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How to inventory tropical flies (Diptera)—One of the megadiverse orders of insects

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Abstract

A new approach to inventory Diptera species in tropical habitats is described. A 150 x 266 m patch of cloud forest at Zurquí de Moravia, Costa Rica (10.047N, 84.008W) at 1585 meters asl was sampled with two Malaise traps for slightly more than one year (Sept. 12, 2012–Oct. 18, 2013). Further concomitant sampling with a variety of trapping methods for three days every month and collecting during a one-week intensive "Diptera Blitz", with 19 collaborators collecting on-site, provided diverse additional samples used in the inventory. Two other Costa Rican sites at Tapantí National Park (9.720N, 83.774W, 1600 m) and Las Alturas (8.951N, 82.834W, 1540 m), 40 and 180 km southeast from Zurquí de Moravia, respectively, were each sampled with a single Malaise trap to allow for beta-diversity assessments. Tapantí National Park was sampled from Oct. 28, 2012–Oct. 13, 2013 and Las Alturas from Oct. 13, 2012–Oct. 13, 2013. A worldwide group of 54 expert systematists are identifying to species level all 72 dipteran families present in the trap samples. Five local technicians sampled and prepared material to the highest curatorial standards, ensuring that collaborator efforts were focused on species identification. This project, currently in its final, third year of operation (to end Sept. 1, 2015), has already recorded 2,348 species and with many more yet expected. Unlike previous All Taxon Biodiversity Inventories, this project has attainable goals and will provide the first complete estimate of species richness for one of the four megadiverse insect orders in a tropical region.

Considering that this is the first complete survey of one of the largest orders of insects within any tropical region of the planet, there is clearly great need for a consistent and feasible protocol for sampling the smaller but markedly more diverse smaller insects in such ecosystems. By weight of their species diversity and remarkable divergence of habit, the Diptera are an excellent model to gauge microhabitat diversity within such systems. Our model appears to be the first to provide a protocol that can realistically be expected to provide a portrayal of the true species diversity of a megadiverse order of insects in the tropics.

Key words: Biodiversity, diversity, tropical, Costa Rica, ATBI, Diptera

Introduction

One of the great gaps in our understanding of biodiversity is our current inability to accurately estimate the number of species of smaller terrestrial invertebrates, particularly in tropical regions where the full species richness of insects is still largely a matter of conjecture. With continuing and increasing pressures on ecosystems due to habitat destruction and global climate change, the importance of understanding biodiversity has become paramount. As pointed out by Terry Erwin (2004), "Considering potential benefits for humanity, not accomplishing an inventory of life on Earth has been the greatest failing of the human race thus far." And as famously noted by Edward O. Wilson (1992) over 20 years ago, we know how many stars are in the Milky Way and the mass of an electron but don't know within an order of magnitude the number of species on our planet. This level of ignorance continues in large measure today and reflects how little we understand, in particular, about the truly megadiverse orders of insects, the beetles (Coleoptera), the butterflies and moths (Lepidoptera), the ants, bees and wasps (Hymenoptera),

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and the true flies (Diptera). Together, these make up about 40% of the described diversity of planetary eukaryotic life (Costello *et al.* 2013; Grimaldi & Engel 2005).

There have been many previous attempts at estimating the diversity of insects in the tropics using various models. Perhaps best known has been the tally of beetles made by Erwin (1982) who sampled all specimens from 19 individuals of a single tree species in Panama and found 955 species of Coleoptera. By adding assessed but unstudied species of weevils and then multiplying estimates of host specificity and other factors with numbers of tropical tree species, he gauged beetle diversity and extrapolated from that to total arthropod diversity, providing a final figure of 30 million arthropods on Earth. This figure has been challenged by subsequent work and estimates of numbers of extant insect species generally vary from 1.8 (Hodkinson & Casson 1991) to 10 million (Gaston 1991), with various authors falling in between these limits (Basset *et al.* 1996; Groombridge & Jenkins 2002; Hammond 1995; May 1990, 2000; Mora *et al.* 2011; Raven & Yeates 2007; Stork & Gaston 1990). These numbers vary so widely that Caley *et al.* (2014) concluded that estimates of numbers of species via modelling are not converging. They suggest that new analytical approaches are needed. We agree with Erwin (2004) that much of the discussion is model rich but data poor. What is needed are far more field investigations of diversity, especially so in the tropics (e.g. Erwin *et al.* 2005). Without substantial investment in firsthand studies, we will never be able to provide a realistic tally of species richness.

Others have taken more inclusive and specific approaches to appraising species diversity. The acronym ATBI refers to an "All Taxon Biodiversity Inventory", a concept originally proposed by Janzen & Hallwachs (1994) that would see all the species in a broad area cataloged and ultimately interpreted ecologically. The reasons to undertake such complete inventories are manifold and are of benefit to both society and science (Janzen 1997, 2003, Janzen & Hallwachs 1994). By recording the species in a given habitat, they provide the tools not only for understanding those species but for interpreting community structure and a host of other biological patterns. ATBIs provide baseline support for the protection of biodiversity. They are of intrinsic and fundamental value to human health and basic to providing a sustainable future for human beings and other living organisms on our planet.

From Janzen's pioneering proposal to complete an ATBI in northwestern Costa Rica (Janzen 1996) to other ambitious projects like the "Arthropods of La Selva" (ALAS) in northeastern Costa Rica (Longino 1994) or that in Great Smoky Mountains National Park (USA) (Sharkey 2001, Parker & Bernard 2006), ATBIs have attempted to determine all the species living in large areas. Similar projects that we know of elsewhere in the USA (Boston Harbor Islands: Rykken 2011, Rykken and Farrell 2013), Germany (Spreewald, http://www.atbi.eu/spreewald/), Slovakia (Gemer area, http://www.atbi.eu/gemer/), (Parc National du Mercantour), Italy (Parco Naturale Alpi Marittime) (http://inpn.mnhn.fr/accueil/a-propos-inpn) and United Arab Emirates (van Harten 2008, 2014), for example, have also bravely tried to sample sizeable areas. In spite of their incredible value, why have ATBIs not been successfully completed? All remain long-term, unfinished projects or have succumbed under the weight of either too large an area being sampled, lack of sufficient sampling, large numbers of samples, huge numbers of uncurated specimens, poor organisation for sample processing and dissemination, a lack of systematists with the expertise to identify the resultant material, or, most usually, a combination of these factors.

At the present time, a total ATBI of any site on the planet is out of reach (except for sites in extreme habitats and on some islands, e.g. Wolfgang 1998). Aside from other considerations, there simply aren't enough systematists to cover all the groups; nor is there the sort of financial support necessary for such a study (Janzen & Hallwachs (1994) suggested $88 million for an ATBI covering at least 50,000-100,000 hectares). This lack of knowledge seriously undermines efforts to preserve the world's biodiversity in light of tremendous anthropogenic pressures, as documentation of biological diversity is a cornerstone of conservation biology (Primack 2014). Further to this, current decisions made for the preservation of natural areas and other conservation priorities are mostly done without information from the vast majority of groups making up biological diversity (such as megadiverse groups like Diptera).

In this paper, we describe a project directed at estimating the total number of fly species (Diptera) at a tropical location. Diptera represent but one subset of the total fauna but they encompass a high percentage of the total invertebrate diversity. Our study has the capacity of rectifying a huge gap in our knowledge base because it employs a unique approach to discovering how many species of a megadiverse order of insects occur at a limited site. Reviewers of our National Science Foundation grant remarked that this project should become "a benchmark for future studies on Diptera", that it is "a 'how to' inventory megadiverse groups in the tropics", and that it "is unprecedented in scope, yet appears feasible in light of the proponents' expertise and design of a comprehensive,

collaborative and expertly thought out research plan. It constitutes big science done by systematists, with excellent prospects for visibility for taxonomy, conservation science, and the general public." These supportive statements and our subsequent experience have led us to consider that a description of our approach and experience would be helpful to others planning to interpret the species level diversity of a megadiverse taxon, particularly in a tropical locale.

Why study flies?

Diptera are remarkably diverse at the species level, with over 155,000 named species, representing, at present, 12% of all named insects on the planet (Grimaldi & Engel 2005). This extraordinary diversity is clearly only the tip of the proverbial iceberg, with every fly systematist recognizing that a truly huge number of additional species in many of the approximately 160 families recognized worldwide are yet unnamed. For example, there are presently 6,224 species of named biting midges (no-see-ums) of the family Ceratopogonidae worldwide (Borkent 2015), but there are likely more than another 9,000 unnamed (e.g. the Andes are virtually unsampled).

In conjunction with this great diversity, the Diptera display a remarkable range of ecological adaptations and are found in virtually every conceivable terrestrial and aquatic (and some marine) microhabitat. Various taxa are predators, parasites (vertebrate and invertebrate), scavengers (of nearly all forms of organic matter), fungivores, commensals, herbivores, pollinators, or gall makers; they occur from the tropics to the high arctic (to the edge of permanent polar ice) and Antarctica (the only free-living insect to do so), from marine shores (some chironomids are pelagic) to the highest altitudes, from lush rainforest to the driest of deserts (Marshall 2012, Yeates & Wiegmann 2005). In short, Diptera have a huge ecological repertoire that makes them excellent candidates for estimating ecological heterogeneity at a fundamentally more accurate and diversified level than other taxon-specific inventories, which are almost always restricted to the study of large organisms such as vertebrates, Odonata (dragonflies and damselflies) and butterflies. Studying the markedly more diverse Diptera, with their vast array of ecological niches, almost certainly provides greater insight into the overall community structure of terrestrial and aquatic ecosystems. There are three other orders of megadiverse insects which might be candidates for inventories involving many hundreds or thousands of species. However, the Lepidoptera are primarily herbivores and Hymenoptera primarily parasitoids, predators or herbivores, providing a substantially smaller window on a given ecosystem. Only the Coleoptera share a diverse ecological repertoire with the Diptera, although beetles have no vertebrate parasites or haematophagous species. Also, with 12,700 aquatic species (Jäch & Balke 2008), beetles are not as diverse in freshwater habitats as are, at a minimum, the 39,000 aquatic species of Diptera (as calculated from Borkent 2012, Pape *et al.* 2011, Wagner *et al.* 2008).

Our project on fly diversity is built on a collaborative venture in which experts for each family of Diptera in Central America cooperated to produce two large volumes of the *Manual of Central American Diptera* (MCAD-Brown *et al.*, 2009, 2011) (Table 1). Authors described, family by family, the genera present in Central America, producing well-illustrated keys to these genera and discussing the known and projected number of species in each genus. These manuals provide an excellent basis for pursuing the next level of systematic sophistication—that of the species level at a given Central American location.

Finally, and intimately tied to the development and writing of the MCAD, there is a remarkable community of Diptera systematists who are able and willing to examine species of each family of Diptera present at our study site (Table 1). This situation is unique within the entomological community. In spite of the lamentable failure of our society to recruit a sufficient number of systematists to broadly interpret the biodiversity that is rapidly disappearing from our planet, the Diptera community yet has a combination of employed, retired, and independent systematists who are actively enthused about pursuing species-level taxonomy in their respective families.

Development of the project

Our project began in the late 1990s with informal discussions about faunal surveys in general. Inventorying insects, in particular, is challenging because of the huge numbers of undescribed or poorly known tropical species, coupled with the relatively low number of systematists available to accomplish the task of identification. Also, technical

support is a limiting factor, as specimens collected in mass sampling devices need to be sorted, prepared, mounted, labeled, databased, and shipped to the appropriate expert. However, in most instances, material is received (and nearly always so for those numerous species small in size and in diverse families) in vials and/or plastic bags and requires a huge amount of curation before they can be adequately studied. The sad fact is that many insects caught during nearly all scientific inventories are merely sorted to varying taxonomic levels, shipped and never looked at again. Instead, they sit in vials and bags whose alcohol is slowly drying while newer, more pressing, material catches the attention of the researcher who at one time promised to look at them. The problem is compounded by the reality that many systematists are singlehandedly (or nearly so) responsible for groups that include thousands of species, equal to that, for example, of all mammals worldwide. There just aren't enough hours in the day to cover all the requests for identification and especially when these first require careful preparation.

These facts have been discussed informally by researchers for decades. It was discussed by us and colleagues at meetings in Costa Rica when we were planning and implementing the MCAD. We roughly calculated how much it would cost to inventory all of the flies (Diptera) in that small country (51,100 sq. km), and came up with a depressingly large amount of money which was clearly not available at the time. Talk inevitably included reference to the other surveys noted above, none of which had led to anything more than a partial list of a few fly families (as well as other non-Dipteran taxa) at their research sites. We recognized that there were four major problems with previous surveys.

1) Too much collecting, not enough preparing. Every entomologist knows that it is easy to collect thousands of insects, but much more difficult to have them prepared for a systematist to examine. Usually, the burden of curation is left to the specialist, who is expected to prepare (mount, label and often dissect) everything gathered by mass collecting methods like Malaise traps, light traps and pan traps, each of which can sample many thousands of Diptera in a week. For example, the ALAS project (Longino 1994) operated 20 Malaise traps at 5 different altitudes during five years (2001-2005) and 16 Malaise traps at La Selva Biological Station in Costa Rica for nearly three years, and despite much energy put into getting the material examined, large numbers of specimens were not studied and still sit in plastic bags full of alcohol.

Our first thought, therefore, was to strongly limit the sampling protocol. Because Malaise traps have been shown worldwide to be one of the most efficient means of sampling diverse Diptera (they basically collect flies that fly into an upright mesh panel and then upward, funnelling these into a collecting bottle), we determined that the minimum prerequisite for a survey would be a single Malaise trap from a single site for one year. This would entail a drastically reduced amount of sampling compared to other surveys with which we had been associated. For instance, a survey of national parks in Thailand used three Malaise traps in 10 parks (30 Malaise traps in total) each year for three years (http://sharkeylab.org/tiger/static.php?app=tiger&page=index) , thus sampling material for about 90 Malaise trap years (MTY). The ALAS project in Costa Rica resulted in about 48 MTY worth of samples. Our one MTY seemed paltry and almost laughable in comparison, but the catch of Malaise traps is composed largely of Diptera (Brown 2005). In particular, large, poorly known families of small flies, such as Cecidomyiidae, Ceratopogonidae, Chironomidae, Sciaridae, and Phoridae dominate the samples. A one-week sample could easily have hundreds or thousands of specimens from any of these families alone, not to mention the approximately 70 other families we would expect to find in a survey.

To address the problem of "not enough preparation", it was apparent that virtually all of the material sampled should be mounted before being sent to collaborators for identification. The responsibility for the most laborious, time-consuming part of the collaborator experience should be taken by the organizers of the project, not the expert systematists who should be devoting their time to determining the identity of the fauna.

A decision to prepare all morphotypes present in the material within a project cannot be taken lightly and is one of the most serious bottlenecks to obtaining accurate identifications (Fig. 10). Smaller flies, in particular, need special methods to prepare specimens for identification and interpretation by systematists. Many need to be mounted on microscope slides, after first being cleared in potassium hydroxide, dehydrated in a series of alcohols, transferred to clove oil, dissected, and mounted parts placed individually under separate coverslips in Canada Balsam. Even highly talented technicians can each prepare only about 30 slides per day. For specimens not requiring such time-consuming work, there is still the need to dry the specimens, using special chemicals that prevent shrivelling, before they are mounted on pins.

2) Too large an area. Surveys of areas on the scale of countries, national parks, and so on sample an extraordinarily large number of habitats across which faunas change markedly. Particularly in tropical regions, the

astronomical number of species one may encounter in such big areas is another, currently insurmountable, impediment to a full inventory. For an ATBI to succeed, the collecting area must be restricted.

3) ATBIs without the "A". ATBIs, as indicated by their name, propose to sample "All" the multicellular species in a given area. For many groups of organisms there simply isn't the expertise of systematists available. Such orphan taxa have no one to interpret the species present or have only one or a few systematists who are committed to other tasks. A vital aspect of a feasible inventory, therefore, is to have systematists available to interpret those collected specimens.

4) Too many undescribed and poorly described species. One of the challenges faced by many systematists studying tropical Diptera is the presence of large numbers of undescribed species and an old taxonomic literature often littered with named but inadequately described species. This is the reason that modern revisions by systematists, carefully comparing freshly collected material with types housed in museums, are of fundamental importance to making scientific progress. We recognized this as a major limiting factor in undertaking a comprehensive interpretation of species collected at a tropical site. Consequently, as a stop-gap measure, if a given morphotype could not be confidently named, it was merely labeled with a distinctive number, so that the survey would yet recognize all distinct species present. It is paramount, therefore, that all material be subsequently housed in museums where future work will allow for these specimens, each with a unique identifying barcode number, to be scientifically named.

Zurquí all Diptera biodiversity inventory (ZADBI)

These perspectives led us to propose an innovative Diptera survey of a mid-elevation tropical cloud forest to the National Science Foundation (USA) (see http://phorid.net/zadbi/ and YouTube link https://www.youtube.com/watch?v=HkROS6-K02U). We chose a study site at Zurquí de Moravia (hereafter "Zurquí), San José Province, Costa Rica, for a number of reasons. From previous prospecting, this site was known among some entomologists as remarkably rich in insect species (Hanson 2000). The site is also easily accessible, about a 30 minute drive northeast of San José and, although privately owned, we had assurances from the owner, Jorge Arturo Lizano, that it will remain protected. Our study site, at 1585 meters elevation, was limited to a 150 x 266 meter area (Fig. 1) that included a small ridge, a permanent stream, a temporary stream and a variety of vegetation, including some disturbed habitat (pasture) (Figs. 2–3). It is abutting the extensive and virtually pristine Braulio Carillo National Park. Our baseline sampling was a single Malaise trap set on the ridge at the edge of the forest (Fig. 2B), to be employed for one year, the results of which were to be studied by all cooperating systematists (Table 1). Supplementary sampling is discussed below.

TABLE 1. Families present at Zurquí de Moravia, Costa Rica with associated collaborating systematists. Families in partial phyletic sequence.

Family	Collaborator
Tipulidae	Jon K. Gelhaus
Bibionidae	Dalton de Souza Amorim
Mycetophilidae	Peter H. Kerr
Ditomyiidae	Peter H. Kerr
Keroplatidae	Peter H. Kerr
Lygistorrhinidae	Peter H. Kerr
Sciaridae	Heikki Hippa, Pekka Vilkamaa
Cecidomyiidae	Mathias Jaschhof
Chironomidae	John H. Epler
Ceratopogonidae	Art Borkent, Gustavo R. Spinelli, Maria M. Ronderos
Simuliidae	Peter Adler
Dixidae	Art Borkent

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TABLE 1. (Continued)

Family	Collaborator
Corethrellidae	Art Borkent
Culicidae	Thomas J. Zavortink
Scatopsidae	Dalton de Souza Amorim
Psychodidae	Gregory R. Curler, Sergio Ibáñez-Bernal, Gunnar M. Kvifte
Anisopodidae	Dalton de Souza Amorim
Xylomyidae	Norman E. Woodley
Stratiomyidae	Norman E. Woodley
Xylophagidae	Norman E. Woodley
Athericidae	Norman E. Woodley
Rhagionidae	Norman E. Woodley
Tabanidae	John F. Burger
Bombyliidae	Carlos Lamas
Asilidae	Eric M. Fisher
Therevidae	Stephen D. Gaimari
Dolichopodidae	Marc Pollet (coord.), Daniel J. Bickel, Scott E. Brooks, Renato Capellari, Neal L. Evenhuis, Stefan Naglis, Justin Runyon
Empididae	Jeffrey M. Cumming, Bradley J. Sinclair
Phoridae	Brian V. Brown
Syrphidae	Christian Thompson, Manuel Zumbado
Pipunculidae	Jeffrey H. Skevington
Agromyzidae	Stephanie Boucher
Milichiidae	John E. Swann
Chloropidae	Terry A. Wheeler
Sphaeroceridae	Stephen A. Marshall
Drosophilidae	David A. Grimaldi
Tephritidae	Allen L. Norrbom
Micropezidae	Stephen A. Marshall
Neriidae	Alessandre Colavite
Pseudopomyzidae	Stephen A. Marshall
Tanypezidae	Owen Lonsdale
Psilidae	John E. Swann
Conopidae	Jeffrey H. Skevington
Lonchaeidae	Cheslavo A. Korytkowski
Ulidiidae	Valery A. Korneyev
Platystomatidae	Valery A. Korneyev
Pyrgotidae	Valery A. Korneyev
Piophilidae	Sabrina Rochefort
Richardiidae	Valery A. Korneyev
Lauxaniidae	Stephen D. Gaimari
Chamaemyiidae	Stephen D. Gaimari
Sepsidae	Vera C. Silva
Clusiidae	Owen Lonsdale

.....continued on the next page

TABLE 1. (Continued)

Family	Collaborator
Anthomyzidae	Kevin N. Barber
Aulacigastridae	Alessandra Rung
Periscelididae	Alessandra Rung
Heleomyzidae	Norman E. Woodley
Curtonotidae	Stephen A. Marshall
Diastatidae	Wayne N. Mathis
Ephydridae	Wayne N. Mathis
Inbiomyiidae	Brian V. Brown
Muscidae	Jade Savage
Tachinidae	D. Monty Wood, Manuel Zumbado
Hippoboscidae	Carl W. Dick
Streblidae	Carl W. Dick
Scathophagidae	Verner Michelsen
Anthomyiidae	Verner Michelsen
Fanniidae	Jade Savage
Calliphoridae	Terry Whitworth
Sarcophagidae	Thomas Pape
Rhinophoridae	Thomas Pape
Oestridae	Thomas Pape

To ensure that our samples would be fully identified we approached over 50 fellow systematists. Nearly all had been involved in the previously published MCAD (Brown *et al.*, 2009, 2011) and they gave enthusiastic endorsement of this new project when we approached them. As a community, Dipterists are in the enviable position of having experts who are available and willing to take on all of the 72 families discovered (Table 1). When we said we wanted to do a Diptera inventory of the site, we meant a full inventory. In the parlance of the survey community, we wanted our project at Zurquí to be an "All Diptera Biodiversity Inventory", which we named ZADBI for short. We incorporated all Diptera groups including those previously considered to be "impossible": Cecidomyiidae, Ceratopogonidae, Chironomidae, Phoridae, Sciaridae, Tachinidae, Tipulidae, and others.

As expected, many of the discovered species proved to be undescribed and, as such, are for the present given a morphospecies code number, entered into the database, and, if not described soon, will be housed in the established collections at the Instituto Nacional de Biodiversidad (INBio), Costa Rica and Natural History Museum of Los Angeles County, California, USA (LACM).

Our project is fortunate to have the support and collaboration of INBio, our "home base" in Costa Rica. The institution provides logistical support and serves as a center for incorporating results into their database and collection. To assist with sampling and curation of material we hired five technicians, all of whom were trained and had extensive previous field and lab experience at INBio (Figs. 4–5). The individual talents of our team—Carolina Avila, Marco Moraga (who moved away during this study), Annia Picado, Wendy Porras, Elena Ulate, and Elvia Zumbado (more recently hired)—made our project possible. At the start of our project, most could already identify Diptera to at least family level and there was a high level of skill in sorting and preparing both slide-mounted and pinned material. Hiring locals had huge advantages in lower costs, less damage to specimens during transport from the field and also contributed to local structure and economy.

We also hired a project manager at the Natural History Museum of Los Angeles County (Anna Holden for the first portion of our project and Estella Hernandez in the latter part) to oversee the mechanical details of our project including ordering supplies, helping to organize the technicians, assisting with the initial set up of sampling equipment, ensuring that specimens flowed well throughout our curatorial system and loans were arranged correctly. Our project manager also provided educational outreach at the Natural History Museum of Los Angeles County. As a strong educational component of our project, the museum promoted flies in educational

programming, offered resources for teachers, collaborated with the Encyclopedia of Life and encouraged the use of social media.

To ensure the highest levels of curation we approached each of our collaborators to ask for their exact requirements. Because of the nature of their groups and the history of their preparation, many had different curatorial requests. Some systematists, in addition, had their own idiosyncratic method for handling material. The result was the preparation of a 33 page protocol manual, detailing the curatorial needs for each family (summarized in Fig. 10). Collaborators needing slide preparations, for example, differed in how they wanted dissected parts arranged and whether they wanted specimens in Euparal or Canada Balsam as the final mounting medium. Individuals requesting pinned specimens wanted their specimens either on points (white or gray paper), glued directly to the pin, pinned through the thorax or on secondary minuten pins. We needed to teach the technicians these various additional protocols. To ensure highest quality, we sent out an initial small batch of prepared specimens to each collaborator for them to give us their appraisal and further instructions. Our goal was to produce "perfect" specimens as far as possible. Generally, the resulting specimens were in very good to excellent condition and for collaborators, pleasurable to study. Each specimen was fully labeled, including a unique barcode number, and entered into a database, to be housed at LACM and duplicated at INBio (ATTA system). The care given to all specimens produced material allowing our collaborating systematists to immediately begin employing their expertise in distinguishing species.

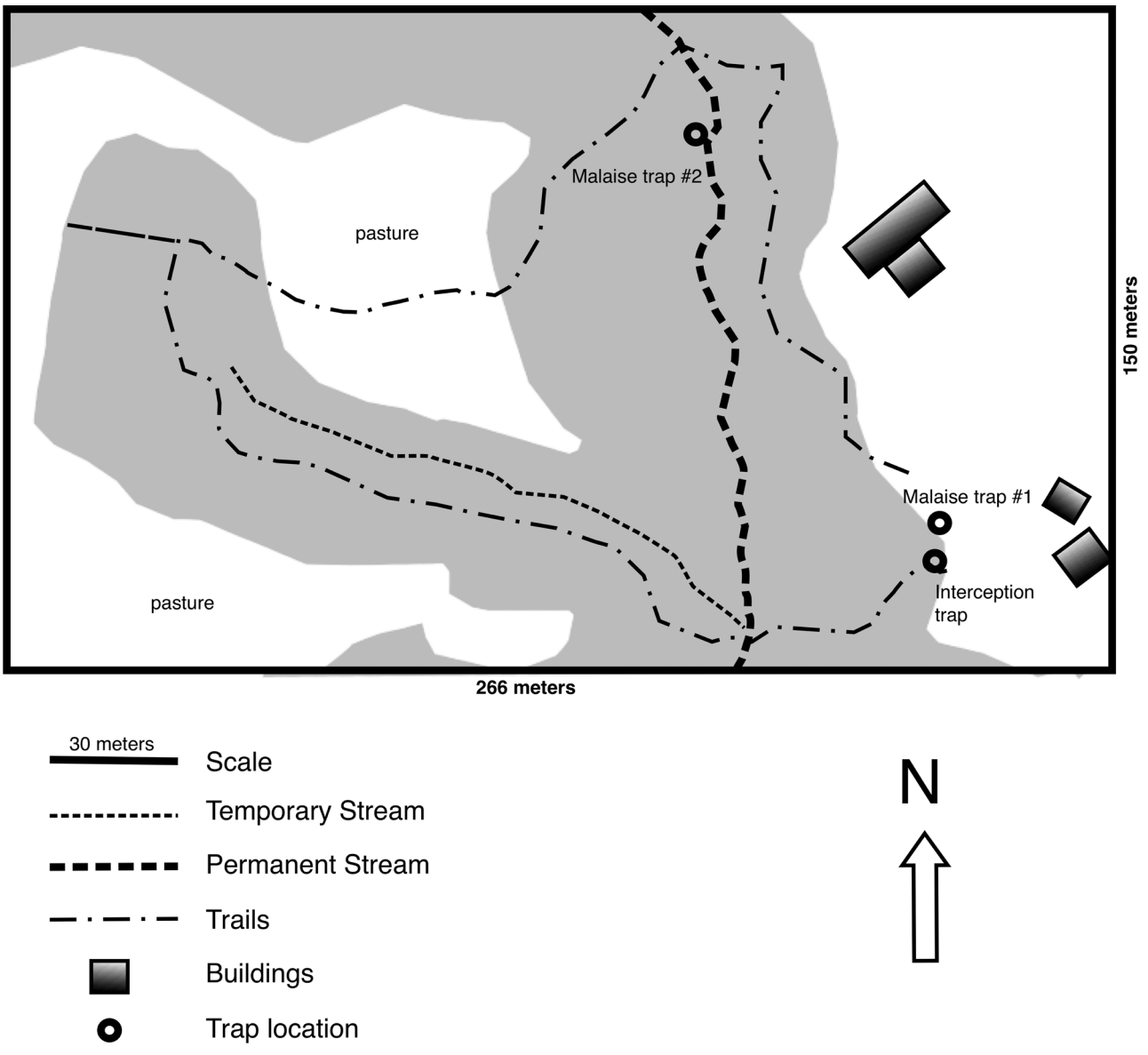

FIGURE 1. Map of study site at Zurquí de Moravia, Costa Rica, with primary features indicated.

FIGURE 2. Details of study site at Zurquí de Moravia, Costa Rica. A. Primary cloud forest with bordering pasture. B. Primary Malaise trap set on ridge indicated with black arrow; red arrow points to temporary black light set over pan with soapy water; large white mass in middle of photo was a piece of plastic garbage.

FIGURE 3. Details of study site at Zurquí de Moravia, Costa Rica. A. Supplementary Malaise trap beside narrow, permanent stream. B. Permanent stream.

FIGURE 4. A. The ZADBI team: Back row, left to right: Manual Zumbado (Coordinator of Biosciences at INBio), Brian Brown (co-PI), Art Borkent (co-PI). Front row, left to right: Elena Ulate (technician), Wendy Porras (technician), Anna Holden (project manager), Carolina Avila (technician), Annia Picado (technician), Marco Moraga (technician). B. Annia Picado (technician) preparing slide mounts.

FIGURE 5. A. Wendy Porras (technician) pinning freshly collected specimens. B. Brian Brown teaching Carolina Avila (technician) how to dry specimens using ethyl acetate.

FIGURE 6. Some supplementary collecting methods utilized at Zurquí de Moravia, Costa Rica. A. Emergence trap over wet vegetation. B. Black light over tray with soapy water.

FIGURE 7. Some supplementary collecting methods utilized at Zurquí de Moravia, Costa Rica. A. bucket light trap (with Wendy Porras and Art Borkent). B. CDC light trap. C. Sweeping at the site (left to right, Marco Moraga, Annia Picado, Art Borkent).

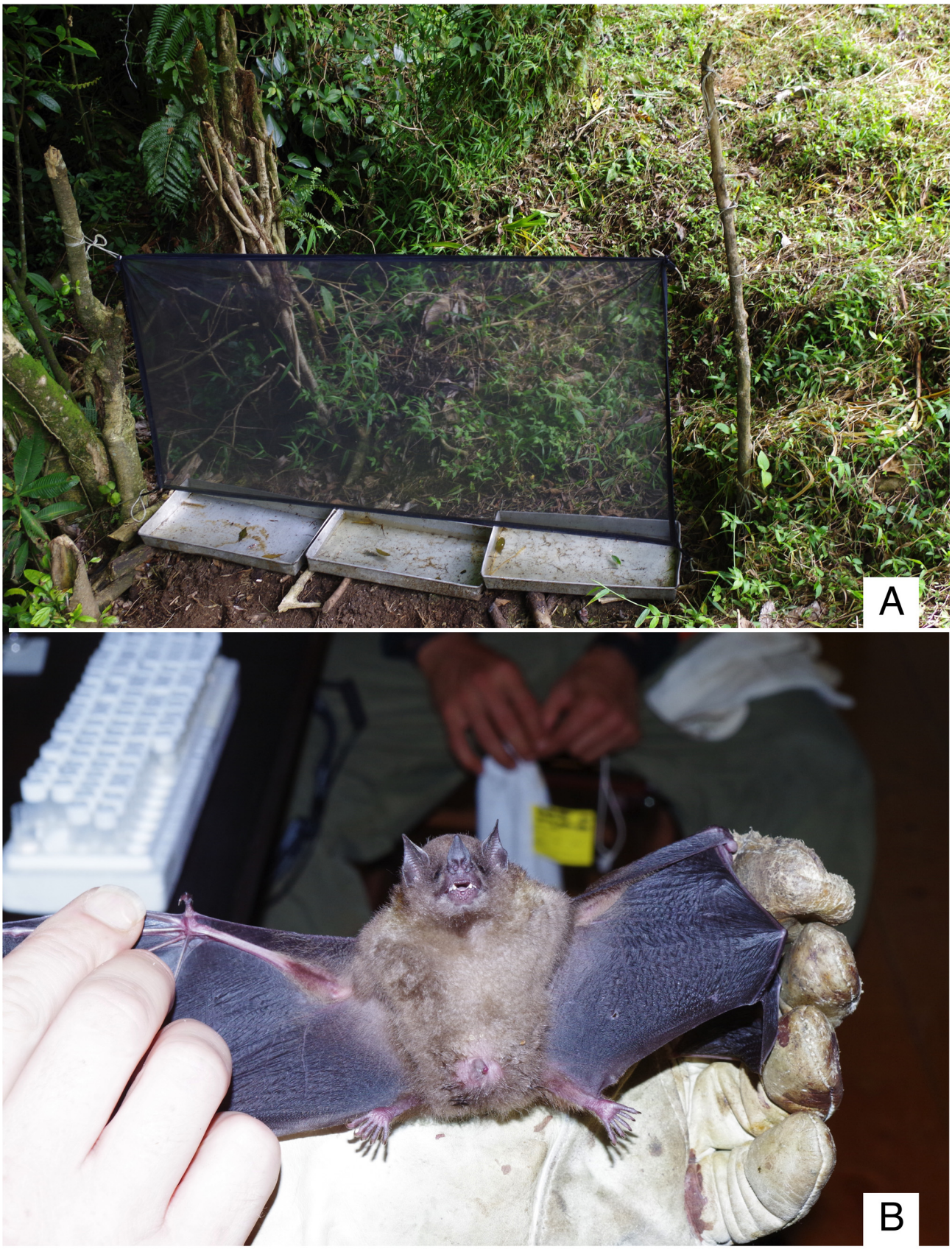

FIGURE 8. Some supplementary collecting methods utilized at Zurquí de Moravia, Costa Rica. A. Flight intercept trap. B. Bat (*Sturnira ludovici* Anthony) trapped with mist net and examined for bat flies (Streblidae) by Carl Dick.

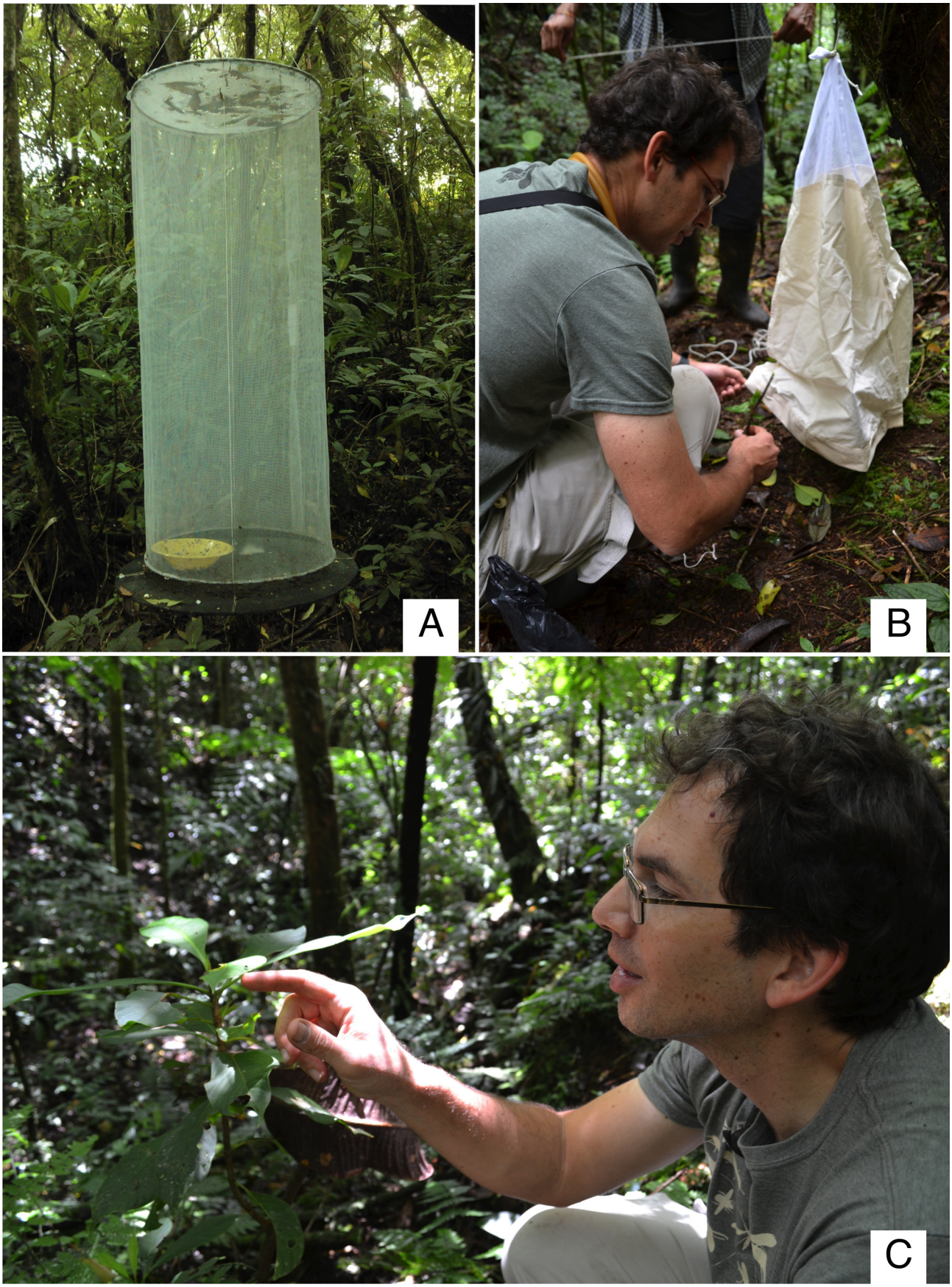

FIGURE 9. Some supplementary collecting methods utilized at Zurquí de Moravia, Costa Rica. A-B. Bait traps. C. Brian Brown observing fly behaviour.

Our sampling with a single Malaise trap at Zurquí (Fig. 2B) was proposed to our collaborators as the minimum requirement for identifying species. We also placed an additional continuous Malaise trap near the permanent stream at the bottom of the ravine, a habitat appearing substantially different to us (Fig. 3A). Although Malaise traps were our primary collecting method, we were keenly aware that some flies would not be collected by this method alone. We therefore had a team of two technicians go to the site once a month for three days (including three nights) to utilize various other methods of collecting (Figs. 6–9). We rented a cabin at the site for accommodation and storage of some traps when these were not in use (e.g. light traps). This effort resulted in numerous additional samples from techniques including hand collecting (Fig. 9C), sweep netting (Fig. 7C), three different types of light trapping (CDC, bucket traps, black light over pans of soapy water) (Figs. 6B, 7A–B), baiting with various attractants (fruit, carrion) (Figs. 9A–B), emergence traps (Fig. 6A), a flight intercept trap (Fig. 8A), yellow pan traps, and a canopy Malaise trap.

To make faunistic comparisons, we ran a Malaise trap at each of two more distant sites in Costa Rica, both at nearly the same elevation, in Tapantí National Park and Las Alturas, 40 and 180 km southeast of Zurquí, respectively, for the same one year period as that at Zurquí. This additional sampling substantially increased the amount of material of most groups. Even so, every collaborator wanted to study all the specimens from the supplementary trapping at Zurquí and nearly all also wanted the additional material from Tapantí National Park and Las Alturas.

Collecting by specialists can often strikingly increase the number of species obtained. A few examples illustrate this point. In the summer of 2013, we held the "Diptera Blitz" at Zurquí (see YouTube link https://www.youtube.com/watch?v=HkROS6-K02U). Nineteen systematists were invited to use their specialized knowledge to obtain species that our trapping program might otherwise miss. Even with the plethora of collecting methods employed, it is often the case that a specialist in a particular group can zero in on those special microhabitats and obtain species not (or rarely) collected by mechanical means. One of these experts, retired Smithsonian Dipterist Wayne Mathis, raised the number of shore flies (family Ephydridae) sampled at the site from three to 26 species. Additionally, he added three entire families to the inventory that our various traps had not collected (Anthomyzidae, Diastatidae, Therevidae). Another obvious specialized approach was by Carl Dick, who along with Kimball Garrett, netted bats and birds during the Diptera Blitz to obtain 10 species of two families of parasitic flies (Streblidae, Hippoboscidae) that lived only on these animals (Fig. 8B). As a final example of very specific collecting, samples of black fly (Simuliidae) larvae were collected from the permanent stream (Fig. 3B) and preserved in Carnoy's solution, allowing their chromosomes to be examined and thereby the species identified by Peter Adler.

The handling of material is a crucial component of a successful inventory (Fig. 10). Samples in the field were nearly entirely preserved in 95% ethanol; only hand-swept specimens of some larger and/or robust specimens were pinned directly after being collected. Alcohol material in variously sized containers was completely topped up with ethanol in the field to ensure that no sloshing occurred during transport to the laboratory. Such groups as Cecidomyiidae, Chironomidae and Tipulidae, amongst others, are often badly damaged in the initial stages of sample collection in other projects, meaning that these taxa are generally in too poor condition to study. Even slight jostling in alcohol can break the legs and antennae of the more sensitive taxa and great care in transport and subsequent handling is critically important. In the laboratory, samples were first databased and separated into fractions: non-Diptera and each of the different families of Diptera (some uncommon families were kept as a group and separated after further curation) (Fig. 10). Material which was to be further prepared as dried, pinned material using either ethyl acetate for larger specimens or HMDS (Heraty & Hawks, 1998) for smaller, more delicate taxa (Fig. 10) included all specimens in the samples. Further specifics of pinning protocol followed our collaborators' wishes, as indicated above. Other taxa, to be dissected and placed on microscope slides, were generally more abundant and were first examined in ethanol and differing morphotypes selected for subsequent mounting in either Canada Balsam or Euparal (Pinder 1989; specific processing varying somewhat by family). For some of these latter families, specimens were selected for slide-mounting by the specialist (all Cecidomyiidae, many Ceratopogonidae and some Psychodidae), in others by the technicians who had been taught to mount every specimen even suspected of being different. Finally, a few researchers wished to receive their specimens in alcohol, ultimately preparing the specimens themselves, or returning these as identified specimens to be pinned or slide mounted at a later date. Each prepared specimen was databased when it was labeled (including a unique barcode number) and subsequently tracked as batches of specimens were sent to our specialists for identification. With each group of fully prepared

specimens sent (and particularly for the first, small batch of specimens) we received feedback on the quality of specimen preparation and in some instances, fine-tuned the subsequent preparation by the technicians.

To track identifications, collaborators were directed to a dedicated website in which collecting data were indicated and species identification and gender could be inserted for each of their specimens. Unnamed morphotypes were indicated with a prefaced number (e.g. "*Forcipomyia* ZADBI-1" for an unidentified species of Ceratopogonidae).

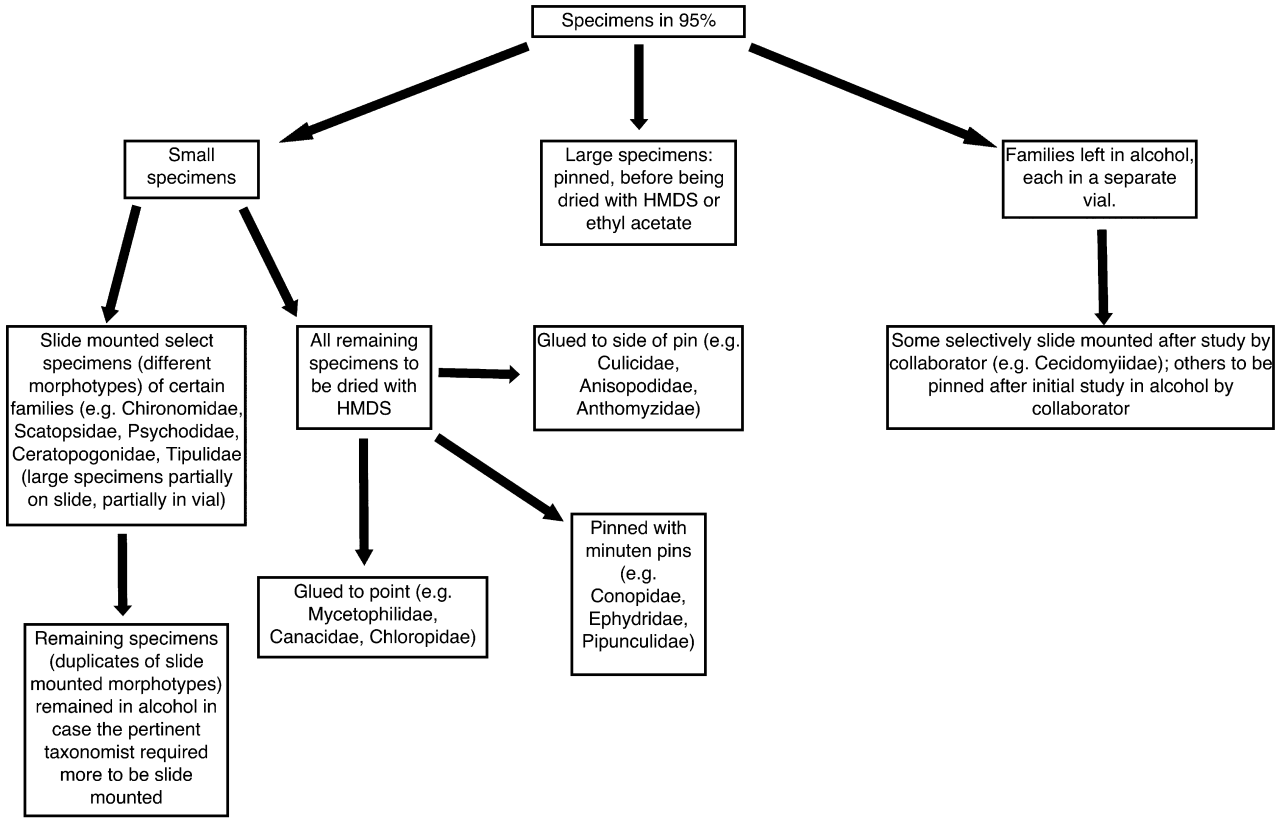

FIGURE 10. Sorting protocol after a sample has been collected and databased (location, date, method of collection). Many families required further specific manipulation or arrangement, depending on the needs of the systematist (e.g. arrangement of parts on a microscope slide; size of pin or specific position on a pin). HMDS (hexamethyldisilazane) is a liquid used to dry insects through immersion and subsequent evaporation (Heraty & Hawks, 1998).

Discussion

At this stage of our project, in our last of three years, initial results have been more than encouraging. Beyond the initial trial set of samples sent shortly after beginning our project, each of our collaborators have now received nearly all curated specimens from Zurquí (only some microscope slides of Chironomidae and Psychodidae are pending). It is still too early to tell how successful our survey has been in providing an estimate of the total diversity of Diptera, although initial results are exciting. Only about one-third (13,381 specimens out of 41,752 curated) in 61 families (out of 72 present) have been identified to date. From these, 2,348 species have already been recognized, equivalent to 33% and 54% of those known for all of Great Britain and Denmark, respectively (Kahanpää 2014), two countries which likely have some of the best known Diptera faunas (no comparable data is available for any tropical region). At this stage, only one of the "big" families from Zurquí has been interpreted in any detail and these initial results are astounding. The Cecidomyiidae (gall midges) expert Mathias Jaschhof and his collaborating wife Catrin Jaschhof have found 812 species and of these, over 750 are undescribed. Jaschhof & Jaschhof (2014) have recently published a description of a new genus, *Zadbimyia* Jaschhof & Jaschhof, including 19 new species from our site at Zurquí. Aside from the impressive numbers, photographic plates of gall midge male genitalic structures show the raw material of evolution: intricate changes in tiny hairs, sclerite sizes, directions of

processes, and thicknesses of membranes that characterize the male and female reproductive structures as shaped by millions of years of change (http://phorid.net/zadbi/research/latest-discoveries/). The Cecidomyiidae are admittedly an unusual example of stupendous diversity because these small, fragile flies have been almost entirely ignored in the tropics and, even within temperate climates, have never been systematically sampled with standard entomological techniques as employed here.

There are several critically important features that have allowed our project to succeed to even this point and which contrast with previous surveys. The limited collecting at a small site, with the minimum contribution by a given collaborator being the identification of specimens from one Malaise trap (although all agreed to examine the material from all traps at Zurquí), guaranteed that the systematists were not overwhelmed by vast numbers of specimens. The full curation of specimens by the project technicians took this time-consuming task out of the hands of our collaborators, removing the common handicap of having to prepare numerous specimens before identification is even possible. Allowing systematists to report some specimen identifications as numbered morphospecies ensured that their skills in species interpretation were fully utilized without them needing to examine the features of previous described type material and interpreting the often antiquated taxonomic literature for a given taxon.

It cannot be stressed enough how crucial systematists are to this kind of project and to science in general. Recently, a technology-based, team approach to solving problems has become fashionable in species determination, phylogenetic and other studies in systematics. In particular, heavy emphasis is placed on training students in genomics to address "larger" issues, such as evolution of higher taxa, and the origin of particular traits or lifestyles. With the large amounts of overhead such expensive projects attract (which brings in more money to support institutions), as well as attention in so-called "high impact" journals, expert systematists, on much smaller budgets, are often perceived as quaint Victorian naturalists, good for providing identifications but not much else. It is clear that genomic studies have contributed in profound and unique ways to our understanding of species and their phylogenetic relationships (although the latter with numerous instances of conflicting results). Barcoding is particularly useful to associate sexes and immatures and has provided insights into both intra- and interspecific variation. Yet, more than ever, there is a vital need to draw students and others towards immersing themselves in the intricacies of their taxa, using whatever appropriate methods are available to learn more about their morphology (including different life stages), life histories, habitats, behaviour, fossils, distributions, and phylogenetic relationships. The richness of this knowledge provides profound insights, with the goal of integrating these various aspects of species and their histories into a unified whole. Investing years of their lives to obtain the deep knowledge necessary to become a broadly based expert, systematists develop an integrated perspective of their groups that cannot be found elsewhere. The firsthand collecting by experts during our Diptera Blitz at our study site provided ample evidence of the value of firsthand knowledge of the taxa being studied. Numbers of additional species were discovered because these experts understood their taxa as whole organisms as they exist in nature and therefore knew where to look for them in the field.

It can be reasonably argued that if our goal was to merely list how many species are present at Zurquí that a DNA barcoding approach might have sufficed and been less costly (but see Yu *et al.* 2012, Cristescu 2014). However, we view our inventory as just the first stage of understanding the diversity of flies as members of complex communities, encompassing a remarkable diversity of form and function and embedded in an historical and phylogenetic context. Our plans are to not only record the number of species of the flies of Zurquí but also their various adaptations and habitats, as understood by the many systematists involved. A further proposal (in prep.) is intended to describe the fauna of Zurquí, at least in part, and place it within a phylogenetic and zoogeographic context.

Even though our study is not yet complete, it is clearly the largest and most comprehensive project ever to attempt to obtain all species of Diptera at a tropical forest site. Of course, no survey ever gets "everything". A casual attempt will collect common species and a selection of rare species, and more intensive studies will collect further rare species, but getting "everything" would require continuous sampling for decades, wading through gallons of samples of common species looking for that elusive novelty that remains to be uncovered. Moreover, extended collecting through time presents difficulties in determining the possibility of species turnover. Realistically, sampling should be done until an asymptote is approached, after which sampling another area becomes more important and informative. One year of sampling is probably not enough time to reach this asymptotic state (see Brown & Feener 1995) and we plan to test this with a variety of the families collected.

Regardless, our sampling program is an important first step towards understanding how diverse the Diptera might be in the tropics and therefore what precious biological wealth is present among the smaller organisms found there. In a time when every major ecosystem on our planet is under duress, understanding such biodiversity can assist in making conscientious choices regarding both our own and the planet's future.

Finally, it is worth pointing out that in large measure, systematists have been hampered for decades by a lack of funding for research describing the biodiversity of life on our planet. Understanding this diversity should (and could) be a cornerstone in our toolbox of methods to gauge the health and future of our planet, to say nothing of the added benefits of describing millions of more species with their presently unknown characteristics and various roles in ecosystem services. This is not due to a lack of money but rather a question of priorities. The recently heralded Rosetta Mission, successfully sending a probe to Comet 67P, was launched on March 2, 2004 at a cost of about €1 billion ($1.25 billion US). We also think the probe is important as a means of exploring our universe but with a comparable amount of funding systematists could describe much of the fauna and flora on our planet and provide powerful tools to assess what is happening to life on earth. It would be money well spent!

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We extend our deepest thanks to all our collaborators (Table 1) who are the backbone of this inventory. Without their expertise, none of this would have been possible. Similarly, our team of technicians at INBio, Carolina Avila, Marco Moraga, Annia Picado, Wendy Porras, Elena Ulate, and Elvia Zumbado sampled, sorted and prepared the many thousands of species that made up our survey with great skill and devotion. Anna Lepiz prepared many, many labels for our specimens and Manuel Zumbado and Carlos Viques, as well as other INBio staff, provided much appreciated logistical support and advice. Muchas gracias a todos! Our program managers Estella Hernandez and Anna Holden helped to keep the flow of our project happening (supplies and specimens) and assisted with numerous other tasks important in a project this size. We extend our deepest thanks to Jorge Arturo Lizano who very kindly allowed us to collect Diptera on his land for a year and provided advice and gracious support whenever we needed it.

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