to be secured to the bottom of the trap base to assist pulling canopy traps down from the canopy position. Looping it through the holes of the wire bait cup retainer works well.

Bait – Traps are baited with locally obtained bananas that are first chopped into 2-3 cm pieces and mashed in a large container (that has a lid) by treading on the chopped bananas (wearing rubber boots is optional but useful). Approximately two large bananas are appropriate for each trap, but prepare 1.5 times the volume needed to initially bait all traps. This will be required for subsequent re-baiting during the sampling period. Depending on the source, bananas may have been sprayed with insecticides and fungicides and should either be peeled or washed prior to mashing. The mashed bananas should be allowed to ferment in the large container with the lid sealed for 48 hours prior to use. The day before trapping approximately 150-200 ml of banana mash is added to the bait receptacle in each trap such that the bait level is below the top of the receptacle. Sampling begins the next day. To keep the bait fresh, on day three of trapping add additional bait from the large container to the remaining bait in the receptacle.
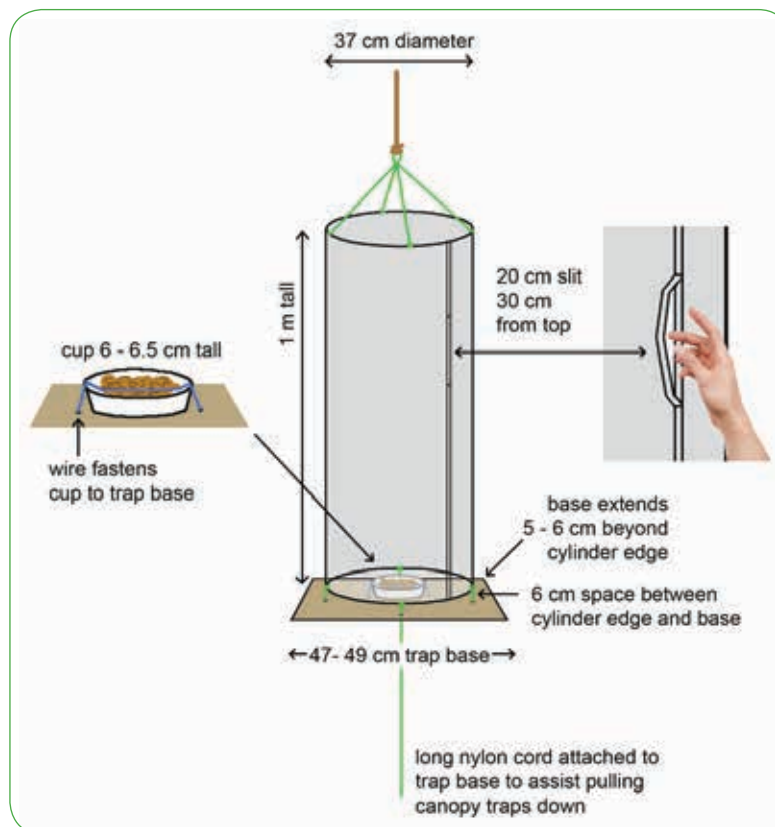

Figure 1 (A)
Standardized butterfly
trap design

Figure 1 (B)
Canopy trap ready to be deployed in lowland rainforest, Ecuador.

Materials and supplies

Equipment list per trap

- 1 m x 1.3 m of mesh material per trap – enough to make the cylinder and the top.

- Two rust-resistant metal rings – 37 cm diameter. These can be made from thick wire obtained at a local hardware store, and welded or taped into the correct diameter.

- 47-49 cm base plate made of 3 mm of durable plastic for wet habitats, or 5 mm plywood for dry habitats.

- 10 cable-ties – to affix base plate to trap. The space between trap bottom and bottom ring will dictate the length of cable-ties.

- 6.5 cm tall, 200 ml volume, plastic receptacle for bait (e.g., the cut base of a plastic water bottle works well).

- 0.5 m of flexible metal wire to affix bait receptacle to base plate.

- 70 m of nylon cord.

- BigShot line catapult – comes with cords and weights.

- Three 3 m poles for tripod construction when placing traps in open habitat, can be locally available materials (e.g. bamboo).

- One large bucket with a sealing lid for banana mash.

- Bananas, approximately two large bananas are required per trap to make the fermented banana mash. The total must be scaled to the size of the study, and additional mash to add during third day of sampling.

Other required equipment

- Indelible ink pens.

- Glassine envelopes: most specimens fit in size #1, large specimens will fit in size #2.

- Waterproof notebook for data entry (e.g., Rite-in-the-Rain).

- GPS device capable of accuracy within 10 m.

- Sealable plastic container for storing specimens.

- Silica gel or similar desiccant.

- Digital camera.

- Device to record minimum and maximum temperature and relative humidity.

Trap Placement — In tropical forest and savanna habitats where tree canopies are at least 8-10 m, it is essential to place traps in the canopy because available evidence indicates that the canopy and understory butterfly communities are distinct (see rainforest studies cited in DeVries *et al* 2012; Freitas *et al* 2014; Fordyce & DeVries, unpublished; Brazilian Cerrado G. Freire Jr., pers. comm.). Canopy trap lines need to be shot over a tree limb with a line catapult such that the trap can be elevated and lowered easily from the ground without hitting other vegetation. This is important as it dictates what individual trees are selected to suspend the canopy traps. Canopy traps should be placed such that each trap is located within, or very close to the canopy of the individual tree selected. The 'Big Shot' brand catapult is very good for this purpose, or if necessary, it can serve as a model to build a similar apparatus from locally available materials. Understory traps are placed with a cord thrown over a convenient limb and suspended such that the trap base is 1 m above the forest floor. Traps need to be uniquely numbered and lettered for easy reference later (e.g., trap 10C, 10U, 5C, 5U, etc.).

To be consistent and comparable with published and future butterfly trap studies, each trapping station (consisting of a paired canopy and understory trap in forest, or single trap in open habitat) should be placed haphazardly within the area of each habitat type to be sampled. Trapping stations should be separated by at least 20 m (e.g., DeVries & Walla 2001). We use a haphazard design because the structure of a particular habitat often precludes using a strict randomization that makes trap placement difficult or impossible (e.g., presence of ravines, rivers, etc). The placement of a canopy trap in forest habitats depends on a suitable canopy tree. Tree selection is dictated by nearby vegetation (liana cover, mid-story palms and trees), inasmuch as not all trees will allow an easy line shot, or space to smoothly run traps up and down. Choosing an appropriate canopy tree will, in turn, determine the placement of the understory trap. In other words, common sense and habitat architecture should be used to facilitate trap placement.

In habitats where there is no forest canopy cover (e.g., grassland-like habitats), traps should be suspended by employing a tripod constructed of poles of sufficient length so the trap bottom is 1 m above the ground (to ensure comparability with forest traps).

For analytical purposes, each individual trap represents an independent sampling unit (i.e., canopy and understory traps of the same trapping station are separate sampling units).

Sampling Effort — In forested habitats a minimum of 5 stations should be established, each with a paired canopy and understory trap. In savanna-like habitats without high canopy cover, a minimum of 10 stations should be established. This maintains parity in minimum sampling effort across habitats.

Since it is not possible to sample the entire butterfly community during a rapid survey, it is important to understand the relationship between sampling effort and the number of species observed (Fig. 2). Sampling effort and observed species richness can be increased either by longer sampling duration or a greater number of traps. Figure 2 demonstrates the relative contribution of each approach to species richness. Given the time available for sampling at each survey site, as well as the availability of materials

and accessible area of the site to be sampled, this relationship can be used to determine how many trap stations should be established. Note that the standardized sampling protocol described herein allows for comparisons among sites with unequal sampling effort using standard rarefaction methods (Gotelli & Colwell 2001).
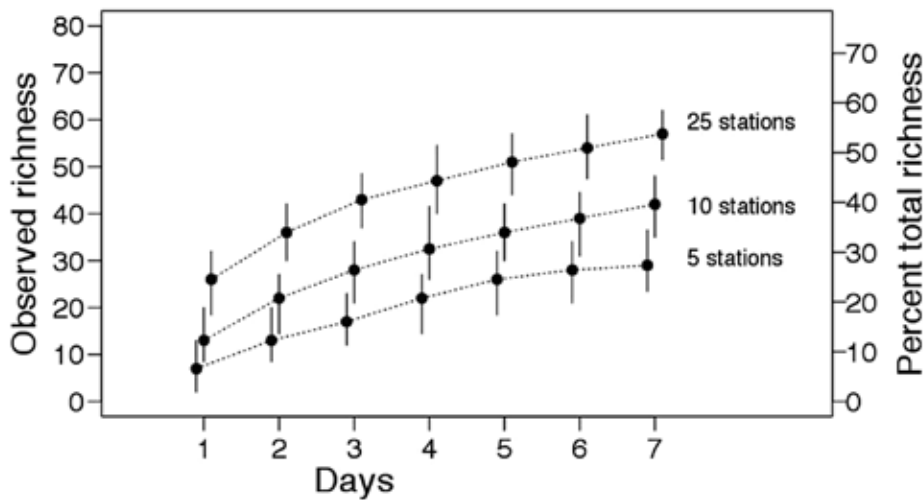

Figure 2
Null expectation of richness captured as a function of sampling effort based on empirical capture probabilities obtained at Reserva Biologica La Tirimbina, Sarapiqui, Costa Rica. Bars indicate 95% highest density interval based on 1000 simulated data sets. Total species richness observed over the course of this 5 year study was 102 species (DeVries et al. 2012). Y-axis indicates raw richness, Z-axis indicates percentage of total richness.

Data Collection and Management — On trapping days each trap needs to be checked at least once a day, sometimes twice, depending on daily capture abundance. In some areas, certain seasons or months may show high species abundances that require checking the traps more frequently. All individual butterflies should be removed, killed, placed in individual glassine envelopes and the relevant data written directly on the envelope with indelible pen (locality, trap number and vertical position, date, etc.). A minimum of two researchers is needed to check the traps at least once (sometimes twice) a day. One person is responsible for removing and processing sampled individuals, while the other records envelope data for each individual butterfly into a field notebook (example below).

Example of field notebook data taken during sampling:

Name	Position	Station ID	Date	Location
Archaeoprepona demophon	Canopy	1	1 jan	Tirimbina
Hamadryas februa	Understory	2	1 jan	Tirimbina

After initiating trap sampling in a new area there will be an initial period when the researchers will need to learn to identify the genera and species in their samples. In areas where field guides are unavailable researchers should make up temporary names for recording in the field notebook (e.g., large orange spot, brown 2 eyes). Eventually the samples will be determined to species by a specialist, at which time the temporary field names in the notebook can be modified.

Specimens should be deposited into an appropriate and curated repository, such as a museum or natural history collection. Data from individual specimens should be digitized as soon as possible and stored in multiple locations. Ideally, the data are stored in some type of database (e.g., SQL), a format that allows for easy hosting and dissemination of data. Data collected by this method should be given a DOI and made publicly available as soon as possible and placed under a Creative Commons license that allows free use as long as proper attribution is given. These data can be hosted free of charge on sites such as FigShare.

Conservation Implications – This sampling protocol provides a standardized method for assessing the species diversity of a butterfly feeding guild. In tropical forests fruit-feeding nymphalid butterflies show fluctuations in abundance and richness, and respond to disturbance (e.g., DeVries, Murray & Lande 1999; Hill & Hamer 2004; Molleman *et al*. 2006; Bossart & Opuni-Frimpong 2009). Using these standardized methods makes it relatively easy to compare results among sites, to understand community-level changes over time, and to evaluate fluctuations in rare and common species within and among sampling sites. For these reasons fruit-feeding nymphalids have great potential as a group for revealing critical patterns for conservation monitoring.

Limitations – All sampling methods have limitations, trade-offs, and biases. Based on the systems that we know well (lowland rain forest), fruit-feeding butterfly richness and abundance are idiosyncratic across time and do not necessarily reflect seasonal trends (Fig. 3; Table 1), thus complicating comparisons where long-term data are not available. For example, Table 1 shows monthly pairwise (dis)similarity of butterfly communities at Reserva Biologica La Tirimbina, Costa Rica. Each month is roughly equally similar to all other months, and there are no obvious seasonal (or temporal) autocorrelations in community composition. This might be advantageous, in that there is no obvious "best time of the year" to assess these communities. However, it also exposes the weakness of short-term studies to capture community composition, as many less-common species will not be detected (see also Fig. 2). Thus, reliable estimates of species richness and records of species occurrence might require long-term trap data, and comparisons among short-term studies should be conducted with a keen awareness of these limitations. Furthermore, testing fruit-feeding butterflies for seasonal effects will be required for other habitats such as savanna, grasslands, paramo and wetlands where there are no data currently available.

While trap-sampling only fruit-feeding butterflies using this standardized protocol provides comparable data across multiple sites, it will not capture nectar-feeding species in the Nymphalidae or other butterfly families. Opportunistic collecting with a hand net should therefore be done to complement trap-sampling. Such hand-collecting will contribute to our understanding of diversity at the site, but due to the biases associated with this method, data cannot be compared across different sites. Alternatively one could conduct Pollard transects (Pollard & Yates 1993), but there are serious drawbacks with this method in tropical habitats that have high richness and low abundance, or where the butterfly fauna is poorly known (Hamm 2013). Moreover, transect-based survey methods cannot account for potential vertical stratification of butterfly communities in lowland tropical forests.
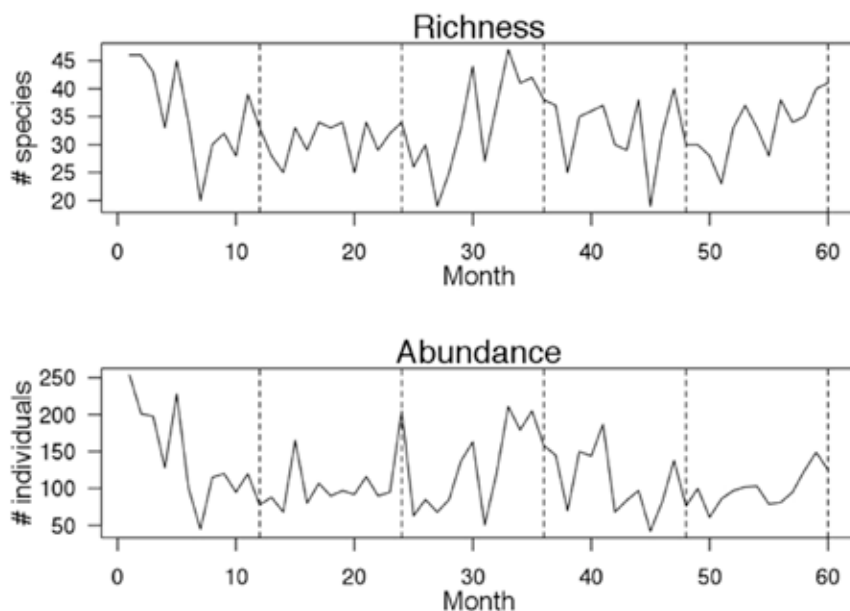
Figure 3
Richness and abundance over time for five year trap study at Reserva Biologica La Tirimbina, Sarapiqui, Costa Rica. Although richness and abundance are idiosyncractic (i.e., showing no seasonality), richness and abundance are highly correlated (r = 0.8).

TABLE 1: Monthly pairwise Jaccard (dis)similarity matrix of butterfly communities based on richness (incidence) data.

Note the idiosyncratic nature of monthly comparisons – monthly comparisons at the same site range between 0.23 and 0.40, with no obvious temporal trend.

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Jan	0.00											
Feb	0.37	0										
Mar	0.36	0.33	0									
Apr	0.31	0.33	0.28	0								
May	0.31	0.38	0.35	0.25	0							
Jun	0.38	0.35	0.32	0.32	0.34	0						
Jul	0.34	0.36	0.35	0.25	0.32	0.23	0					
Aug	0.34	0.40	0.39	0.35	0.32	0.37	0.31	0				
Sep	0.33	0.35	0.34	0.27	0.27	0.29	0.25	0.25	0			
Oct	0.30	0.39	0.32	0.33	0.31	0.31	0.24	0.38	0.26	0		
Nov	0.32	0.36	0.36	0.33	0.36	0.29	0.27	0.36	0.29	0.30	0	
Dec	0.28	0.30	0.35	0.30	0.24	0.32	0.28	0.28	0.29	0.33	0.33	0

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DUNG BEETLES

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STANDARDIZED METHODS FOR RAPID ASSESSMENTS USING DUNG BEETLES

Trond H. Larsen[1], Adrian Forsyth[2] and Alejandro Lopera-Toro[3]

Introduction

Why are dung beetles useful for rapid assessments?

Due to the tremendous diversity of invertebrates, particularly insects, it is not feasible to sample all groups during rapid surveys. Virtually every biologist touts the advantages of examining their study organisms, particularly when it comes to choosing among taxonomic groups of insects. However, dung beetles (Coleoptera: Scarabaeidae: Scarabaeinae) really do provide an ideal, cost-effective model taxon for understanding wider patterns of biodiversity (Larsen and Forsyth 2005; Spector 2006; Nichols, Larsen *et al*. 2007). This has been demonstrated by objective comparative studies of multiple taxa (Gardner, Barlow *et al*. 2008).

Dung beetles 1) can be sampled rapidly and inexpensively using standardized traps; 2) readily show graded responses to many kinds of environmental change; 3) are linked with large vertebrate, especially mammal, communities, therefore providing an indicator of hunting pressure; 4) play important but varied functional and ecological roles (e.g., seed dispersal, parasite regulation); 5) are usually abundant, especially in tropical forests; and 6) are relatively diverse and widespread.

Dung beetle species richness and abundance decline sharply in response to virtually every type of environmental disturbance, which also alters species composition. Habitat loss, fragmentation and degradation have especially strong impacts on dung beetle communities (Nichols, Larsen *et al*. 2007; Larsen, Lopera *et al*. 2008; Edwards online early). Because dung beetles are tropical ectotherms, they exhibit a very narrow thermal tolerance, making them especially sensitive to climate change (Larsen, Brehm *et al*. 2011; Larsen 2012). Our studies have already shown that species' ranges are moving upslope in response to climatic warming (Larsen, unpub. data). Dung beetles can be effectively used to indicate hunting pressure on large vertebrates since dung beetle biomass is directly derived from the food they ingest, yet dung beetles can be comprehensively sampled in a fraction of the time it would take to sample large vertebrate communities themselves (Larsen 2011). The many ways in which diverse dung beetle communities segregate ecologically result in high Beta-diversity and high variability of species traits (Edwards 2013). We know of no other invertebrate taxon offering such holistic advantages for rapid assessment and monitoring, particularly with respect to sampling efficiency and standardization.

[1] Director, Rapid Assessment Program
Conservation International
2011 Crystal Drive, Suite 500
Arlington, VA 22202 USA

[2] Executive Director
The Andes Amazon Fund
1633 Connecticut Ave NW, 3rd Floor,
Washington, DC 20009 USA

[3] Coordinador Investigaciones
Asociacion GAICA
Calle 11A # 32-21
Pasto, Narino, Colombia

Core Methods

Dung-baited pitfall traps

To exhaustively sample the Scarabaeinae community, a range of methods will be needed. The minimum standardized sampling approach for rapid, quantitative studies consists of linear transects of pitfall traps baited with human dung. These provide the most effective and standardized way to sample the majority of dung beetle species, and quickly yield a wealth of data.

Bait preparation. Dung can be deposited directly onto a large leaf or, if traps are not to be set right away, can be put straight into a large tupperware or other plastic food storage container (lined with large leaves). Do not store bait outside of a sealed container, as beetles, flies, etc. will quickly invade it, and it will become ineffective. If the bait must be stored, place it in a relatively cool place in the shade for up to 24 hours.

When you are ready to set the traps, prepare the individual baits. Cut a square of nylon fine mesh fabric (e.g., mosquito netting or bridal tulle), approximately 6 inches (15 cm) per side, and a piece of cotton string approximately 8 inches (20 cm) long for each trap. Lay the fabric onto a flat surface, clear of debris (e.g., on a large leaf or rock). Use two sticks to transfer the bait from the tupperware onto the center of the tulle. A dung bait about half to two thirds the size of a chicken egg is effective (25 g or 2-4 cc). A typical production by an adult human can be subdivided to provide baits for about ten traps. Some dung beetle species may prefer dung baits of a certain size. Keep the bait size roughly constant between traps. Pull the four corners of the tulle together and tie tightly with string. Place the baits back into a tupperware (a shallow tupperware works best), with the loose end of the string hanging out, for easy transport to the traps.

Fill a large plastic bottle (you may need more than one), such as a 2 liter soda bottle, with water and a small amount of detergent (unscented variety is best), which acts to quickly drown the beetles. A pinch of salt can also be added to the water to slow the decay of beetles, especially at sites with very high temperatures, but in some areas this can attract inordinate numbers of bees and other non-target insects.

Setting the traps. Choose an approximately linear transect through each focal habitat and select five to ten evenly spaced trap locations (more replicates are better, but can be constrained by bait availability or habitat area, e.g. small forest remnants). A transect should be placed in each habitat type of interest, and each habitat type will contain a different species composition. A minimum of 50 m spacing between traps should be sufficient to eliminate trap interference, but greater distances (e.g., 100 m) are preferable when possible. Use flagging tape to mark trap location and trap number. If possible, record GPS coordinates for each trap.

Figure 1
Dung beetle pitfall trap design

Equipment needed

- Plastic cups/containers (≥16 oz)
- Dish detergent/soap
- Salt
- Tupperware
- Ziplock bags (gallon)
- Scissors
- Nylon mesh or tulle
- Strainer/sieve
- String
- Machete
- 1-2 liter water bottles

- Datasheets/notebook
- Label paper (preferably bonded)
- Alcohol (≥70%)
- Soft forceps
- Sorting tray/dish (optional)
- 10x hand lens
- Whirlpaks (4 oz) or other non-leaking storage
- Pencils or alcohol-proof pens
- Permanent marker
- Flagging tape
- GPS

Use a machete or trowel to dig the pitfall holes (a machete is useful for cutting through roots), without digging a hole much wider than the pitfall cup in order to avoid flooding and collapse. We use the 16 fl. oz. variety of plastic cups that can be bought cheaply at many convenience and grocery stores, although these can get quite full with beetles in just 24 hours; larger containers, such as large yogurt containers work very well. Save the excavated soil to one side. Place two stacked plastic cups in the excavated hole so that the rim of the cups is slightly less than an inch above the surrounding soil level (elevating the cups slightly helps to avoid flooding from heavy rain). Repack the soil around the outside of the cups, so that the dirt is flush with the rim of the top cup. Be sure to repack the dirt firmly around the rim to avoid the dirt falling away, which can prevent small species from entering the trap. Remove the top cup to dump out the dirt that falls in during backfilling. By using stacked cups, it is simple to remove the upper cup during each collection period without the entire hole collapsing.

Fill the cup two thirds full with the mixture of water and detergent. Shove a relatively sturdy stick into the ground at a 45 degree angle near the pitfall. Tie a bait ball to the stick so that it is suspended above the cups, but not close to touching the rim. Place 2-3 large leaves above the bait as a roof – understory palms work well, and do not curl up as they dry. If no large leaves are available, plastic plates or cardboard can be used, and supported with additional small sticks found nearby or small barbeque sticks. Beetles attracted to the bait will land in the vicinity of the trap and walk towards the bait, falling into the cup. A well constructed trap is important not only to keep out sun and rain, but also to prevent the more agile beetles from landing on the bait itself.

For long-term monitoring or population studies, beetles can be collected using non-lethal traps. These are the same design as described above, but without any water. A funnel, such as the cut off top of a soda bottle, works well to keep beetles from flying out of the trap. Beetles can be marked and released using a permanent marker or a mototool with a fine tip to engrave a unique number on the pronotum. This method is ideal for studying population and/or community changes over time without altering abundance due to repeated sampling, especially in small habitat remnants.

Collecting the traps. Traps are generally set and baited in the morning, so that the fresh samples can be sorted during the day. If logistics and timing require the traps to be set and collected in the afternoon, beetles should be immediately placed in alcohol overnight to prevent decay. Traps should be collected at least every 24 hours so that each trap sample includes species active during each period of the day, and represents the minimum sampling unit for statistical analysis. Daily collection also prevents decaying insects in the trap from attracting necrophagous dung beetle species and altering the results. To retrieve the samples, remove the upper cup and pour the contents through a sieve/strainer. A sieve with small holes should be used so as not to lose small-bodied beetle species (the smallest dung beetles are only 1.2 mm long). Transfer the contents of the sieve to a large ziplock bag (one for each trap). Be sure that all the beetles fall into the bag and that no specimen remains in the sieve. Add a label to the ziplock with the trap number and date (written with pencil or waterproof pen). Replace the top cup and refill with soapy water for the next collection period. Dung baits should be replaced at least every two days (but ideally every 24 hours), at which point they rapidly become less attractive. In especially arid habitats, dung baits may need to be replaced daily.

Sampling Effort – Generally, a minimum sampling effort at one site consists of 20 trap samples based on 24 hour collections. For example, ten trap stations sampled for two days, or five stations sampled for four days (more time helps to avoid the effects of daily weather patterns, and we recommend at least four days). The most effective design is probably ten traps sampled for six days (60 trap samples). If the objective is to compare more than one site simultaneously, five traps per site might be more tractable. However, sufficient sampling effort will depend on the diversity and abundance of dung beetles at any given site. Plan ahead if possible based on the available literature for the study site. Once in the field, the best way to determine sampling completeness is to construct species accumulation curves (number of species vs. number of samples or individuals), and to examine whether these curves reach a plateau (if possible, richness estimators calculated using statistical software are also very useful) (Fig. 2). If multiple sites or habitats are being compared, try to maintain similar sampling effort for each site. While the work can be done by a single person in the field, two people are ideal for dividing tasks and sampling multiple sites at the same time.
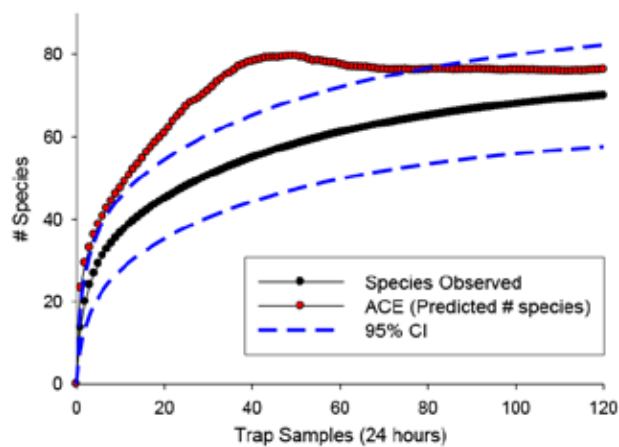

Figure 2
Species accumulation curve demonstrating sampling completeness according to sampling effort data from Los Amigos, Peru, which is probably the most diverse site for dung beetles in the world.

Processing specimens – Back at camp, after collecting all the traps, pour water into each ziplock bag, swish around, and pour the entire contents back into the sieve. Use forceps to remove debris and unwanted material, being careful not to miss any small dung beetle species. Soft, round-tipped forceps are best for sorting small species without damaging them. Beetles can be placed directly into alcohol, or sorted and identified to the morphospecies level as they are collected. A 10x hand lens (we like the Coddington variety) is appropriate for identifying most species in the field. Record the number of individuals of each beetle species captured in each trap on each day on a data sheet (Appendix 1). Alternatively, beetles can be sorted back in the lab with a dissecting scope. If beetles are not identified immediately, maintain separate trap samples (especially for statistical purposes) and place a single 24-hour trap sample into a whirlpack (or similar container) with alcohol, making sure to include a label with collecting data (written with alcohol-proof pen or pencil). 96% ethanol from a local pharmacy or lab equipment store works well and preserves genetic material, but 70% can be used if it is the only thing available. Since dung beetles from the pitfall traps will contain a lot of water, the alcohol should be changed after 5-7 days to prevent decay.

You may find identification assistance from specialized beetle taxonomists, but do not count on it - they are overworked and under-resourced. If you do find someone willing to look at your specimens make sure they are well-curated and preserved, following standard entomological procedures for pinning and mounting. Small beetles may need to be glued to a cardboard point, rather than pinned directly (Fig. 3a). Include a locality label under each specimen with all relevant collecting data. Difficult identifications can often be aided by extraction of the male genitalia (aedeagus). Use very fine-tipped, hard forceps to pry open the pygidium and pull out the heavily sclerotized aedeagus, which can then be glued to a small cardboard point on the same pin with the beetle (Fig. 3b). Ideally the aedeagus should be stored in very small plastic vials in glycerin, which preserves the soft inner parts of the genitalia. If the beetle has dried, soak it first for at least 30 minutes in warm water. It is always important to maintain voucher specimens and a synoptic collection, especially so that species can later be compared with those from other studies, and the value of the data preserved. Specimens should be deposited in a local museum collection in addition to other final destinations.

Figure 3a
Example of a small dung beetle glued to a cardboard point.

Figure 3b
Example of a male dung beetle aedeagus glued to a point.

Context-Dependent Sampling Considerations

Seasonality does not seem to affect dung beetle species composition in most places around the world, and most species in tropical areas can be sampled year-round. However, sampling effectiveness and overall abundance is greatest during the wet season, as many species seem to emerge with the initial rains. Daily weather patterns have a weak to moderate effect on dung beetle activity and trapping success. Fewer beetles will be captured during heavy rains or periods of extreme cold. However, at least some beetles are active even during heavy rain, and with at least four days of continuous trapping at each site, the results should not be affected strongly by daily weather. In arid environments or harsh dry seasons, baits may need to be replaced more frequently as they dry out and become less effective.

Supplementary Sampling Methods

Passive sampling with FITs – Flight intercept traps (FITs) are used to catch beetles passively, and are most useful as a complementary strategy to capture species that are not attracted to the usual baits. Unlike baited traps, FITs will yield relatively low numbers of beetles, but will collect species not found in the baited traps. Flight intercept traps are simply rectangular sheets of netting (dark green nylon works well) that are stretched out perpendicularly to the ground (Fig. 4). We recommend a screen size of about 5 ft (1.5m) wide by 3 ft (1m) high. A sleeve can optionally be sewn on either side of the mesh so that a strong, straight stick can be placed through each sleeve. The sticks can then be pushed into the ground, and the mesh pulled tight by tying string between the tops of the sticks and nearby trees. Below the screen, place pans of the same kind of soapy water used in the pitfall traps. Types of pans that can be used include aluminum roaster pans (turkey size), plastic humidity lids for seed starter trays, and other types of plastic trays. Since beetles, with their heavy elytra, are relatively clumsy fliers, they will tend to fall into the water, while many other insects will fly around or over the netting. Another option is to dig a small trench beneath the screen and line it with plastic trash bags, then fill the trench with soapy water (however bags are very susceptible to holes). FITs should be checked daily since heavy rain can flood the beetles from the traps and because decaying insects can become attractants for necrophagous species that are not targets for passive sampling.

Additional bait-trapping – Although human dung is the most effective in standardized trap studies, sampling beetles with other dung types can be useful depending on the questions to be addressed (Larsen, Lopera *et al*. 2006). Some beetle species have strong preferences for dung of different animals, even though they appear not to be exclusively specialized to any one species. In general, primate, pig and canid dung tend to be highly effective. However, some ungulate dung, such as cattle and horse, contains an especially high proportion of undigested plant material and tends to be less effective in the Neotropics, although is effective in Old World environments. Wild feline dung is also not especially effective, perhaps due to its high nitrogen content. However, all types of dung will attract dung beetles, even dung from birds, lizards, insects and other invertebrates.

Dung beetles feed on many types of decomposing organic material that is rich in bacteria, such as carrion, rotting fruit, and rotting fungus. To capture these species, use the same type of pitfall trap described earlier and prepare the bait in the same way, using a piece of nylon mesh. Some dung beetle species are specialized exclusively on one of these types of food, while others are generalists. After dung, carrion is the most effective bait, and anything available in the field can be used (fish, rats, left-over scraps from dinner, etc.). In remote sites where carrion cannot be found, we have found that dead invertebrates from a previous day's pitfall traps provide a good alternative. As with carrion, they should be allowed to rot for 1-2 days in a sealed plastic bag.

Traps baited with dead insects (e.g., a single katydid, millipede or cockroach) often attract dung beetle species that appear to specialize on small invertebrates, in addition to generalist necrophages. This habit is similar to feeding on carrion, but probably enables species to exploit a slightly different ecological niche.

Some species can be captured in pitfall traps baited with rotting fruit or fungus, although frugivorous and fungivorous species are sometimes more easily found by searching directly at their food source, such as under fruiting trees or at the decaying fungus by rotting logs. If wild fruits cannot easily be found, tomatoes, bananas, and other domestic fruits provide a good alternative, but should be allowed to rot slightly beforehand (e.g., in a bag or sealed container).

Figure 4
Dung beetle pitfall trap design

Other collecting methods – Several dung beetle species are specialized to forage in the forest canopy. These can be sampled using baited pitfall traps similar to those previously described, but with the cup supported in a platform which can be suspended from a high branch. Some species specialize in unusually restricted habitat types, such as narrow riverine beaches or small habitat patches.

Hand sampling of certain microhabitats is also useful for finding certain specialized dung beetle species. A variety of unrelated dung beetle species live in ant nests, and can sometimes be found by excavating the nest. It is not always necessary to dig very deeply, and some leaf-cutter species (e.g., *Atta colombica*) have an external refuse pile where scarabs can be found.

Other species can be collected by sampling leaf litter, or by searching aerial detritus (by hand, or by beating above a sheet), such as that contained in bromeliads or in vine tangles. Many species can be found perching on leaves on low vegetation during day or night, and sometimes these species are difficult or impossible to find in any other way. Searching directly underneath rotting fruit, fungus, carrion and dung is also effective. Blacklighting is not very effective in the Neotropics, attracting just a few species, but is highly effective in Africa, especially for attracting species not captured in pitfall traps.

Identification – Most dung beetle species show high intraspecific variation, and it is best to use a combination of characters in identification. Body size provides a guideline, but varies tremendously within species, depending heavily on the amount of food resources available to the developing larva. In general, color can provide a useful first approximation for separating species, but is not a good character to rely upon on its own. Many species show distinct color forms even at the same locality. Furthermore, adult beetles undergo a 'teneral' period after emerging, during which the exoskeleton remains soft and undarkened, and these individuals should not be confused as separate species. For example, dung beetle species typically black in coloration may appear red while teneral (including many individuals imaged in this book). After emerging as adults, dung beetles do not change in size; however, the exoskeleton often becomes highly worn with age, and this can alter characters such as clypeal teeth and leg shape.

Learning to distinguish males and females is an important step towards identifying species. Sexual dimorphism, where males and females differ morphologically, is common among dung beetles, although sexual dimorphism in body size and color is rare. Most sexually dimorphic dung beetle species differ in the presence and shape of secondary sexual characters, such as horns and pronotal armaments (most commonly, but not always, associated with males). For some closely related sister species, females of two species can be virtually indistinguishable, while species differences are quite obvious in males. However, small (minor) males often lack these traits entirely, making this separation more difficult. A variety of less obvious characters can be used to distinguish sexes, although these vary by species group. In many species, the last male abdominal segments are narrower than in the female, and/or the pygidium is longer. The shape of the foretibia and tibial claw also differs often between males and females.

Certain characters are much less variable within species and between sexes. To give just a few examples, these often include features such as the density, shape and size of punctures and hairs, differences in microsculpturing on various parts of the body (often apparent without magnification due to differences in 'shininess'), shape of the margin of the head, eyes, pronotum or pygidium, and many other characters. A scope with diffuse light (basic tracing paper or opaque plastic work well to diffuse the light source) is useful for comparing features such as punctures, although with experience, it is possible to identify and count species as they are collected in the field using just a 10x hand lens.

Size and shape of male genitalia can be very useful for separating closely related species, and genitalia size does not vary much within species, even among individuals of differing body size. However, most species can be identified without the need to dissect genitalia. Finally, in conjunction with morphological characters, ecological preferences, such as habitat type, diet, and elevational distribution, are important for guiding identification.

Analysis – There are several great references that describe various analytical methods for biodiversity studies in detail, so we will not discuss them here (we strongly recommend 'Measuring Biological Diversity' by Anne Magurran). Dung beetle sampling yields tremendous amounts of data in very little time, which are the envy of ornithologists and mammalogists who struggle with small sample sizes. Furthermore, standardized trapping provides excellent abundance information which strengthens diversity analyses. The software program 'EstimateS' by Rob Colwell is available for free online and provides the basic tools for many of the most useful diversity analyses.

We recommend that basic analyses should include the construction of species accumulation curves and extrapolation of estimated species richness and sampling completeness. A variety of diversity indices exists, and these can be used to describe not just the number of species, but the distribution of abundance among those species ('evenness'). In addition to number of individuals, biomass measurements are very useful for dung beetle studies. Biomass is even more ecologically relevant than number of individuals, since it describes the amount of energy flowing through the ecosystem, and is correlated with the biomass of the large vertebrate community. As for diversity, several indices exist to compare species composition and community structure among sites. The most effective analyses of species similarity include species abundance distributions. More complicated methods, such as correspondence analysis and principle components analysis as well as other multivariate analyses based on similarity indices such as ANOSYM and PERMANOVA tests will help compare compositional species similarity and community structure among sites.

Conservation Implications

Dung beetles provide an excellent model invertebrate taxon for rapid surveys, especially in tropical and subtropical forests and savannas. Perhaps most importantly, they can be consistently and thoroughly sampled in a very cost-effective manner in a short period of time, and the subsequent data can be used to assess changes in mammal communities (e.g., due to hunting), impacts of climate change, and many other types of environmental change. Rapid dung beetle surveys therefore provide an ideal baseline for ongoing monitoring purposes. Two limitations to dung beetle research are that their taxonomy is incomplete in many parts of the world and their abundance in extremely xeric and high elevation habitats can be low.

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Appendix 1: Data sheet

Site/locality					
Coordinates					
Elevation (m)	100	150	200	75	100
Notes/weather	Heavy rain				
Collector	T. Larsen	T. Larsen	T. Larsen	T. Larsen	T. Larsen
Date	3/23/16	3/23/16	3/23/16	3/23/16	3/23/16
Bait type	Dung	Dung	Dung	Fruit	FIT
Trap number	1	2	3	4	5

_____

 # individuals by species

Anomiopus andrei	3	1	4	8	2
Canthon luteicollis	2	5	3	7	1

Photo © Trond H. Larsen

ANTS

LEAF LITTER (GROUND-DWELLING) ANTS

Leeanne E. Alonso[1] and Donat Agosti[2]

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.

[1] Global Wildlife Conservation
  PO Box 129, Austin, TX 78767, USA
  Email: leeannealonso@yahoo.com

[2] Plazi
  Zinggstrasse 16, 3007 Bern, Switzerland
  Email: agosti@amnh.org

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&sciodt=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 m^2) 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 m^2 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 m² 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

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**

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 m^2 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 m^2 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 m^2 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 to 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 emptly 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 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/m^2) 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 and 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.

Acknowledgments

Odontomachus hastatus. Photo © Trond H. Larsen

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FRESHWATER MOLLUSCS

RAPID BIOASSESSMENT METHODS FOR FRESHWATER MOLLUSCS

Kevin S. Cummings[1], Hugh A. Jones[2] and Manuel Lopes-Lima[3]

Introduction

Freshwater molluscs are found worldwide, occurring on all continents except Antarctica. There are approximately 1,200 species of freshwater bivalves, 97% of which belong to eight primary freshwater families: Unionidae, Margaritiferidae, Hyriidae, Mycetopodidae, Iridinidae, and Etheriidae (all Unionoida or freshwater mussels), Sphaeriidae, and Cyrenidae (both Veneroida) (Graf 2013). The world's freshwater gastropod fauna comprises approximately 4,000 described species (Strong *et al*. 2008). Many species are globally imperiled and freshwater molluscs are considered to be the most threatened group of animals in the world (Williams *et al*. 1993; Lydeard *et al*. 2004; Johnson *et al*. 2013).

Freshwater mussels (unionoids) are an integral component of aquatic ecosystems. Freshwater mussels can comprise >90% of the benthic biomass of rivers and an individual mussel can filter 40 L of water each day (Tankersley & Dimock 1993; Pusch *et al*. 2001; Strayer 2008). In addition, their shells function as substrate for many organisms including caddisflies, mayflies and other aquatic insects. Unionoids are often described as ecosystem engineers due to the direct and indirect physical effects that they have on freshwater ecosystems (Gutiérrez *et al*. 2003). Freshwater mussels also provide important direct services to humans, such as water purification, serving as an important prey for several mammals and commercial fishes, and providing a direct source of protein. Given their importance within aquatic ecosystems, the cascading consequences of unionoid declines can be considerable (Haag 2012; Vaughn *et al*. 2015).

Freshwater snails graze on biofilms on rocks and vegetation, and some are suspension or deposit feeders. Gastropods can numerically dominate benthic stream communities and may exceed 50% of the invertebrate biomass. Gastropods are the principal grazers in many aquatic habitats and significantly influence algal primary productivity, playing a pivotal role in aquatic food webs and nutrient cycling (Johnson *et al*. 2013; Pyron & Brown 2015).

[1] Illinois Natural History Survey - University of Illinois at Urbana-Champaign
607 E. Peabody Dr.
Champaign, IL 61820 USA

[2] NSW Office of Environment and Heritage, Sydney 2000 NSW, Australia
Centre for Ecosystem Science, School of BEES,

The University of New South Wales Sydney 2052 NSW, Australia

[3] CIIMAR-UP Interdisciplinary Centre of Marine and Environmental Research, University of Porto, Rua dos Bragas 289, 4050-123 Porto,

Portugal.IUCN-SSC Mollusc Specialist Group, c/o IUCN, 219 Huntingdon Road, Cambridge, UK.

Freshwater molluscs are ideal organisms for rapid biological surveys. Many are conspicuous and for the most part are easily and inexpensively sampled. They are sensitive to anthropogenic disturbance and are considered excellent indicator species. Freshwater mussels are sometimes colloquially referred to as "aquatic canaries in the coalmine" or "livers of the rivers" due to their sensitivity to changes in the environment and water quality and their water filtering capacity.

Freshwater mussels have been harvested for a variety of purposes, including for food, buttons, natural pearls, and as seed material for the commercial production of marine pearls. They were collected and utilized by indigenous people, particularly the mound-building tribes of North America, at least as early as 5400 years ago (Saunders *et al*. 1997). Mussels were not only eaten, but also used for tempering pottery and for making utensils, tools, and jewelry (Lucey 2000; Serrand & Cummings 2014). Freshwater snails also serve as a food source for humans in many parts of the world.

Some species of freshwater molluscs are highly invasive and can change the functioning of ecosystems, cause considerable damage to crops (e.g., some *Pomacea* spp.), spread diseases like schistosomiasis or liver flukes, are biofoulers that impact industry (e.g., zebra, quagga and golden mussels in the genera *Dreissena* and *Limnoperna*), or are detrimental to other wildlife (e.g., New Zealand mudsnails, *Potamopyrgus antipodarum* impacting trout) (Bequaert 1928; Strayer 2010; Sousa *et al*. 2014; Van Bocxlaer *et al*. 2014; Cummings & Graf 2015; Pyron & Brown 2015).

Figure 1
An example of a large river. The Rio Amazonas just outside of Iquitos, Peru.

Core Standardized Methods

Because of the wide variety of habitats occupied by freshwater snails and bivalves, no single sampling method is applicable across all species. The methods presented here will work on all continents and in both temperate and tropical ecosystems. Before fieldwork commences it is essential to do a thorough review of museum collections, the literature and to contact experts to compile data on what species are known from, or could potentially occur, in the study area. A comprehensive risk assessment should be carried out to obviate health and safety issues.

The definition of what constitutes a site varies, but in general, a site is typically an area that can be reasonably searched without traveling a great distance in a relatively short period of time. An area of about 100-300 meters of stream encompassing most of the habitats (i.e., pools, riffles, runs) is a good rule of thumb.

We highlight some basic safety rules for any mollusk survey:

- Avoid working alone, particularly in remote regions.
- Avoid sampling rivers that are in spate.
- Avoid sampling from steep or unstable banks unless equipped with appropriate safety gear, and always test the depth and stability of waterbodies before entering the water.
- Be careful when transporting and handling flammable or toxic liquids (e.g. formaldehyde and ethanol).
- Beware of potential hazards including broken glass, needles, discarded medical equipment, etc., especially when sampling urban rivers.
- Wash hands carefully with soap or a sanitizing spray after the work and before drinking or eating.
- Wear protective equipment (e.g. wet-suits, waders, rock-fishing boots, and gloves to prevent cuts and abrasions).
- Carry at least one first-aid kit.
- Let someone else know where you are going, and carry an Emergency position indicating Radio
- Beacon (EPIRB), mobile phone or satellite phone. Establish a reporting protocol for checking in at the end of each day.
- Be mindful of potential infectious diseases; in case of any eventual symptoms the surveyor should seek medical attention.

In some countries (e.g., Australia) protocols have been developed for sampling in waterbodies containing crocodiles (e.g., DERM 2011) and training is available via crocodile awareness programs to prepare people for fieldwork in waterbodies where these reptiles may be present. Precautions for working in crocodile-infested waterbodies include:

- Using local knowledge whenever possible – local inhabitants will often know if there are large crocodiles in the area.
- Always work in teams of two or more people: one person samples while another keeps watch, holding a whistle to use as an alarm if a crocodile is sighted.
- Avoid sampling sites with treacherous terrain such as steep, muddy banks and turbid waters
- Set up defensive barriers such as sturdy nets around the sampling area.

For rapid surveys, the methods used are habitat dependent. We have identified 5 major habitat types where freshwater molluscs are most frequently found: large rivers, medium-sized rivers and creeks (wadeable streams), lakes (natural and artificial impoundments), wetlands, springs and caves (Fig. 1-4). The following protocols are applicable to all of the habitat types with some modification. However, as most surveys will be conducted in wadeable streams and to a lesser extent on large rivers, the following methods were developed with those habitats in mind.

A wide variety of techniques are used to survey freshwater bivalves and large gastropods, and the method used will depend on the goals of the study and the resources available (Strayer & Smith 2003). The method chosen will influence and limit the way data can be interpreted so it is important to be clear on the objectives of the biodiversity assessment. Sampling methods for molluscs can be categorized as either qualitative or quantitative.

Qualitative sampling includes visual and tactile searches of the streambed, dip net sweeps, use of brail hooks for mussels, searches of the stream bank for shells, and under rocks for gastropods or inside dead bivalves for some fingernail clams (Fig. 5-8). Quantitative methods may include dredging, use of grab samplers or, more usually, quadrats or linear transects distributed over a defined area according to a defined sampling strategy. Quadrats require excavation of the substrate combined with sieving so that buried mussels, and small or cryptic molluscs are not overlooked.

Figure 2
An example of a medium to large river. The Rio Xingu, Para, Brazil.

Surveys for molluscs based on visual or tactile searches of quadrats or a fixed area of stream bed, usually for a set time, are best considered to be semi-quantitative since detectability will vary with water clarity and substrate type, and they will be biased against small individuals and species (Hornbach & Deneka 1996).

If the main goals of a survey are to develop a species inventory or to detect rare or threatened taxa, then a qualitative survey should be adequate. However, where estimates of population density or age structure are required, quantitative methods will need to be used. Quantitative sampling should be used in baseline or monitoring studies where the objective is to assess changes in populations over time. Probability-based designs and quadrat sampling are recommended to provide estimates of uncertainty for abundance estimates and greater power for detecting change (Lindenmayer & Likens 2010; Downes *et al*. 2002). The most common method for collecting freshwater mussels and conspicuous gastropods is simply tactile sampling in the substrate or picking up shells along the shoreline. Viewing buckets and snorkeling can supplement hand sampling, but they are only effective in clear water. Rakes or dredges can be used in shallow water with sandy or fine substrates to bring bivalves to the surface. Snails on vegetation and floating debris can easily be sampled, shaking them in to a white bucket or pan. Small snails and bivalves can be detected using stacked sieves or a kitchen strainer to process small amounts of detritus or sediment throughout the sampling site.

As stated above, no single sampling method is applicable for all species or groups due to the wide variety of habitats occupied by freshwater molluscs. Therefore, for rapid assessments we recommend the following four-step approach.

1. Reconnaissance surveys

A good indicator of current or past presence of molluscs in a waterbody is to search for stranded shells and shell middens along the stream bank and on logs and boulders in the waterbody. Often gastropod and bivalve shells accumulate in debris piles left by floods. The composition of shell middens can provide an indication of changes in the composition of the molluscan community over hundreds or thousands of years (Walker *et al*. 2001, Haag 2012). Consultation with local people can also be helpful in locating where to conduct surveys (Fig. 9-10).

Reconnaissance surveys are essentially exploratory with no set time limit. The intention is to determine presence and spatial distribution of molluscs at a site. A reconnaissance survey of the site will provide data on what molluscs occupy the area and the locations of mussel concentrations within a site, information that can be used to decide upon a more robust sampling method.
If the reconnaissance survey indicates that molluscs are clustered into particular habitats or areas, a stratified search should be done, with greater emphasis given to those areas where they appear to be most abundant. If the site is large then stratify the site by habitat or set out equally spaced transects or cells to ensure coverage of the entire site.

Figure 3
An example of a medium to small river. The Paterson River, a tributary of the Hunter River, Australia.

The available range of substrates should be explored, (e.g. mud, sand, rock, submerged logs, vegetation, and floating debris). In both large rivers and small streams efforts should be made to cover all available habitat types present at the site including riffles, pools, slack water, including searching along the banks versus center of the channel; lakes should be surveyed in quiet, protected bays as well as on exposed shores. Some ampulariid, lymnaeid and pomatiopsid snails can be often found at a considerable distance from the water, so the floodplain area should also be checked.

2. Timed searches for conspicuous bivalves and gastropods

A timed search across the range of habitat types is a rapid and effective technique for determining the species present at a site. The area to be searched depends on the habitats present but a length of about 100-300 m is a good rule of thumb. A variety of search methods may be used, depending on conditions. Viewing boxes are useful in clear, wadeable streams whereas tactile searches to a depth of 40 cm are appropriate in turbid water. Snorkeling, SCUBA or a surface supplied airline (hookah) are necessary for sampling in deeper water, especially in large rivers or lakes (Fig. 11-12).

Figure 4
An example of a small river. An unnamed tributary of the Luangua River, Zambia, Africa.

Rainfall generally suspends sediments in the water increasing turbidity, so surveys should be avoided in the period during or immediately following the rainy season. Additionally, water level is generally higher and the flow stronger during these periods, hampering the actions of the surveyors and decreasing efficiency. Surveys should preferably be undertaken in the dry season when water levels and water clarity are optimal for detecting molluscs. Whilst the time of day is generally not critical for mollusc activity, surveys should be conducted when light availability is good. Surveying during periods with low water clarity, high flow or high water levels will result in detecting fewer species, lower abundances and bias in species composition for the most conspicuous taxa. The suggested conditions for conducting the assessment applies for all steps (sections 2, 3, and 4).

We recommend undertaking timed searches at a site for a minimum of 4 person-hours. However, it has been demonstrated that, in North America, 4 person-hours detects about 60% of expected species. Ten person-hour searches captured more than 70% of all species at over 70% of the sites tested (Huang *et al*. 2011). No studies have been conducted in tropical streams to assess the time required to collect percentages approaching 70%.

Specimens should be placed in separate mesh bags (colors work well) at 1 person-hour intervals as they are collected so that sampling adequacy can be estimated from species accumulation curves. For a 4-person survey team this would require changing storage bags at 15 minute intervals.

The search times suggested here should be reviewed following analysis of species accumulation curves and adjusted accordingly to ensure that the majority of species are collected. The point of diminishing returns, where the curve flattens out, is a sensible time to cease sampling. An estimate of the total number of species present should be made using an estimator of species richness such as the Chao-1 estimator (Gotelli & Chao 2013).

3. Timed searches for small bivalves and gastropods (<2 cm length)

For this method we recommend undertaking timed searches in each habitat type for a minimum of 2 person-hours. A complete survey for freshwater snails and small bivalves will include sampling both benthic surveys and a variety of other substrates including macrophytes, crevices of rocks and wood, other types of floating debris and leaf litter. For sediment sampling, a "kick net" Surber bottom sampler with a rectangular or triangular opening should be used in flowing water. Save the sediments from each sample into lidded buckets for lab analysis or dump the sediments into white trays for sorting and identification in the field, collecting all specimens with forceps or plastic Pasteur pipettes.

For aquatic vegetation and other loose debris, flush the sample into a bucket or run a dip-net several times through it, examining the net contents carefully for small snails such as the hydrobiids, limpets, and small planorbids. Small kitchen strainers and white trays or buckets can be used as cheap and effective alternatives. For strainers, the mesh should have a maximum diameter of 1 mm to capture small or newly-hatched gastropods.

Figure 5
A brail (also called a crowfoot bar) with mussels attached to the hooks. Mississipp River, Illinois, USA.

4. Quantitative Sampling

The methods described above work well for answering basic questions about species presence or absence and richness, but timed searches tend to miss small individuals buried in the sediment and may give biased estimates of absolute abundance, proportional composition of species, and size structure owing to differences in detectability among species and individuals of different sizes. While not completely eliminating bias, implementation of strict search protocols will greatly reduce sampling bias and improve the precision of counts, allowing comparisons among sites.

Quantitative sampling overcomes these shortcomings, providing unbiased estimates of population parameters but it is time-consuming and increases survey costs considerably (Miller & Payne, 1988). Quantitative methods are usually conducted in the form of line transects or quadrat samples, although mark and recapture methods are occasionally used to estimate population abundance and other demographic parameters (Villella *et al.* 2004).

Typically, quadrats are placed on the bottom, and all substrate is removed to a depth of about 10 centimeters and passed through a series of sieves. This method is especially effective in recovering juvenile mussels and small species that are easily missed by hand grabbing (Fig. 13).

Molluscs, especially freshwater mussels, are often spatially aggregated in waterbodies. In this situation, stratified random sampling is a good choice for estimating abundance, especially when combined with an initial reconnaissance survey to delineate areas where molluscs are clustered at a site (Christman 2000). Systematic sampling is also a good choice as it is easy to implement and ensures that quadrats are spatially distributed throughout the site. The number of quadrats required to achieve a desired precision (d) is often expressed as the percentage deviation (p) from the mean ($\bar{x}$) and it depends on the variability of the count data among quadrats (s^2). The required sample size is expressed as:

$$n = \frac{(4s^2)}{(p\bar{x})^2} = \frac{(4s^2)}{d^2}$$

(Thompson 2012). At low densities (e.g. $< 1\,m^{-2}$) the number of samples required achieve a precision of 25% of the mean may exceed $100 \times 0.25\,m^2$ quadrats (Dunn 2000).

A large survey effort is required to establish the presence of rare taxa at a site. This is exacerbated for small or cryptic species that have low detectability (λ). Assuming that rare species are randomly dispersed, the following relationship can be used to estimate the power of a sampling program for the species for a given number of quadrats (n).

$$\text{Sampling power} = 1 - \exp(-\bar{x}\lambda n)$$

Figure 6
A dredge (modified Missouri trawl) used to sample for large molluscs in the deep water (~25 m) of the Rio Xingu, Para, Brazil.

For example, to have a 90% chance of detecting an uncommon species occurring at a mean density of $\bar{x} = 0.1$ individuals/m^2 with a detectability of $\lambda = 0.9$, a sample size of 100 quadrats (size 0.25 m^2) would be required. However, if the aim is to assess regional molluscan diversity, it should be remembered that for rare species it is often more effective to survey more sampling sites less intensively than to spend a lot of time searching a limited number of sites (Mackenzie & Royle 2005). For further details on quantitative sampling methods and different probability sampling designs, see Strayer and Smith's book 'A Guide to Sampling Freshwater Mussel Populations'.

Innovative Methods

A number of innovative methods are now available which can be used to maximize the number of species collected. The use of remote operated vehicles (ROV) equipped with a camera might be useful for visual searches of lakes and big rivers in deep water. The use of side-scan sonar can also be a valuable tool to detect the location of mussel aggregations (Powers *et al*. 2015).

The use of metabarcoding techniques with Environmental DNA (eDNA) water samples can be an alternative tool for the detection and quantification of molluscs in distinct freshwater habitats (Bronnenhuber & Wilson 2013; Goldberg *et al*. 2013; Deiner & Altermatt 2014; Mächler *et al*. 2014). Although these techniques have not yet been mastered, further technological development should increase their accuracy and importance for aquatic surveys in the very near future.

Supplemental and Habitat Dependent Methods

Large Rivers & Lakes

Large rivers present a challenge to sampling benthic organisms like molluscs. Murky water, strong currents and water depths limit tactile sampling to river margins, point bars and shallow side channels, in the absence of SCUBA or surface supplied air. Many sampling regimes involving diving and transect sampling have been developed in North America for use in large rivers (Smith *et al.* 2001; Villella & Smith 2005). Dredges are also an important tool for sampling benthic animals in deepwater habitats (Miller *et al.* 1989; Herzog *et al.* 2009). Additionally, dredges may allow for spatial and temporal comparisons and in some cases assessment of secondary production of molluscan species (Sousa *et al.* 2005, 2007, 2008). The Mini-Missouri Trawl has been used in collecting mussels in the Rio Xingu, Brazil with great success. The efficacy has not been tested and further studies are needed to assess their overall ability to capture and detect a representative sample of those habitats.

Crocodilians are a serious hazard in many tropical rivers throughout Africa, southeast Asia, South America, and northern Australia and entering the water is not always possible. In these circumstances, dredges dragged behind boats or thrown from the shore can provide qualitative samples of the benthos, although these are only effective on sandy or soft mud substrates. Samples from replicate runs or throws should be kept separate so that sampling efficiency can be estimated, and allow statistical comparisons. Trawls should be standardized by trawling set distances or times so that replicate trawls can be compared.

Figure 7
Conspicuous gastropods (Family Viviparidae) living on the underside of a large flat rock in the Wabash River, Illinois, USA.

TABLE 1: Equipment

	Small River		Large River		Lakes	Wetlands	Springs
	Timed	Quant.	Timed	Quant.			
Mesh bags (xx mm mesh)	x	x	x	x	x	x	x
Photo camera	x	x	x	x	x	x	x
GPS	x	x	x	x	x	x	x
Large-mouth double lid plastic Jars (var. sizes, 15-1000 ml)	x	x	x	x	x	x	x
5L buckets with lids	x	x	x	x	x	x	x
Reversing pliers	x	x	x	x	x		
1 mm sieve	x		x		x	x	x
Kitchen strainer	x		x		x	x	x
Waders	x	x			x	x	
Viewing scopes	x	x			x		
Snorkeling equipment	x	x			x		x
Surber benthic sampler	x	x	x	x	x	x	
Quadrats (50 x 50 cm)		x		x	x	x	
Boat			x	x	x		
Dredge			x	x	x		
Scuba or Hookah Diving Gear			x	x	x		

For genetic studies the following supplies are needed:

Ethanol/RNA later
1. 5 ml Micro-centrifuge tubes with O-ring seal screw cap
Dissection kits (including scissors, pincers and scalpels)
Vernier calipers
Paper towels
Lighters or matches
Plastic Disposable Pipettes
Disposable swabs

Figure 8
Fingernail clams (Family Sphaeriidae) living inside a large dead shell of a freshwater mussel (Family Unionidae).

Wetlands, Springs, and Caves

These are specialized habitats and methods for standard sampling in these environments are nonexistent. Protocols 1 & 3 for wadeable streams should be used and modified as needed.

Seasonal and Biogeographical Considerations

Other factors that affect sampling besides stream size are flooding and droughts. We recommend sampling at or near the dry season. High water levels increases turbidity preventing effective visual sampling, and areas that are usually dry may be inundated. High flows also make it difficult to operate dredges or safely employ SCUBA in larger rivers or snorkeling in smaller streams.

Biogeographical or regional considerations to consider include the numbers of species found in temperate as opposed to tropical systems. The temperate systems of North America and tropical Asia are hotspots of freshwater molluscan diversity and the number of person-hours spent searching sites in these regions should be increased to improve sampling adequacy.

Spatial scale of surveys

The geographical scope of the biodiversity survey will be set by the program goals. Biodiversity surveys may be focused on a single reach of a small stream or lake, river basin or the geographic range of a species spanning multiple drainage basins. The spatial design is critical and site selection needs to be spatially distributed, covering all likely habitats and watersheds. Use of Geographic Information Systems (GIS) and advanced eco-informatics models such as Ecological Niche Modelling (ENM), which combines statistical or machine-learning algorithms with spatially geo-referenced environmental data and information contained in historical records, may aid in site selection (Daniel & Brown 2013; Prié *et al*. 2014). A limitation of ENMs is that they identify regions of potential habitat suitability for a species based on its realized ecological niche in relation to environmental (usually climatic) predictors. However, some freshwater mussels and gastropods have restricted geographic ranges that are a consequence of past climatic or geological events (e.g. Ponder 1991, Strong *et al*. 2008) and their distributions may not be accurately modelled by ENMs. In addition, and for unionoids, the life cycle depends on fish hosts. Therefore, ENM models should also include data on host distribution and density. When assessing regional molluscan diversity, specialized habitats characterized by long-term hydrological stability, including the headwaters of streams and spring-fed waterbodies, should be targeted as these are often favored by gastropod groups such as hydrobiids (e.g. Ponder 1991).

Vouchers, Identification, and Data Management

It is extremely important to document species occurrences with vouchers, if at all possible, and to deposit then in an established museum to allow verification of the identification of specimens found in the study. Coordination with the host country museum should be made for depositing vouchers. Data without vouchers have far less value and are more often ignored by researchers than those documented by specimens. At a bare minimum, photographs of all target taxa collected should be taken. Small specimens that cannot be determined in the field should be returned to the lab for identification.

Accurate locality data are essential. A global positioning system (GPS) unit should be employed to record the geospatial coordinates of the samples. Field notes, including detailed ecological observations or demographic data, (sizes, ages, sex ratios, etc.), are also desirable to fully document the survey. Specimens should always be labeled in the field with complete and clear locality data using a pencil or indelible ink and waterproof paper. At a minimum, labels should include the following data: Body of Water (e.g., Stream or Lake); Country; Latitude/Longitude; Date of Collection; and Collector(s). Other data that are helpful include Drainage; State (or Province / Department, etc.), Secondary political divisions; and Common Location (Distance, Direction, and Location – i.e. 5 km SSE Manaus).

The objectives of the study will dictate the number of specimens to be collected, and in some cases a single voucher will suffice. A study on variation in shell shape, size, etc. may require retention of more specimens. If the specimens are to be used for anatomical studies, they should be narcotized and relaxed, if possible, before being placed in fixative. Commonly used relaxing agents include MS-222, chloroform, menthol crystals, and phenobarbital. Placing live molluscs directly into a fixative solution causes the animals to tightly close their shell and prevents the fluid from fully penetrating the tissues. Small wedges or pegs should be inserted between the shells of bivalves or opercula of snails to allow the fixative to enter. With the increased interest in molecular genetics, specimens should be fixed in 95% ethanol in the field, if possible. Distilled spirits may also work if ethanol is unavailable. Denatured ethanol should be avoided. It may be sufficient to voucher shells instead of live animals to document occurrences. Additional references and curatorial methods for bivalves and gastropods can be found in Cummings and Bogan (2006) and Dillon (2006) respectively.

Non-lethal sampling, in lieu of whole animal preservation, for genetic research can be done if large numbers of individuals are required or the target species is rare. This is done by swabbing the mantle cavity of unionoids or taking tissue clips from either the mantle or the foot for genetic analysis. A protocol developed for collecting genetic samples of bivalves and gastropods is given below. The equipment needed is listed in Table 1. The examples shown are for freshwater mussels.

Figure 9
A large shell deposit along the banks of the Illinois River, Illinois, USA.

Protocol for taking Tissue Samples for Genetic Analysis

If the specimen is not vouchered, take a photo of the specimen with a locality label beside it and a reference for size (e.g., a coin or ruler). For whole animals collect 3-6 animals from each population (river or lake) to use as vouchers and place each specimen in a separate vial of an appropriate volume (>5x the total volume of the specimen/clip) with ethanol (>95%). For bivalves and operculate gastropods the following surgical procedures should be applied to allow the ethanol to enter and preserve the tissue. Anesthetize the animals (e.g., in a 2-phenoxyethanol solution 0.4%): for bivalves, while holding the specimen, insert a knife or scalpel between the shell valves and sever the muscles (one on each side), thus opening the mussel. Using the point of the knife, separate the mantle a little on each shell valve and place the shells on a cloth or paper for about 15 minutes to dry them a little. For operculate gastropods, it is necessary to puncture the operculum.

For small tissues take 20-25 clips (one per specimen) per species at each site. Hold the shell in your hand and, using reverse pliers, open the valves wide enough to insert small scissors and pincers (Fig. 14). Cut a small (0.5 cm) piece off of the tip of the foot or remove a small piece of the mantle. Place the tissue clip in a labeled tube filled with ethanol. Replace the ethanol after a couple of days. Return the animals to the exact same places where they were caught. After processing each specimen, clean the scalpel blade with paper, rinse it with ethanol, and carefully cauterize the tips of scissors and pincers with a lighter.

Figure 10
Local men showing large colonies of the cementing bivalve *Etheria elliptica* Lamarck, 1807 (Family Etheriidae) in Zambia, Africa.

Tissue snips and swab tips are then placed in 1.5 ml plastic tubes with RNAlater or high concentration ethanol to be returned to the lab. Population size can be estimated using genetic analysis if a sample of at least 25 individuals is taken.

Conservation Significance of Molluscs

Freshwater molluscs can be used as sentinel species since they are among the most sensitive species in fresh waters, especially the younger life stages such as the larvae and juveniles. Larvae of most species spend a variable amount of time in the water column and the duration of the larval stage is quite sensitive to the chemistry and physical characteristics of the water. For instance, an increase in water temperature is often correlated with a dramatic decrease of the larval lifespan in many species of bivalves (Taeubert *et al*. 2014). Increased levels of pollutants, metals, nutrients, such as ammonia and other nitrogen compounds, may accumulate in the sediments inhabited by benthic molluscs. These are generally deleterious to juveniles and may cause recruitment failure in the population. Consequently, the U.S. Environmental Protection Agency bases the acceptable threshold value of ammonia for good water quality on the tolerance level of freshwater mussels (EPA 2013). In this context, freshwater molluscs are generally threatened by any major change in hydrology and channel geomorphology, water quality or other kind of disturbance. It has been observed that in many streams where habitat is apparently intact, (i.e. there are no obvious impacts to the water body, and the streams continue to support relatively healthy fish, and insect faunas), the mollusc fauna is declining, especially the bivalves (Haag & Williams, 2014).

Figure 11
A diver, using surface supplied air commonly referred to as a "Hookah rig", to sample in large, deep rivers. Mississippi River, Illinois, USA.

Given the high conservation importance of freshwater molluscs and their global decline, it is imperative to implement a standardized sampling protocol. This chapter is an attempt to cover this gap and will permit comparisons at different spatial and temporal scales, providing the basic information needed to assess the conservation status of molluscs.

Acknowledgments

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Figure 12
A cleaning station set up to wash and sort molluscs collected by divers.

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Figure 13
A quadrat frame used to conduct quantitative surveys for freshwater mussels and large gastropods.

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Figure 14
Collection of tissue clips
of freshwater mussels for
genetic analysis in the Oued
Noun, Morocco.