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TERRESTRIAL LEECHES, iDNA SURVEYS, AND BEYOND

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ABSTRACT

Terrestrial leeches have long been famous for their blood-feeding behavior, feeding on humans who venture into wet forests across much of the Indo-Pacific. More recently these leeches have emerged as a tool for detecting vertebrate diversity in conservation studies. Specifically, scientists use DNA from the bloodmeals found in these leeches' digestive tracts to identify mammals, reptiles, amphibians, and birds they have fed on. Although this so-called invertebrate-derived DNA (iDNA) technique has grown in popularity, few resources exist for researchers to gain familiarity with these methods. Most researchers using these methods have a vertebrate conservation focus and therefore may benefit from learning more about the leeches, which are variable and also potentially in need of conservation. For this review, we focused on providing basic information on iDNA best practices and precautions, and on Haemadipsidae leech background and taxonomy. We hope this information empowers more people who live and study in the Indo-Pacific to work with these blood-feeding worms.

Terrestrial blood-feeding leeches in the family Haemadipsidae have recently been especially useful for surveying elusive vertebrates (Fig. 1) (Weiskopf et al., 2018; Nguyen et al., 2021). The method at a glance is as follows: leeches find us, we sequence DNA from blood in their digestive tracts, and we detect vertebrates from the general area in which we sampled—this research is a form of invertebrate-derived DNA (iDNA) study. Over the last decade, this method has turned the study of haemadipsid leeches from a fringe topic (mostly pest control and systematics) to an important matter for conservation surveys in the Indo-Pacific. This review is a primer on Haemadipsidae leeches to better equip researchers in leech-derived iDNA surveys of vertebrate diversity and to encourage the study of these diverse and often more colorful worms. We hope this information provides the knowledge required for more non-specialists in countries across the Indo-Pacific to perform this type of work.

iDNA

Blood-feeding invertebrates have emerged as attractive options for sampling the DNA of the hosts on which they feed. In addition to leeches, flies, mosquitoes, and ticks have been tested for their ability to retain host DNA and for their utility as tools for conservation (Calvignac-Spencer et al., 2013; Gogarten et al., 2020; Danabalan et al., 2023; Fernandes et al., 2023). Digestive tract contents from any blood-feeding leeches, aquatic or terrestrial, can be used to determine the most recent meal or meals that the leech has consumed (Williams et al., 2020) and probably remain viable for several months (Fogden and Proctor, 1985; Schnell et al., 2012). However, terrestrial leeches possess a number of traits that make them especially effective for iDNA surveys of vertebrates. These leeches can be abundant and easy to sample; they find you as you walk (Fahmy et al., 2019). Because terrestrial leeches move only short distances, a researcher can be fairly confident that the host species was, at some point, nearby (Tessler et al., 2018c). Most haemadipsids seem to be generalists, feeding on tetrapods both large and small (Schnell et al., 2018).

VERTEBRATE SURVEYS AND CONSERVATION

Haemadipsid iDNA is a targeted form of environmental DNA (eDNA), DNA that exists in the environment rather than being extracted from tissue samples (Tessler et al., 2023). Like other conservation-based eDNA methods (Beng and Corlett, 2020), iDNA has its strong points. Leeches actively pursue a broad array of vertebrates to feed on, "collecting" and conveniently "sealing" the vertebrate DNA sample within their digestive tracts (Wilting et al., 2022). Typically the leeches feed on host species that spend time on the ground or in the understory, although at least 1 lemur that was primarily arboreal was also detected by using the iDNA method (Fahmy et al., 2023). Where leeches are abundant, collection is easy and fast. The presence data collected

Figure 1. Photos from Madagascar showing a typical experience in the field with leeches in the family Haemadipsidae. (**A**) *Chtonobdella* leeches in a plastic bag, (**B**) Mai Fahmy walking through typical leech habitat in humid rainforest, (**C**) *Chtonobdella* leech looking for a bloodmeal. Color version available online.

from these leeches are a valuable conservation metric for evaluating protected-area management (Ji et al., 2022).

In a study in Bangladesh, camera traps and leech iDNA were complementary methods for collecting data (Weiskopf et al., 2018). Leech iDNA is especially helpful for getting clear species identifications for cryptic or well-camouflaged species (Schnell et al., 2012) and works equally well for large and small species (Weiskopf et al., 2018). Camera traps often work well for only large or small animals, depending on their setup (Glen et al., 2013). Approximately 200 leeches collected across 4 days in Bangladesh equaled the number collected across 99 nights of camera trapping (Weiskopf et al., 2018). The Bangladesh study used Sanger sequencing, and newer studies using metabarcoding would surely increase the efficiency of bloodmeals detected per leech further.

Of course, there are tradeoffs between camera traps and iDNA. Cameras are often stolen and require at least 2 visits to an area (setup and take down) to get data (Weiskopf et al., 2018; Meek et al., 2019). However, camera traps can be set up anywhere and at any time of year, and data analysis does not require molecular facilities (Sollmann, 2018; Kays et al., 2020). Leeches are easy to collect (limited principally by how far you can walk or drive and how many people are in the field), but data analysis requires laboratories and computational capacity (e.g., GenBank's Basic Local Alignment Search Tool) (Schnell et al., 2018). Although many institutions are capable of sequencing and data processing, less well-funded institutions may not be (Dalal et al., 2023; Auge et al., 2024). Collection and export of leeches often require permits from a given country, which can be challenging (Hamer et al., 2021).

Although most iDNA work has been conducted on new specimens collected for the task, other opportunities for use of iDNA have been identified. Natural history museums contain biodiverse collections ranging back 100 or more years (Krishtalka and Humphrey, 2000; Winker, 2004). Although most specimens in historic collections have been preserved in formaldehyde or formalin, specimens collected in the last 20+ yr are often in ethanol, which preserves DNA. Ethanol-stored specimens can contain viable iDNA (Siddall et al., 2019) that can be used for iDNA studies of organisms from localities where appropriate habitat no longer exists due to expanding development. This approach may be particularly useful for evaluating historic tetrapod losses, although we are unaware of studies focused on this issue.

Haemadipsid bloodmeals may be used to detect more than just vertebrates. For instance, 6 virus families associated with humans were detected from bloodmeal sequence pools from 2 terrestrial leech species, suggesting that analysis of these leeches may be a good way to survey for vertebrate viruses without having to disturb or find the vertebrate hosts (Alfano et al., 2021). Other researchers have also successfully used leech-derived iDNA to track viral infections, including those from African swine fever virus and mammalian viruses (Kampmann et al., 2017; Karalyan et al., 2019).

FIELD AND LAB PROTOCOLS

Collecting specimens

In some places, terrestrial leeches can be abundant to the point of overwhelming, whereas in other places they are hard to find (Fig. 1A, C) (Fahmy et al., 2019). The diversity of leech species may be high or low in either scenario (Nakano, 2017; Tessler et al., 2018c). Season, elevation, forest type, and other biotic and abiotic factors determine the abundance and species richness of these leeches (Drinkwater et al., 2019). In 1 of the few studies on the subject of abundance in *Haemadipsa,* higher humidity, being closer to a river, and being on a trail were associated with a higher number of individuals (Fogden and Proctor, 1985; Jambari et al., 2022). Haemadipsids are found in the rainy season in wet forests; dry forests make poor leech habitat (Fig. 1B) (Kendall, 2012). Like many organisms (Lomolino, 2001), terrestrial leech species often segregate by elevation. In our experience, mid- to high-elevation areas have high leech species richness, but lower

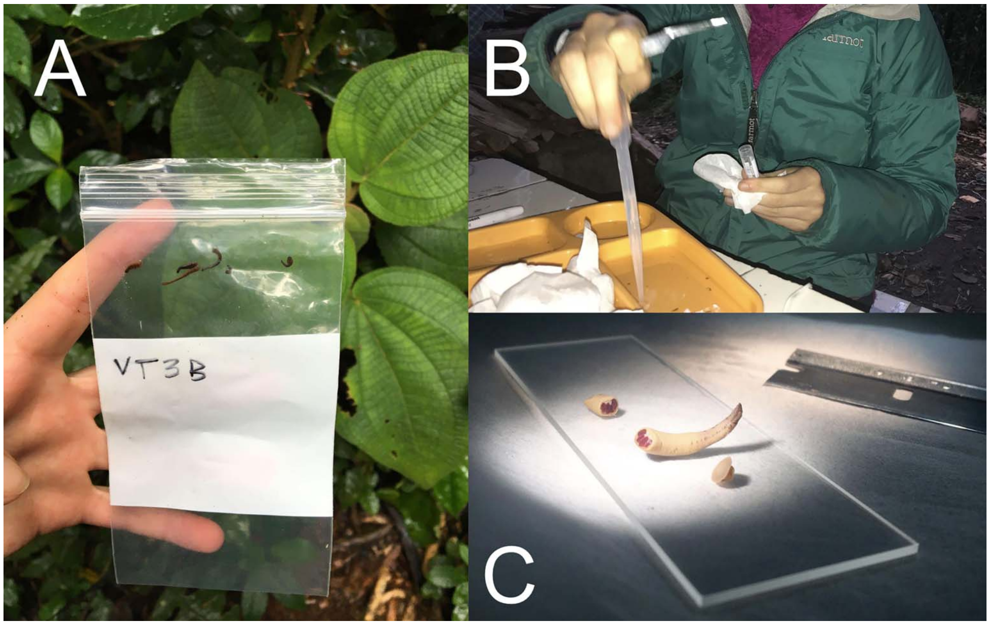

Figure 2. Photos of several key leech processing steps in the field and lab. (**A**) Haemadipsid leeches collected into a small plastic bag, (**B**) leeches being preserved in RNAlater, (**C**) leech digestive tract being dissected out (keeping caudal sucker and upper portion of leech as a voucher) for later DNA extraction. Color version available online.

elevations can have higher abundance at least for certain species (Nelaballi et al., 2022). Although haemadipsids avoid entering water, they often aggregate near smaller streams or water sources that are small enough not to disrupt contiguous canopy cover (Jambari et al., 2022). Still, it is often unclear why they are abundant or sparse.

While walking through the forest, watch the ground and low vegetation for leeches (Drinkwater et al., 2019; Miler et al., 2019; Nelaballi et al., 2022). Based on our experiences, we also suggest the following tips. Boots provide some protection against snake bites (Malhotra et al., 2021). Check your boots and clothing regularly for leeches while walking (Kvist et al., 2014). Walking with multiple people can help, especially when leeches are scarce. Researchers walking at the back of the group will often encounter more leeches, likely because the lead walker alerts dormant leeches and continued movement attracts them. When leeches are abundant, additional hands can help place the leeches that are hard to handle into collection bags. Working with a local team helps avoid undue risks, identify prime field sites, and promote equitable fieldwork (Ramírez-Castañeda et al., 2022).

Leeches can be collected using formal sampling protocols (e.g., transects) or opportunistically (Tessler et al., 2018c; Fahmy et al., 2019), depending on the research goal. Transects work where leeches are abundant and relatively evenly distributed, such as in wet forests with large and intact canopies and moist understories (Drinkwater et al., 2020b). Forests with large populations of domesticated animals similarly may have abundant leeches; however, most sequences will accordingly be of those domesticated animals (Tessler et al., 2018c). Opportunistic sampling may be better for habitats with fewer leeches (Fahmy et al., 2023). Ultimately, the study question will dictate the best approach.

While collecting in the field, leeches should be placed in small plastic bags for storage until they can be preserved in a fixative such as RNAlater or ethanol (Fig. 2A) (Abrams et al., 2019). Leeches are often hard to handle; when you unstick 1 sucker—oral (anterior) or caudal—they may stick the other sucker back on. One trick is to gently roll the leech into a ball between your index finger and thumb and then quickly place it in the bag. Leeches are remarkably adept at escaping bags that are open for short periods, such as when you open a bag to put the next specimen in. Although leeches can be collected in a variety of containers other than bags, we have found that plastic bags work well because they are cheap, make it more difficult for leeches to escape, and take up little space.

Human DNA contamination, such as from handling leeches, can be a major problem when collecting leeches for iDNA studies (Hanya et al., 2019). If bitten, do not use the leech for iDNA. With Sanger sequencing, you will get human sequence only, wasting time, reagents, and samples. With metabarcoding, you may still get host animal sequences, but any human sequences will use up valuable sequencing reads, including redundant sequences that build confidence in your findings (Sims et al., 2014; Gruber, 2015). Human DNA contamination is equally if not more likely to occur in the lab (Weyrich et al., 2019). Wearing nitrile or latex lab gloves helps reduce human DNA exposure (Llamas et al., 2017). Most studies do not report the percentage of sequences from leech iDNA that are actually from humans; most researchers simply remove these data (e.g., Drinkwater et al., 2020a) or use human-blocking probes to avoid human results (Schnell et al., 2015). However, 1 study did include more explicit details of the human sequences; most samples were either sika deer or humans (Hayna et al., 2019). Although these researchers tried to determine whether the human sequences were the result of human bites or contamination, they could not fully disentangle the real results.

When collecting leeches, be prepared for leech bites (Fig. 1C). Shirts and pants should be tucked in to reduce bites (Parson, 1990). Bites are often painless, at worst resembling an itchy pinprick (Eom et al., 2023). After a haemadipsid leech has latched, it will feed for a few to many minutes; it can consume up to 10+ times its weight in blood (Phillips et al., 2020). The incision is small but will bleed disproportionately because of the leech anticoagulants (Iwama et al., 2022). The leech will drop off after feeding, but you can also remove it by sliding your fingernail or credit card under the feeding sucker to release the suction (Joslin et al. 2017). Do not pull the leech aggressively because this can prompt regurgitation into the incision (O'Dempsey, 2012).

Many countries require collection and specimen export permits (Hamer et al., 2021). However, it is often unclear where to find country, province, or state rules on permitting. Laws often do not explicitly cover leeches, making this task more difficult. The best solution is often to contact government officials or local collaborators to ask for help. Permits also take time to be approved, so apply months to 1 year in advance. The Nagoya Protocol is also a valuable resource for those conducting biodiversity research and should be consulted for recommended practices on data sharing and transparency (United Nations, 2010).

Preserving specimens

Leeches can be preserved in a variety of fluids (Fig. 2B). Since DNA sequencing technology became widely available, ethanol has been considered the most flexible storage material (Shokralla et al., 2010). For sequencing leech DNA, $\geq$95% ethanol is ideal (Bely and Weisblat, 2006; Tessler et al., 2016); although suboptimal, drinking alcohol (e.g., 40% alcohol vodka) also works (Pérez-Flores et al., 2016).

For morphological work, leeches require special care. Storage in 70% ethanol provides greater specimen mobility for dissections while preserving DNA and body shape (Marquina et al., 2021). Before this final preservation, leeches should be relaxed by moving them slowly from water with a small amount of alcohol up to a high concentration of alcohol, waiting for the leech to release its muscular tension (Lai et al., 2011). As the leech relaxes, it should be gently stretched out to reach its more normal (i.e., neither contracted nor extended) body length. Ideally, this is done with soft forceps by carefully securing an end of the leech and gently straightening the body.

For dissections or scanning electron microscopy, the leeches are often transferred to 10% buffered ($CaCl_2$) formalin and then ultimately stored in 70% ethanol (Borda, 2006); however, formalin-fixed specimens will not work well for DNA or RNA sequencing.

In iDNA work, alternative preservatives such as RNAlater provide consistently high-quality DNA (and even RNA for transcriptomics) (Macagno et al., 2010; Fahmy et al., 2020). These preservatives are not flammable, making shipping safer. Specimens preserved in ethanol and RNAlater can vary in their viability for iDNA (Tessler et al., 2018c; Fahmy et al., 2019, 2020). Sanger sequencing may be best with ethanol specimens because only the dominant bloodmeal is saved (Fahmy et al., 2019). However, with metabarcoding, specimens preserved in either ethanol or RNAlater can be used, but RNAlater is usually a more attractive option because it better preserves multiple bloodmeals—up to 4 have been found preserved in 1 leech (Fahmy et al., 2020).

Whenever possible, store well-preserved leech voucher specimens at a museum or another collection that is available to researchers (Huber, 1998). Metadata must include locality and date, and information about habitat or even weather can be helpful (Phillips et al., 2019). Lack of voucher specimens or DNA barcodes for leech study systems has been problematic, such as for lab studies on *Helobdella* specimens that turned out to be genetically divergent (Bely and Weisblat, 2006). Researchers should refer to the Nagoya Protocol (United Nations, 2010), which lays a framework for fair and equitable sharing of genetic materials, to guide data-sharing practices. Doing so promotes respect and inclusivity in biodiversity science.

Lab protocols and processing

Some iDNA projects may monitor just mammals, whereas others may survey across vertebrates. These decisions influence lab workflows, including tissue selection, prep, sample pooling, primers, and sequencing methods (Fahmy et al., 2019, 2020; Drinkwater et al., 2020a; Williams et al., 2020). For all protocols, contamination avoidance should be taken seriously (Tessler et al., 2023). DNA contamination can arise from human handling of the leech, so researchers must wear gloves at all times. DNA contamination across leech samples is also a serious issue because it reduces the reliability of the results, as it does for any DNA-based study (Ballenghien et al., 2017). The risk of cross-contamination is particularly high while processing individual leeches for DNA extraction because more handling of leeches, reagents, and tools is required. Researchers must take care before any work to thoroughly clean lab surfaces with DNAaway or bleach, use UV radiation on their tools, and use filter pipette tips (Tessler et al., 2023). Similar caution must be taken between samples, and negative controls should be incorporated to minimize and track contamination issues (Dickie et al., 2018).

Although time-consuming, removal of host DNA from leech tissue improves results and keeps morphologically diagnostic portions of the leech that can be vouchered (Fig. 2C) (Tessler et al., 2018b). To remove host DNA, use a sterile blade, soft forceps, and a dissecting microscope to slice off the posterior sucker. Then, remove the posterior third of the leech anterior to this cut. Bisect this segment, preserving half for future analyses and half for DNA extraction (Fahmy et al., 2019). Re-sterilize surfaces and tools between each leech dissection. When the dissection technique is not used, digesting entire leeches for DNA extraction works, and the DNA can still be used to identify the host and leech (Schnell et al., 2012; Drinkwater et al., 2020a). Researchers typically use Qiagen's DNeasy Blood and Tissue Kit for iDNA digestions before extraction (Williams et al., 2020; Lynggaard et al., 2022; Saranholi et al., 2024), but other options are available (Casquet et al., 2012; Kocher et al., 2017). In the Qiagen kit, for instance, proteinase K is used for digestion.

Dissection and digestion are followed by any type of traditional DNA extraction, such as Qiagen's DNeasy Blood and Tissue Kit or other extraction methods used for eDNA (Cunningham et al., 2024). Extracting bloodmeal DNA from individual leeches separately (Weiskopf et al., 2018; Schnell et al., 2018) or by combining tissues and extracting DNA from pooled samples have both been tested (Drinkwater et al., 2020a; Williams et al., 2020; Fahmy et al., 2023). Both methods work, but for larger studies blending numerous dissected leech tissues (a form of pooling) prior to extraction may be most efficient (Fahmy et al., 2023). Of course, there is a trade-off when it comes to pooling samples. Researchers risk losing the ability to detect DNA found in small quantities at the cost of being able to process a higher quantity of samples. Hanya et al. (2019) discussed this trade-off in the context of iDNA, stating that the ability to track leech bloodmeals from individuals is lost when pooling samples for next-generation sequencing. Sanger sequencing or individual tagging with short nucleotide identifiers may be options depending on the research question (Shokralla et al., 2014).

Once bloodmeal DNA is extracted, various genomic regions are amplified by PCR, purified, and sequenced. In early studies, Sanger-based sequencing was used, which must be done on extractions from single leeches (Schnell et al., 2012; Weiskopf et al., 2018). In more recent studies, next-generation sequencing typically has been used; metabarcoding of pooled samples generates far more data per DNA sequencing run and works better for the typically degraded iDNA (Schnell et al., 2018; Fahmy et al., 2020; Wilting et al., 2022). More data are efficiently generated when using metabarcoding rather than Sanger sequencing. Pooling multiple samples for sequencing in a meaningful way (i.e., with respect to leech species or locality) reduces the need for tracking specimens in a sample (Fahmy et al., 2020).

Any primer used should amplify only a short region of DNA (typically less than 250 base pairs) because (1) bloodmeal data are likely degraded and (2) short fragments work best with Illumina sequencing (Tessler et al., 2023). We are unaware of long-read next-generation sequencing technologies being used for leech iDNA studies. Primer selection is mainly dictated by the study objectives. Some researchers may be primarily interested in surveying mammals, in which case they will use a mammal-specific primer set. Primer sets thus far have often focused on the following loci: 16S for mammals (Tessler et al., 2018c; Weiskopf et al., 2018), 12S for vertebrates (Siddall et al., 2019), cytochrome oxidase I (COI) for reptiles (Nagy et al., 2012), CytB for mammals (Abrams et al., 2019; Axtner et al., 2019), and ND2 for birds (Payne and Sorenson, 2007; Fahmy et al., 2020). With next-generation sequencing, multiple primers can be pooled for sequencing, and the data can be separated bioinformatically (Axtner et al., 2019; Fahmy et al., 2020). Although some researchers have effectively blocked a portion of human DNA contamination with human-blocking probes (Boessenkool et al., 2012; Schnell et al., 2018), this method takes time and may co-block target taxa such as primates (Piñol et al., 2014). Primers for Illumina sequencing have added adapter tags that are needed for sequencing setup. The primers may also have nucleotide barcodes for multiplexing of samples, which allows multiple samples (pooled or unpooled) to be used in a single sequencing run (Drinkwater et al., 2020a).

After sequence data have been generated, basic steps must be followed for data cleaning and assembling contigs. For Sanger sequence data, traditional protocols are well established, and programs such as Geneious make this workflow straightforward (Tessler et al., 2018c). For next-generation sequencing reads and the large quantity of data produced, bioinformatic strategies are needed (Fahmy et al., 2020).

For the type of next-generation sequencing amplicon data typically generated for iDNA, the first step is to remove low-quality sequences and trim off adaptors and low-quality reads at either end of otherwise high-quality sequences (e.g., using Trimmomatic) (Bolger et al., 2014). These data are then merged, assuming paired-end reads were produced, and often clustered by similarity (e.g., using VSEARCH) (Rognes et al., 2016). The processed data are then compared with databases to determine what species the sequences most likely match. Comparisons can range from straightforward queries on GenBank or the International Barcode of Life (Lynggaard et al., 2022) to queries against custom databases using more advanced protocols such as ObiTools (Boyer et al., 2016). Much of this setup has been described in other articles that focus on the metabarcoding of animals or microbes (Axtner et al., 2019; Hakimzadeh et al., 2023; Tessler et al., 2023).

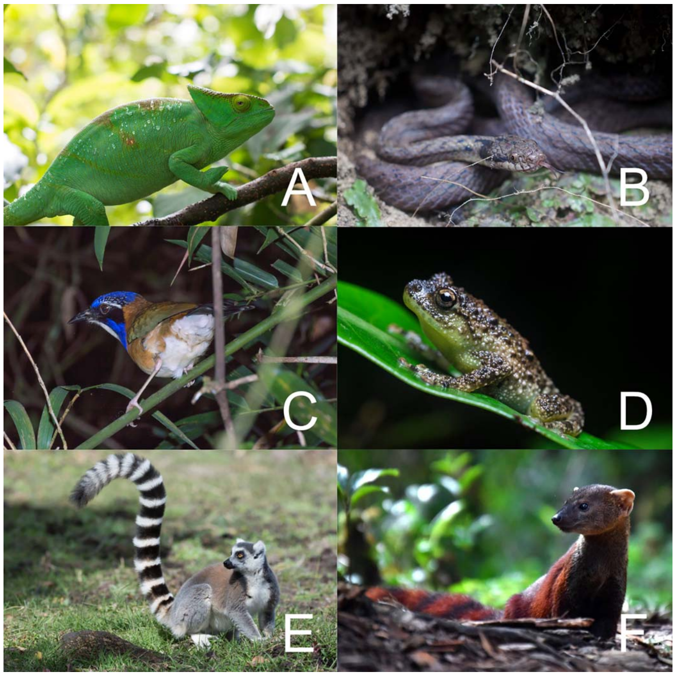

Figure 3. Photos by Franck Rabenahy of some of the vertebrates found in leech iDNA surveys of Madagascar. (**A**) *Calumma oshaugnessyi,* (**B**) *Compsophis infralineatus,* (**C**) *Atelornis pittoides,* (**D**) *Gephyromantis spiniferus,* (**E**) *Lemur catta,* and (**F**) *Galidia elegans*. Color version available online.

ECOLOGY AND BIOLOGY

We know little about the ecology or biology of terrestrial leeches (Drinkwater et al., 2020b; Nelaballi et al., 2022; Fahmy and Tessler, 2024). Questions remain regarding aspects such as life histories, reproduction, intraspecific interactions, dispersal abilities, interactions with other organisms that they do not feed on, and feeding behaviors. With iDNA we are learning more, especially about leech diets (Tessler et al., 2018c). We suspect that iDNA has also raised interest in terrestrial leeches; more recent studies on basic behaviors such as locomotion, respiration, and sensory mechanisms have been published (Phillips et al., 2020; Fahmy and Tessler, 2024).

Diet

Haemadipsids seem to vary somewhat in their host preferences (Fig. 3). For many if not most *Haemadipsa* species, the most frequent meal is mammals (Schnell et al., 2018; Tessler et al., 2018c). Some species occasionally feed on birds, reptiles, and amphibians, but this behavior may be common for *Chtonobdella* species (Rocha et al., 2012; Schnell et al., 2018; Fahmy et al., 2020). Malagasy *Chtonobdella* feed broadly across vertebrate taxonomic groups (Fahmy et al., 2019, 2020). Records exist of *Tritetrabdella* feeding on frogs (Nakano and Sung, 2014) along with terrestrial leeches from the Philippines (Maglangit et al., 2020). Some haemadipsids are specialists, such as *Sinospelaeobdella* species that feed on bats (Yang et al., 2009; Huang et al., 2019) or *Chtonobdella* c.f. *grandidieri* that acts as an endoparasite of frogs in Papua New Guinea (Mann and Tyler, 1963). However, some species that feed on multiple hosts may have strong host preferences. *Haemadipsa japonica* seemingly favors Sika deer and may have spread along with its host (Hanya et al., 2019; Morishima et al., 2020). Two sympatric *Haemadipsa* species from Borneo appear to partition their feeding preferences; *Haemadipsa picta* feeds on a variety of hosts, whereas *Haemadipsa sumatrana* focuses on rodents (Drinkwater et al., 2019).

Behavior

Haemadipsids thrive in moist environments, moving in a looping or inchworm-like motion (Fahmy and Tessler, 2024). They move quickly in this way but still take a long time to traverse the terrain. Although debates continued for over a century as to whether these leeches jump, we captured video showing that at least 1 species, *Chtonobdella fallax,* can jump (Fahmy and Tessler, 2024). Haemadipsids also cannot swim but sink to the bottom and then crawl out of the water (Phillips et al., 2020).

Some work has been done on the feeding behavior of haemadipsids. *Haemadipsa* species gained up to 14 times their body weight in human blood; could be kept in captivity for over 200 days, suggesting that they are not short-lived; and had a dormant period after eating, sometimes laying eggs during this period (Fogden and Proctor, 1985). In 1 study, *H. picta* seemed to wrestle to deal with territorial disputes (Peryga and Miler, 2019). In other studies on *H. picta,* larger individuals climbed higher on vegetation in a quest for prey but were found more consistently on man-made trails than off trails (Gąsiorek and Różycka, 2017; Miler et al., 2019). *Haemadipsa picta* feeding time also appears to be partitioned by size, with larger individuals tending to hunt in the morning and the smaller juveniles hunting consistently throughout the day (Gąsiorek and Różycka, 2017). In an 8 yr study of 2 *Haemadipsa* species, species tended to separate largely based on soil moisture and other moisture-based factors such as rainfall history from lowland to upland forests (Nelaballi et al., 2022). Soil moisture is linked to canopy cover, and regardless of season detection probability for *Haemadipsa* species is positively correlated with canopy height (Drinkwater et al., 2020b).

Disease and endosymbionts

Leeches have close relationships with many organisms. Like other blood-feeding parasites, leeches are known to be a vector for other parasites (Siddall and Desser, 1992, 2001; Karlsbakk, 2004). Leeches also harbor specialized bacteria that digest bloodmeals (Neupane et al., 2019). Parasites of terrestrial leeches are generally less well studied than those of aquatic leeches, but terrestrial leeches certainly do have these symbioses.

Haemadipsids are associated with a unique lineage of *Trypanosoma* parasites (Hamilton and Stevens, 2011; Siddall et al., 2019). Leeches appear to be the only known organisms to house certain mammal trypanosomes and thus are the most probable vector (Hamilton and Stevens, 2011). These trypanosomes are not closely related to species vectored by aquatic leeches (Hamilton et al., 2007).

Haemadipsids are also linked to a variety of bacteria, such as the gut-associated Rikenellaceae, alpha- and beta-proteobacteria, Corynebacteriales, Firmicutes, and Prevotellaceae (Siddall et al., 2019). Terrestrial leeches are linked to at least 1 bacterial genus known to cause human illness. *Bartonella* was found in several surveyed *Haemadipsa rjukjuana* specimens (Kang et al., 2016). However, it is unclear whether these leeches can vector these bacteria. One bacterial infection caused by *Rickettsia japonica* seems to have been acquired from a terrestrial leech (Sando et al., 2019).

Haemadipsid leeches also can attach in unpleasant places. Ocular attachments have been reported for haemadipsids and other leeches, albeit infrequently (Lewis and Coombes, 2006; Phillips et al., 2020). However, we have heard of other ocular attachments by terrestrial leeches, suggesting this phenomenon is more common than reported. There is a published case of a haemadipsid attaching to both the eye and the rectum of a patient in China (Xu, 1995). *Chtonobdella palmyrae* has been found in seabird eyes, suggesting a mechanism for the broad distribution of *Chtonobdella* (Nakano et al., 2020).

Anatomy

Studies of terrestrial leech anatomy have focused on identifying species and clades (Borda et al., 2008; Tessler et al., 2018b). However, some work has been done on aspects of the reproductive anatomy and blood of haemadipsids. One study covered the hermaphroditic reproductive anatomy of terrestrial leeches in the families Haemadipsidae and Xerobdellidae (Borda et al., 2008). In another study, the female anatomy of *H. japonica* appeared to generally be consistent with that of other haemadipsids and even members of the Clitellata generally (Urbisz et al., 2020). A study on leech spermatozoa revealed similarities but also notable differences in structure between *Haemadipsa zeylanica* and 2 distantly related glossiphoniiform leeches (Ahmed et al., 2015). As for leech blood, efforts have focused on the primary structure of leech hemoglobins (Shishikura, 2004).

Salivary compounds

Leech salivary compounds have also been studied, largely because leech saliva contains bioactive compounds such as anticoagulants that make leech bites bleed for long periods of time (Iwama et al., 2021, 2022). Leech anticoagulants furthermore are rather diverse and appear to be ancestral (Iwama et al., 2021, 2022). In an early study of anticoagulant peptides in leeches, haemadin, which inhibits thrombin, was isolated from *Haemadipsa sylvestris,* and antithrombin research with this species continues (Strube et al., 1993; Lai et al., 2019). Another molecule isolated from *H. sylvestris* inhibits inflammation in mice by reducing cytokine production (Liu et al., 2016). A transcriptomic study of salivary compounds from *Haemadipsa interrupta* revealed 20 peptides of interest, some of which may block coagulation along several pathways (Kvist et al., 2014). In another transcriptomic study, differential regulation of genes compared with 2 other leeches was found (Liu et al., 2018). A study of *H. sylvestris* saliva revealed sodium channel inhibitors that may act as an analgesic, preventing both host pain and host detection of the leech (Wang et al., 2018), a property that is often considered but has been difficult to prove for leeches generally.

SYSTEMATICS

Phylogenetics overview

The broad relationships within terrestrial leeches appear to be reasonably well established, although much work remains in understanding the relationships across the Haemadipsidae (Borda et al., 2008; Borda and Siddall, 2010; Tessler et al., 2016, 2018a). Molecular phylogenetics has helped to clear up some of the confusion that had existed for the classification of these leeches. For instance, the first major study revealed that several genera had incorrectly been considered haemadipsids, and these genera were moved to the family Xerobdellidae (Borda et al., 2008). The next large effort at molecular phylogenetics helped establish that 2-jawed (duognathous) leeches form a clade that evolved from 3-jawed (trignathous) leeches (Borda and Siddall, 2010). Those study results further clarified that there are 2 (3-jawed) haemadipsid clades: 1 for *Haemadipsa* and 1 for *Tritetrabdella* plus "*Haemadipsa*" *cavatuses* (Borda and Siddall, 2010). Given that this phylogeny made *Haemadipsa* non-monophyletic, *H. cavatuses* along with another new species were put into the genus *Sinospelaeobdella* (Huang et al., 2019).

A subsequent phylogenetic effort focused on the 2-jawed leeches and established a new taxonomy: a single genus—*Chtonobdella*—is now used instead of the prior 31 genera that were either monotypic or non-monophyletic (Tessler et al., 2016). The next substantial study focused on the genus *Haemadipsa* but produced more questions than answers, indicating a large amount of undescribed diversity and the importance of revisionary work being done at the species level (Tessler et al., 2018c). Those results also suggested that at least for the most well-sampled species (*Haemadipsa trimaculosa*), geographically separated populations are genetically distinct (Tessler et al., 2018c). Other researchers have also found population-level distinctions among *H. japonica* (Morishima and Aizawa, 2019; Sato et al., 2019). Several primer sets have been used for phylogenetic studies on leeches or for barcoding leeches for identification, although COI is often the most useful when focusing on only 1 locus. A longer primer set was used to amplify longer COI fragment in leeches (Tessler et al., 2018a, 2018c).

Taxonomy

The taxonomic ranks of class through order for haemadipsid leeches are based on the taxonomy outlined by Tessler et al. (2018a). This classification does not include Rhynchobdellida and Arhynchobdellida because Rhynchobdellida is likely paraphyletic (Tessler et al., 2018a).

Class: Clitellata Michaelsen, 1919.

Subclass: Hirudinea Lamarck, 1818.

Order: Hirudinida Siddall et al., 2001.

Suborder: Hirudiniformes Caballero, 1952.

Family: Haemadipsidae Blanchard, 1893. The family comprises 4 accepted genera: *Chtonobdella* Grube, 1866; *Haemadipsa* Tennent, 1859; *Sinospelaeobdella* Liu, Huang & Liu, 2019; and *Tritetrabdella* Moore, 1938.

Chtonobdella: All 40+ described 2-jawed leeches were recently placed into the genus *Chtonobdella* (Tessler et al., 2016). The members of the genus are morphologically variable and geographically dispersed, which is likely why members of this now single genus used to be in 31 mostly monotypic genera (sometimes further divided into multiple families, up to 3, and subfamilies, up to 4). Although other genera in the Haemadipsidae have a fixed number of annuli per mid-body somite, *Chtonobdella* species vary significantly (4–7) in annuli per mid-body somite (Borda and Siddall, 2010). The molecular data suggest that these morphological characters in this genus are simply more variable than they are for other genera (Tessler et al., 2016). Having 1 genus for 2-jawed leeches made this genus more comparable to *Haemadipsa,* the other species-rich genus in the Haemadipsidae (Tessler et al., 2016).

Haemadipsa: This genus is the largest group of 3-jawed leeches, all of which have 5 annuli per mid-body somite. Over 25 species have been described, which is an underestimate of the true diversity (Borda and Siddall, 2010; Lai et al., 2011; Tessler et al., 2018c). A number of species appear to be difficult to identify, having similar external appearances despite being genetically distant (Tessler et al., 2018b). Gonopores are separated by 5 annuli (Borda and Siddall, 2010).

Sinospelaeobdella: This 3-jawed genus includes 2 species, *Sinospelaeobdella cavatuses* and *Sinospelaeobdella wulingensis*. Both species live in caves in China (and likely elsewhere, such as Laos) and feed on bats (Borda and Siddall, 2010; Huang et al., 2019). Gonopores are separated by 2 annuli, but as in *Haemadipsa* these species have 5 annuli per mid-body somite (Huang et al., 2019).

Tritetrabdella: This 3-jawed genus consists of 4 species: *Tritetrabdella kinabaluensis* found in Borneo; *Tritetrabdella longiducta* found in Thailand and Vietnam; *Tritetrabdella taiwana* found in China, Taiwan, and Hong Kong; and *Tritetrabdella scandens* found in Malaysia and Thailand (Kappes, 2013; Nakano et al., 2016). These leeches are generally known to feed on amphibians and mammals (Lai et al., 2011). Species in this genus are distinct from other 3-jawed haemadipsids in having 4 annuli per mid-body somite. The location of gonopores and number of annuli separating gonopores ranges from 3.5 to 4 (Nakano et al., 2016; Huang et al., 2019).

FUTURE DIRECTIONS

iDNA

Many creative studies have been done and continue to be done relating to leech iDNA. At this point we think much of the work is simply applying what is known to a broader swath of habitats and questions, essentially doing the conservation work with iDNA rather than figuring out how iDNA works as a tool. Still, use of iDNA presents an opportunity to obtain more locality data for difficult-to-study or even common taxa (Morishima et al., 2020), which can then be used to better estimate distributions of these vertebrate species through methods such as species distribution modeling (Tilker et al., 2020). These data also can be leveraged in other creative conservation pursuits, such as determining the effectiveness of given preserves (Ji et al., 2022). Although more complex, shotgun metagenomics could be used in creative ways to further explore the leech, its symbionts, and its bloodmeals in unison (Siddall et al., 2019).

Revision of *Haemadipsa*

There are many possible future directions for studies of terrestrial leeches. We believe the most critical need is work on the species-level taxonomy within the genus *Haemadipsa*. *Haemadipsa* is

1 of the 2 most species-rich genera in the family Haemadipsidae, is especially widespread, and is the focus of most iDNA work, and yet many of the morphological descriptions are problematic for *Haemadipsa* leeches (Tessler et al., 2018c). In addition to traditional dissections, CT and histology can be used to help discern morphology (Tessler et al., 2016). Even basic external morphology that is consistently documented will help, especially when coupled with DNA sequences (e.g., COI barcodes). When these morphological efforts are insufficient to discern species or clades, then molecular morphology may be employed (Tessler et al., 2022).

Genomes

Two transcriptomes now exist (Kvist et al., 2014; Liu et al., 2018) for haemadipsid species. These transcriptomes coupled with the recently sequenced draft genomes of *Hirudo medicinalis* (Babenko et al., 2020; Kvist et al., 2020), *Hirudo verbana* (Paulsen et al., 2020), and *Hirudinaria manillensis* (Guan et al., 2020) put researchers in a good position to start sequencing and annotating more leech genomes. Haemadipsid species are good candidates, given their utility for conservation and unique attributes (e.g., often mammal-focused diets and terrestrial lifestyles). Sequencing of genomes and transcriptomes facilitates the study of leech adaptations and responses to climate change, helps disentangle their evolutionary relationships, and improves our understanding of leech biology overall (Dunn and Ryan, 2015). These data are also critical for making informed conservation decisions (McMahon et al., 2014).

Community science

A great deal of background information can be obtained on the distribution and morphology of haemadipsids using records collected by non-academics. People who encounter a leech during fieldwork, vacation, or local outings can take pictures with GPS coordinates and upload the data to websites such as iNaturalist (inaturalist.org) (Unger et al., 2020). This type of data will surely better our understanding of these leeches, because their distributions have been studied by only a handful of specialists doing patchwork surveys. Currently, over 3,500 observations of Haemadipsidae leeches have been added to iNaturalist, covering a portion of the species and distributions. People living near forests in the range of these leeches spend a significant amount of time interacting with these blood feeders, and we expect that the increasing numbers of leech observations obtained through community science will be important for this understudied group (Fahmy and Tessler, 2024).

Conservation

Although terrestrial leeches are often reviled and several studies have focused on how to exterminate them (Sasaki and Tani, 1997; Kirton, 2005; Vongsombath et al., 2011; Watanabe, 2018, 2019), they are a common component of biodiversity in many regions of the world, and it is wise to conserve them much as we conserve other organisms (Carlson et al., 2017, 2020). This conservation is important given that these animals are increasingly collected for the iDNA surveys described above, which after sustained collection could impact leech numbers (Drinkwater et al., 2019). Although many haemadipsid species are widespread, most are poorly studied and many are uncommon and restricted geographically (Borda and Siddall, 2010; Tessler et al., 2016, 2018c). There appear to be many species and many distinct genetic lineages that we have yet to discover (Eom et al., 2023).

Few of the rarer leech species are well documented enough to have a sense of their population stability. Some leeches may have gone extinct since their description; the increased numbers of extinctions in the Anthropocene are an important topic of study and concern (Turvey and Crees, 2019). We are aware of only 1 study in which a quantitative attempt was made to assess whether a leech was extinct, along with the implications for conservation (Carlson and Phillips, 2020). The species studied (*Macrobdella sestertia*), if extant, has been given protection status in some US states. In most other studies, examinations of leech rarity have focused on the so-called medicinal leeches in the genus *Hirudo,* which were extensively harvested for medical practices (Trontelj and Utevsky, 2005; Utevsky et al., 2010). Both *H. verbena* and *H. medicinalis* are indeed under protection by CITES and some European countries (Carlson et al., 2020). *Xerobdella lecomtei,* a leech in another family of terrestrial leeches that lives in Europe, appears to be exceptionally rare and may be endangered (Kutschera et al., 2007).

Parasite conservation has begun shifting from a joke to a serious matter. Articles now highlight the need for the conservation of parasites, how climate change is adversely impacting these species, and how to determine best conservation practices (Carlson et al., 2017, 2020; Eom et al., 2023).

Preliminary work indicates that terrestrial leeches differ based on human-modified habitat (Kendall, 2012). However, without a variety of studies across both common and rare species of terrestrial leeches, we are largely unaware of their standing. It is most likely that leeches reliant on pristine habitats are experiencing population declines or worse. We strongly hope that further research is conducted on this topic.

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