

Chapter 1

COLLECTING ARTHROPOD AND AMPHIBIAN SECRETIONS FOR CHEMICAL ANALYSES

*Valerie C. Clark**

Queens University Belfast, Belfast, Northern Ireland, UK

ABSTRACT

A variety of chemical compounds secreted by amphibians and arthropods are used in their defense and communication. These semiochemicals include proteins and peptides, as well as small molecules such as steroids, biogenic amines, alkaloids, quinones, formic acid, etc. Methods are outlined for collecting secretions from various amphibians and arthropods to yield samples that are clean/uncontaminated, concentrated, resistant to degradation under field conditions, and permit easy transportation from remote field sites to the laboratory. The types and quantities of chemicals anticipated in a sample dictate what analytical techniques should be used, and thus how the samples should be prepared when collecting. Select sample collection methods from the literature are reviewed along with additional suggestions to serve as an overall guide for making quality secretion collections for chemical analyses.

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* E-mail: frogchemistry@gmail.com, <http://www.frogchemistry.com/>

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1. INTRODUCTION

Amphibians and arthropods have evolved a great diversity of chemical compounds to attract mates and ward off predators both large and small, along with many examples of these animals that acquire chemical defenses from other organisms. Natural products chemists generally focus on the biosynthesis and pharmacology of such secondary metabolites [Clardy & Walsh 2004, Cragg *et al.* 2009], whereas chemical ecologists focus on how these compounds function in an organism's communication and defense [Eisner & Meinwald 1995, Meinwald & Eisner 2008]. The critical first step for any study of naturally occurring chemical compounds involves securing uncontaminated samples in a stable condition for chemical analysis. Thus, this chapter focuses on specific methods for collecting stable, uncontaminated samples of secretions from amphibians. Techniques and chemical principles are similar for arthropod secretion and venom collection and are thus only briefly outlined.

1.1. Arthropod Secretions and Venoms

Arthropods evolved from aquatic ancestors to colonize terrestrial habitats over 400 million years ago [Pisani *et al.* 2004], and ever since have been further diversifying in form, niches, and chemistry in an evolutionary arms race with their competitors, predators, and prey. Armed with specialized glands, certain beetles can shoot out a super-heated spray of quinones [Aneshansley *et al.* 1969], whereas most arthropods have needle-like spines, hairs, and/or fangs to deliver venoms and poisons [Eisner 2003, Eisner *et al.* 2007]. The remarkable diversity of small molecules and specialized glands found in arthropods has been documented in a number of reviews that focus on particular taxa including: millipedes [Eisner *et al.* 1978], ants [Leclercq *et al.* 2000], beetles [Dettner 1987, King & Meinwald 1996], walking sticks [Dossey *et al.* 2006, 2007, 2008], aphids [Birkett & Pickett 2003], and insects in general [Eisner 2003, Laurent *et al.* 2005, Eisner *et al.* 2007].

A variety of peptides has also been discovered in arthropods [Pimenta & Lima 2005]. The majority of this venom research has focused on scorpions [du Plessis *et al.* 2008], spiders [De Lima *et al.* 2007], and hymenopterans [Piek 1990, Palma 2006] including bees, wasps, and ants. Several arthropod peptides also occur in amphibian skin secretions; for example, kinins from wasps (e.g., *Vespa* and *Polistes* spp.) [Toki *et al.* 1988, Cerovsky *et al.* 2007] exhibit almost complete amino acid sequence identity with bradykinins from frogs' skin secretions (e.g., *Bombina* and *Rana* spp.) [Chen *et al.* 2002, Sin *et al.* 2008].

1.2. Amphibian Skin Secretions

Over 5,600 species of frogs have been described [Frost 2009], and all of these anuran species have permeable skin with cutaneous (slime) and glandular (poison) glands [Neuwirth *et al.* 1979]. Their skin secretions function in defense against micro- and macro-predators [Erspamer 1994, Daly *et al.* 1999, Zasloff 2002, Saporito *et al.* 2007], in communication among conspecifics [Wabnitz *et al.* 1999], and even as glues [Beneski 1989, Graham *et al.* 2005, Means *et al.* 2008]. In addition to some 800 alkaloids reported to occur in 'tropical poison frogs' [Myers & Daly 1993, Daly *et al.* 2005] other classes of bioactive skin compounds serve important roles in the chemical ecology of many other frogs. These include the neutral compound hydroquinone [Mebs *et al.* 2005], biogenic amines [Roseghini *et al.* 1988], steroids such as bufadienolides [Krenn & Kopp 1998, Hutchinson *et al.* 2007], and hundreds of peptides and proteins [Pukala *et al.* 2006] (Fig. 1 & Table 1). Alkaloids and hydroquinones are generally sequestered unchanged from dietary sources [Clark *et al.* 2005, Daly *et al.* 2005, Mebs *et al.* 2005] but in some cases do undergo metabolism prior to glandular storage [Smith *et al.* 2002, Daly *et al.* 2003]. Conversely, bufadienolides, biogenic amines, and peptides appear to be produced by the amphibians themselves.

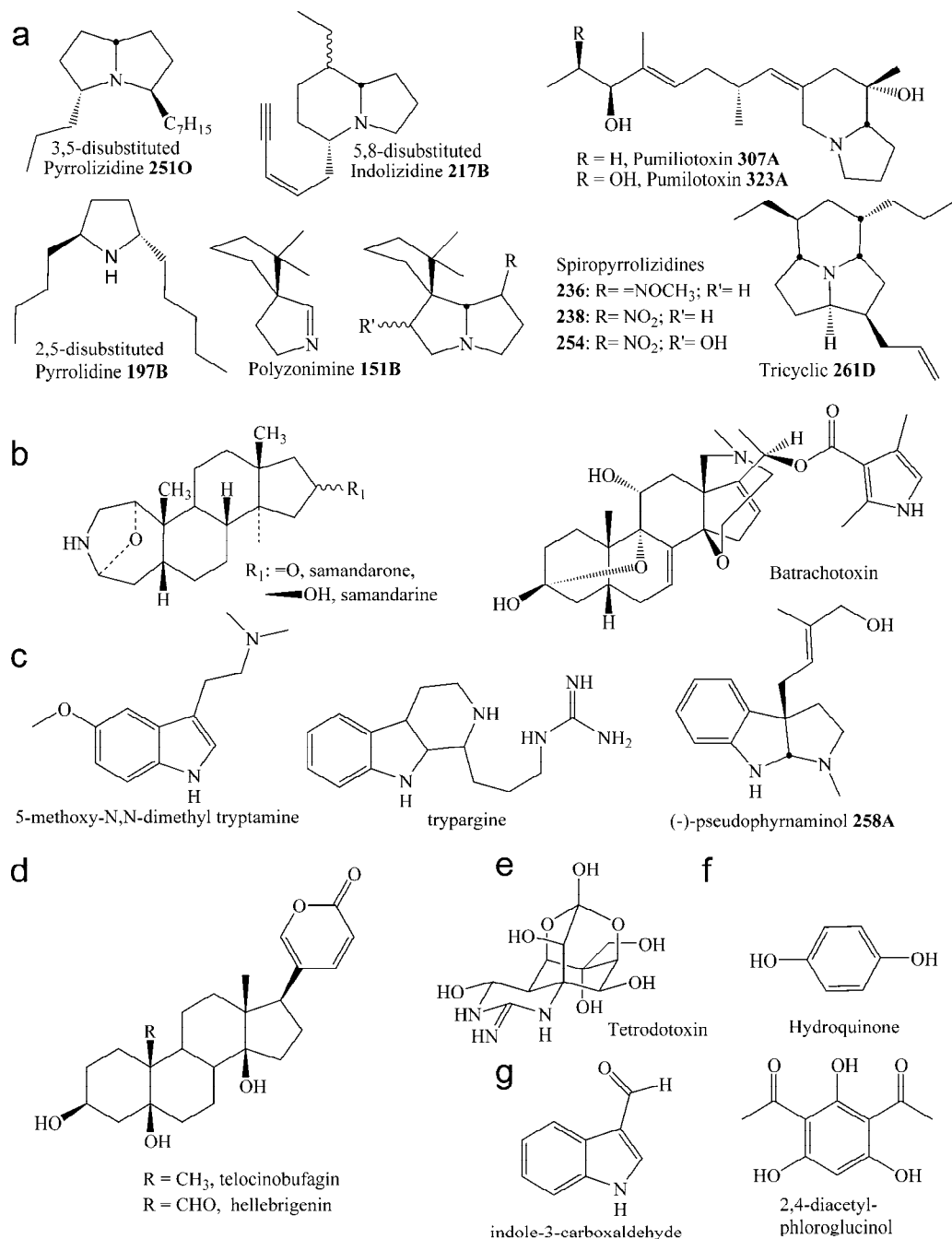


Figure 1. Representative structures of small molecules isolated from amphibian skin secretions. **a**. Alkaloids known from both poison frogs and sympatric dietary arthropods [Clark *et al.* 2005, see also ‘unpublished data’ and references in Daly *et al.* 2005], **b**. Steroidal alkaloids [Daly *et al.* 2005, Mebs *et al.* 2005], **c**. Biogenic amines: 5-methoxy DMT [Erspamer 1994], tryptargine [Rosegheni *et al.* 1988] and pseudophyrnaminol [Daly *et al.* 1990, Smith *et al.* 2002], **d**. Bufadienolides [Hutchinson *et al.* 2007, Tempone *et al.* 2008], **e**. Tetrodotoxin, **f**. Hydroquinone [Mebs *et al.* 2005], and **g**. Antifungal metabolites from salamander skin bacteria [Brucker *et al.* 2008ab].

A multitude of peptides has been identified and cloned from skin secretions of various anurans [Chen *et al.* 2006]; however, no skin peptides have yet been described from caecilians, the limbless amphibians (Table 1). Some skin chemistry has been described for salamanders, including steroids from *Salamandra* [Schöpf & Bayerle 1934], tetrodotoxins from various newts [Brodie *et al.* 1974], fungicidal small molecules produced by microbial skin symbionts [Brucker *et al.* 2008ab] of nest-guarding salamanders, and pheromonal peptides from *Cynops* newts [Kikuyama *et al.* 1995; Nakada *et al.* 2007] and *Plethodon* salamanders [Rollmann *et al.* 1999].

Table 1. Representative peptides from amphibians that established new peptide classes.

Peptide family	Amphibian species of first isolation	Amino acid sequence	Reference
Physalaemin	<i>Physalaemus fuscomaculatus</i>	pEADPNKFYGLMamide	Erspamer <i>et al.</i> 1964
Bradykinin	<i>Rana temporaria</i>	RPPGFSPFR	Anastasi <i>et al.</i> 1965
Caerulein	<i>Litoria caerulea</i>	pEQDYsTGWMDFamide	Anastasi <i>et al.</i> 1968
Bombesin	<i>Bombina bombina</i>	pEQRLGNQWAVGHLMamide	Anastasi <i>et al.</i> 1972
Dermorphin	<i>Phyllomedusa sauvagei</i>	YdAFGYPSamide	Montecucchi <i>et al.</i> 1981
Sodefrin	<i>Cynops pyrrhogaster</i>	SIPSKDALLK	Kikuyama <i>et al.</i> 1995

pE = pyroglutamyl residue (cyclization of N-terminal Q residue at low pH);
Ys = O-sulfated tyrosyl residue; dA = D-alanyl residue.

1.3. Sources of Defensive Chemicals Known from Amphibian Skin Secretions

1.3.1. Microsymbionts that Produce Defensive Chemicals

In most cases the verified biosynthetic sources of amphibian toxins remain a mystery. For example, the tetrodotoxins, a class of alkaloids that blocks membrane sodium channels [Gusovsky *et al.* 1986], are known from *Taricha* and *Notophthalmus* newts, *Atelopus* and other frogs, marine *Fugu* puffer fish, *Vibrio* bacteria, and a stunning variety of other organisms [reviewed in Daly 2004]; however, likely skin bacterial symbionts have not yet been identified from any amphibian, and the biosynthetic enzymes that produce tetrodotoxins have not yet been reported. When *Atelopus* frogs were bred and reared in captivity, the offspring had no detectable tetrodotoxins in skin [Daly *et al.* 1997]. Once the biosynthetic genes and their encoded enzymes are discovered, isotopically labeled tetrodotoxin precursors could be used to probe the biosynthetic pathways.

In the case of certain salamanders (e.g., *Plethodon cinereus*), two species of skin bacterial symbionts have been isolated, cultured, and shown to produce small molecules (Fig. 1g) that prevent growth of fungus on the eggs of the salamanders' nests [Brucker *et al.* 2008ab]. Naturally occurring concentrations of the antifungal metabolite 2,4-diacetylphloroglucinol (Fig. 1g) produced by the *Plethodon* skin bacterium *Lysobacter gummosus* also proved lethal against the chytrid fungus, *Batrachochytrium dendrobatidis* [Brucker *et al.* 2008a]. It seems likely that other mutualisms and symbioses await discovery and characterization, and could even explain the resistance of some amphibians in zones where most species had been affected by the chytrid fungus. However, a number of peptides synthesized in frog skin also show activity against the chytrid fungus [Rollins-Smith & Conlon 2005], and could also possibly explain the resistance of certain frog species to chytrid fungus.

1.3.2. Dietary Sequestration of Toxins

Tropical poison frogs known to contain lipophilic, basic alkaloids in skin include several dendrobatid genera from Central and South America [Daly *et al.* 2005, Grant *et al.* 2006], *Mantella* (Mantellidae) from Madagascar [Daly *et al.* 1996, Clark *et al.* 2005, 2006], *Melanophryniscus* (Bufonidae) from South America [Garaffo *et al.* 1993], *Pseudophryne* and *Geocrinia* (Myobatrachidae) from Australia [Daly *et al.* 1990, Smith *et al.* 2002], and probably also *Limnonectes* (Ranidae) from Asia [Daly *et al.* 2004] (Fig. 2). So far, dendrobatids [Daly *et al.* 1980, Daly *et al.* 1992], *Mantella* [Daly *et al.* 1997], and *Pseudophryne* [Smith *et al.* 2002] have been raised in captivity to reveal that only the *Pseudophryne* are able to make their own alkaloids *de novo* (i.e., only the indolic pseudophrynamines) and that both *Pseudophryne* and *Dendrobates* were able to modify certain pumiliotoxins that were fed to them [Daly *et al.* 2003]. Intensive surveys of frog prey have revealed that certain ants and a millipede have the same alkaloids in common with the tropical poison frogs that prey upon them [Clark *et al.* 2005, Saporito *et al.* 2003, 2004]. Mites might be another major source of alkaloids found in frog skin [Takeda *et al.* 2005]; however, one study [Saporito *et al.* 2007b] that surveyed mites for alkaloids appeared flawed in methods and is therefore addressed in this chapter's section on contamination (part 4.3).

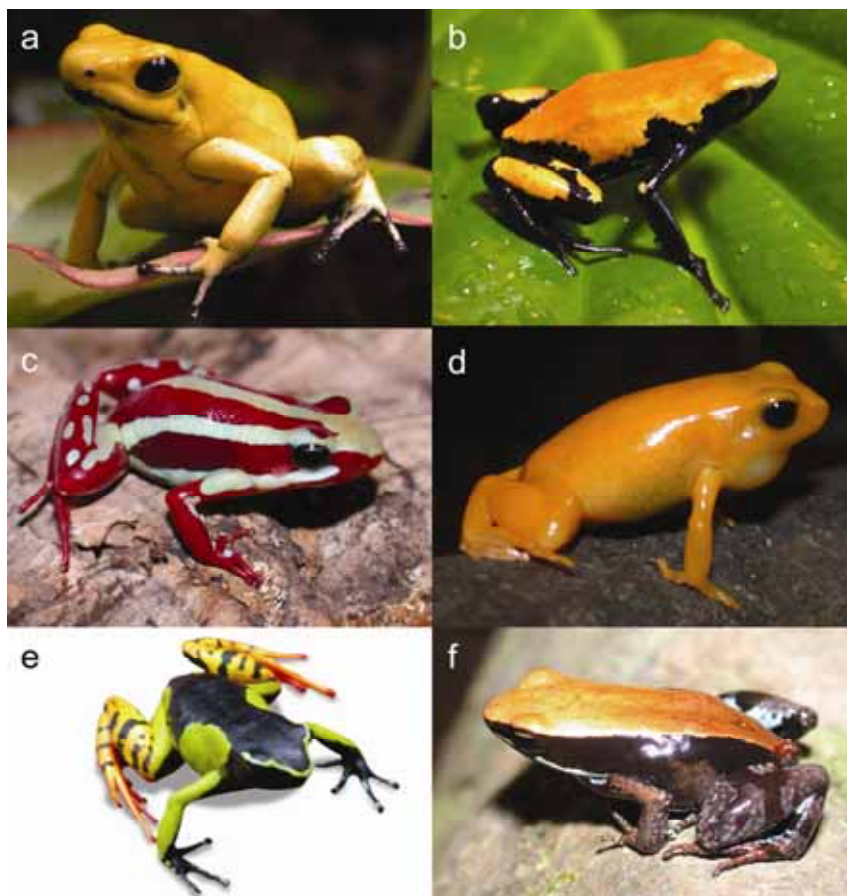


Figure 2. Representative tropical poison frogs. **a.** *Phyllobates terribilis*, **b.** *Dendrobates galactonotus* (*Adelphobates galactonotus* [Grant *et al.* 2006]), **c.** *Epipedobates tricolor*, **d.** *Mantella aurantiaca*, **e.** *Mantella baroni*, **f.** *Mantella betsileo*. Photographs copyright Valerie C. Clark.

Tropical poison frogs are not unique in their ability to sequester chemicals from prey for their own defense. In Papua New Guinea, passerine birds of genera *Pitohui* and *Ifrita* contain batrachotoxin alkaloids in skin and feathers [Dumbacher *et al.* 1992, 2000; Fig. 1b]. The presence of batrachotoxins in sympatric melyrid beetles, coupled with presence of such beetles in stomachs of *Pitohui* birds, suggest that these birds' batrachotoxins are acquired from their beetle prey [Dumbacher *et al.* 2004].

Toads synthesize bufadienolides for protection against predators; however, in one case an Asian snake, namely *Rhabdophis tigrinus*, consumes toads and sequesters their bufadienolides into specialized nuchal glands [Hutchinson *et al.* 2007; Fig. 1d]. A number of arthropods acquire chemical defenses from their plant diet, such as insect herbivores that acquire pyrrolizidine alkaloids [Hartmann & Ober 2000, 2008] and *Manduca* moths that sequester the pyridyl alkaloid nicotine [Gaertner *et al.* 1998]. Dietary sequestration also occurs in the marine world—for example, nudibranchs acquire alkaloids and other secondary metabolites from cyanobacteria that they graze upon [Dalisay *et al.* 2009].

It certainly makes sense from an energetic standpoint to steal and sequester natural compounds, rather than to spend metabolic energy making secondary metabolites. However, relying on the environment for needed defensive chemicals can be risky—it already has been reported that *Mantella* poison frogs in disturbed habitats have fewer sequestered arthropod alkaloids than counterparts in relatively undisturbed forests [Clark *et al.* 2006]. Furthermore, some of the chemicals (e.g., pyridyl alkaloids) recovered from poison frog skin secretions could originate in plants consumed by the frogs' arthropod prey.

1.3.3. Biosynthesis of Defensive Chemicals

Biogenic amines (Fig. 1c) appear to be synthesized by toads of several genera; for example, myobatrachid toads of genus *Pseudophryne* raised in captivity contained indolic pseudophrynamines in skin, whereas lipophilic alkaloids (e.g., pumiliotoxins) characteristic of wild toads were lacking in these captive-born *Pseudophryne* [Smith *et al.* 2002]. Detailed studies on the biosynthesis of biogenic amines and bufadienolides in toads could be accomplished with isotopically labeled precursors dusted on prey items. Although it has been suggested that the production of bufadienolides begins with cholesterol in the liver [Santa Coloma *et al.* 1984], it remains possible that steroid biosynthesis could begin with Acetyl CoA in the parotid glands of toads. Therefore, studies are currently underway to probe both the biosynthesis of bufadienolides and the uptake and metabolism of alkaloids by certain anurans.

Whereas determining the biosynthetic origin of small molecules isolated from amphibian skin secretions requires much further investigation, the genetic machinery for peptide production has been well studied in frogs [Shaw & Chen 2008 and references therein]. The sequence prior to the region encoding for any mature frog skin peptide, namely the biosynthetic precursor or prepro sequence, typically consists of an N-terminal hydrophobic signal peptide (≈ 20 -22 residues) followed by an acidic spacer domain (≈ 20 -22 residues) that often contains propeptide processing sites. The full construct then terminates with a mature peptide-encoding domain including a post-translational amidation sequence on the carboxy-terminus. Some precursors contain two or more tandem repeats of such acidic spacer/mature peptide domains.

Molecular cloning of frog skin peptides has revealed that these biosynthetic precursor regions are highly conserved for any given class of peptides (e.g., bradykinins, caeruleins,

tachykinins) within each frog family, and sometimes are also well conserved between members of different frog families. This conservation (i.e., of both primary structure and nucleotide sequence) of signal peptide domains has allowed the development of numerous primers to effectively “shotgun” clone several peptide classes from frogs’ secretions [Chen *et al.* 2003ab, Chen & Shaw 2003, Chen *et al.* 2005, Zhou *et al.* 2006ab, Thompson *et al.* 2007, Quan *et al.* 2008]. This rapid method to survey peptidomes of amphibians has facilitated studies on the pharmacology of frog skin peptides [e.g., Chen *et al.* 2004, Marenah *et al.* 2004, O’Rourke *et al.* 2004].

After the primary sequence of a peptide is translated by the ribosome, modifications essential for bioactivity are often made to the primary amino acid sequences. A number of such post-translational modifications of peptides from frog skin secretions have been detected using mass spectrometry. Some examples of post-translational modifications reported in frog peptides include: 1) C-terminal amidation (i.e., the glycyl residue distal to the last residue of the mature peptide is hydrolyzed to form the amide) [Molay *et al.* 1986], 2) N-terminal pyroglutamyl residues that block aminopeptidase attack (see Table 1), 3) hydroxylation of prolyl residues, and 4) *O*-sulfation of tyrosyl residues [see Table 1 and Bevin & Erspamer 1990, Chen & Shaw 2003].

A rarely reported modification of frog peptides is the enzyme-mediated conversion of L-amino acids to the corresponding D-isomer in the second position in the peptide chain [Richter *et al.* 1987, Jilek *et al.* 2005]. For example, this phenomenon was discovered in dermorphins (D-Ala²) [Montecucchi *et al.* 1981], is known to occur in deltorphins (D-Met² and D-Leu²) [Erspamer *et al.* 1989 and Barra *et al.* 1994, respectively] and bombinins (D-allolle²) [Mignogna *et al.* 1993], and may be much more common than so far reported. The isomerase that converts L to D-amino acids has been sequenced and demonstrated to isomerize the second amino acid of a model frog peptide [Jilek *et al.* 2005]. However, enzymes mediating the other post-translational modifications of frog peptides mentioned above have not yet been characterized.

1.4. Chemicals used in Communication

1.4.1. Pheromones and Allelochemicals

Semiochemicals are natural compounds that function in communication, including toxins that serve defensive roles as described above. Such chemical signals also include pheromones that mediate interactions involving sex, aggregation, trails, and/or alarms to other members of the *same* species, and allelochemicals that mediate interactions between different species. Such interspecific interactions fall into three categories: allomones benefit the originator but not the receiver (e.g., alkaloids of poison frogs), kairomones benefit the receiver but not the originator, and synomones benefit both originator and receiver. A wide variety of semiochemicals are documented at www.pherobase.com.

Whereas a great diversity of semiochemicals that function in defense has been documented in amphibians [Daly *et al.* 2005], only a few compounds have been shown to act as pheromones [e.g., Rollmann *et al.* 1999] despite a wealth of circumstantial behavioral and morphological (e.g., sexually dimorphic glands [Goncalves 2008]) evidence suggesting that pheromones occur in a number of amphibians [Houck 2009]. However, pheromones have been extensively studied in arthropods, and such research is integral to the design of pest control agents [Pickett *et al.* 1997].

1.4.2. Behavioral Studies in the Field and Laboratory

An odd behavior or bright coloration observed in an animal can alert the field biologist that there may be some associated chemistry to explore. Each possible study subject may require a specialized approach to secretion collection— after careful observation, the glands storing chemicals can often be located and selectively sampled. Later in this chapter, several approaches are outlined to serve as a general guide for collecting secretions.

Once the secretion is collected and analyzed to reveal a chemical structure, the compound's function should be investigated with a variety of studies in the laboratory and/or field. In the case of defensive compounds, a range of pharmacological assays can be used to determine the receptors on which specific compounds act. Often times, a toxic molecule can be tweaked slightly in chemical structure to retain bioactivity while at the same time reducing toxicity, thus leading to drug candidates. Ethological studies have been used to investigate how predators respond to prey [Kutcha *et al.* 2008] and to painted clay models made to resemble their prey [Brodie 1993, Kutcha 2005, Saporito *et al.* 2007b]. Behavioral studies have also revealed much about chemically mediated attraction [Haynes & Yeorgan 1999].

Whereas one approach is to identify all secretion components and then test each compound for function, another approach is bioactivity-guided fractionation. In the latter, the secretion is separated into fractions (e.g., via some form of chromatography), these fractions are each tested for bioactivity, and bioactive fractions are then subjected to further separation/purification. Bioactive compounds tend to be potent and specific; therefore, they are usually present only in minute amounts that may be best isolated by following activity in a bioassay. If HPLC or MS of a bioactive fraction shows relatively few chemical components, then all compounds could be isolated and tested individually to see which one(s) demonstrate the activity. Sometimes activity is greatest for a combination of compounds that act in synergy.

1.5. Aim of this Chapter

This chapter, aimed at field biologists who are interested in chemical ecology, reviews a variety of methods that have and have not been published for the collection and preparation of secretions from amphibians and arthropods. Many of the principles presented could be applied to secretion collection from other animals as well. After establishing a field plan that includes in-country collaborators, one should choose methods for secretion collection based on the types of compounds one expects to analyze.

Many of the guidelines and precautions recommended in this chapter are based on the author's personal experiences. It should be emphasized, however, that circumstances can vary drastically from one environment and country to another. It is the responsibility of each investigator undertaking collection expeditions to fully acquaint themselves with the potential dangers and rules/laws of their selected areas, and to adapt procedures and precautions to their particular needs.

2. COLLECTION PREPARATION AND LOGISTICS

At least one year prior to your field expedition, contact potential collaborators in the country of interest and begin the permit process with the appropriate collaborators and government agencies in the host-country. Some countries have non-governmental organizations (NGOs) that will, for a small fee, facilitate the establishment of collaborations and acquisition of permits. For example, in Madagascar, the Madagascar Institute for the Conservation of Tropical Environments (MICT-ICTE) is an NGO that provides logistical support for researchers working in most areas of that island nation. You will need collecting and/or research permits before heading to your study sites, and to request export permits you must provide a list of all specimens collected with locale details. It is common and recommended to donate half of your collection to a museum based in the host-country (e.g., donate 70 frogs if you collect 140 total).

2.1. In-Country Collaborators and Local Support

Garnering and maintaining local approval for your expedition and subsequent laboratory analysis is as important as having solid scientific goals and experimental plans. One of your priorities should focus on local education and involvement, starting by establishing mutually beneficial collaborations with scientists from national universities who share similar research interests. Ideally, your field team should consist of at least one national collaborator for each foreign researcher on your expedition. Students recommended by national faculty to accompany you in the field can help not only with your sample collection, but also by providing knowledge of travel and living logistics, and of local languages and customs.

Academic colleagues who accompany you to the field will also help negotiate fair prices for porters and local assistants. Services are usually paid for in cash but bartering is also common—supplies such as tarps, cooking pots, and medical supplies can be bartered or generously donated upon leaving your sites. The daily or per-hike cost of porters varies considerably based on host-country and the distance/difficulty of the hike to camp, ranging from about one to 20 USD.

However, the first step is for you and your team to meet the mayor/president/chief/leader of the village closest to your study site, and request local support. You should hire as a full-time team member at least one person from this village-- this person is usually chosen by the village leader to act as cook and guardian for the camp. Additional local assistants can be hired on the usual full-time daily rate basis that includes meals with your team, or on a per-item-collected basis. The latter scenario has proven especially productive in the author's expeditions for rapidly locating rare and/or secretive species in both Madagascar and Guyana. Whenever interested villagers approach your team, share your goals with them. Show them photographs of the species you seek and demonstrate your collection techniques (e.g., do not touch the frogs, make frogs hop into clean plastic bags, and field tape to mark point of capture). Then make a clear offer of how many individuals of each species and the price per collected animal that you are willing to pay. Offer more for species that are particularly difficult to find. If you still have not found your target species by the last day, increase the

reward substantially or risk leaving empty-handed. Usually, an offer of five USD for a frog will make elusive species appear within 20 minutes.

Prior to leaving your field sites, pack out all of your waste and then meet with the village leader again. Ask your guide if common items such as empty bottles should be left as resources or disposed of as trash. Thank your hosts by sharing food and materials, and if you had an enjoyable stay, give a t-shirt or other useful materials to the most helpful people, and obtain a local mailing address so you can later mail photographs with a thank you note. Record names of all local assistants so that you can credit them properly when you publish. If a local assistant provided especially useful insight, consider including them as an author on resulting publications. A good example of this is the properly-credited local man of the Herowana village in Papua New Guinea, who led Dumbacher's team directly to beetles that had the same toxin in common with birds that consumed them [Dumbacher *et al.* 2004].

2.2. Research/Collecting and Export/Import Permits

Research and collecting permit applications should be submitted to the responsible government agency with local collaborators one year prior to traveling to the field. All locales that might be visited should be listed—it is better to list more areas than you might actually visit, so that your plans can be flexible. A road may be blocked or flooded, there may be local political problems, the rainy season you planned for may be late, etc.; therefore, it is advantageous to have several extra options outlined/preapproved as study sites. Adding a study site to issued research permits can sometime take several weeks, so be sure that your initial permit application includes all possibilities.

If it is your first time collecting in a particular country, then plan a week in the port of export with your colleagues upon both arrival and departure. Buy bulky and heavy materials locally. Check your permits and submit requests for any critical changes. Visit the national university with your collaborators and offer to give a formal talk about your previous work and what you plan to do with your new colleagues, including any special field methods.

While conducting fieldwork, keep a running list of all specimens collected and digitize this list early and often, into a program such as Excel[®]. Your table should include the following details for each sample collected: field tag number, genus, species, local place name(s), Province/State, country, GPS coordinates, collection date, collector name(s), and notes on habitat, sex, size, voucher/sample treatment (e.g., skinned, photographed), etc.

To apply for export permits, clearly outline all collections made, what you request to export, and what you suggest to donate to the appropriate museum in the country of origin. Also provide brief descriptions of all sites visited and any major research findings in your exit report. These expedition results should be ready upon return to the port of export so that you can efficiently submit the export permit application. Once again, it is likely to take about a week of visits with collaborators to government agencies to acquire export permits. If you are working with endangered wildlife, CITES permits can take even longer. If you must depart prior to obtaining export permits, leave your collections behind for later shipping or transport by a colleague. In order to achieve legal entry of collections into your own country, be prepared to give a copy of export permits to Fish & Wildlife (USA) or related agents who will inspect your collections upon arrival home. These officials will grant your import permit and should be contacted in advance with details of your arriving flight.

2.3. Tropical Field Work

These are suggestions based on the author's tropical field work experiences. The lighter you pack the easier transport will be, but do come fully prepared. An example packing list for a month long tropical field expedition is included in this chapter's Appendix. Pack your materials in large duffel bags rather than hard luggage. Always maintain control of your bags.

Medical preparation: Consult with a travel nurse and ensure that you are vaccinated for all tropical diseases present in the area(s) that you will visit. Start taking a malarial prophylactic (e.g., malarone) a few days prior to departure, and bring ample supplies in case your departure is delayed. If venturing to remote areas, then consider travelling with a satellite phone and taking out medical evacuation insurance for emergencies.

Your personal medical kit should include ibuprofen (e.g., AdvilTM), feminine products (i.e., for any form of major bleeding), mole foam, small scissors, antibiotic ointment (e.g., NeosporinTM), bandages, iodine tablets (i.e., for emergency water sterilization), anti-itch cream, anti-fungal cream, nasal decongestants and antihistamines (e.g., Claritin), insect repellent, sunscreen, and a compact reflective emergency blanket. It is possible to suffer from hypothermia in the tropics, especially at higher elevations! Some useful medications that you should have with you on your travels (e.g., ciproflaxin and codeine) may be available without prescriptions in your destination country. Do not expect to find any of these materials in your host-country, although many tropical destinations will have major supermarkets and/or pharmacies that offer these products for a price.

In-country purchases: Topographical maps are sometimes only available in-country, if at all. Some bulky, heavy materials that you will need for camping can be purchased locally upon your arrival, and should be left behind upon your departure. Heavy cooking pots, bulky buckets, food, rice bags, machetes, and specimen alcohol can be bought locally.

Local pharmacies are an excellent source for useful medications, and often also sell formalin and 95% alcohol that can be used for voucher preservation. Sometimes alcohol is only available at local distilleries, and this alcohol may or may not be of sufficient quality/purity for collecting genetic samples, and is definitely *not* acceptable for chemical samples. Analytical grade alcohol suitable for chemical samples is expensive and/or unavailable in tropical countries, so arrangements must be made with in-country collaborators several months in advance to secure and/or ship such quality solvents. Your local collaborators can advise you on the best places to find good deals and local chemical suppliers, and will often kindly accompany you to stores and markets.

Clothing: Pack your clothes in zip-top bags and then in a medium duffel or water-tight sac. Bring the following clothing: long sleeve nylon shirts, nylon zip-off pants, a fleece jacket, t-shirts, long-sleeve shirt and pants for night-time, bandana, wool hiking socks, rubber boots, flip-flops, bathing suit, and if female, a sarong, nylon sports bras and bikini bottoms. You can wash your clothes in a bucket using biodegradable soap such as Dr. Bronner's, although it may be a challenge to dry them. Choose your quick-dry nylon pants carefully so that the pockets can not be easily accessed in crowded areas such as the marketplace. Stash your cash in several different hiding places.

Rain gear and daypack: If you will be working at higher elevations, a full waterproof rain suit is advised, and in any case, bring a quality poncho, such as the type that can double as a tarp. Bring a daypack that (1) is small enough to keep with you at all times, (2) fits under your

poncho when full, and (3) is still large enough to carry your camera gear. Keep a Swiss army knife attached to your pants at all times or else it may disappear.

Keep it dry: To keep clothes and cameras dry, put drierite desiccants or silica in pantyhose-fee obtained from shoe stores. These drierite bundles can be used to dry out a camera that has taken on moisture, even in cloud forests. Keep one drierite bundle in each zip-top bag containing your clothes and 2-3 bundles in watertight cases or bags containing cameras. Package your reservoir of drierite bundles so that they will not all be exposed to moist air at once.

Blistering hikes: If hiking steep slopes for several hours, then one is likely to experience chaffing, blisters, and insect bites. On such hikes, you should bring along baby powder, mole foam, antibacterial ointment, and bandages. Use the baby powder liberally in sweaty areas prone to chaffing before it becomes a problem. *Do not puncture blisters.* As soon as you realize that you have a blister, cut a hole in mole foam to fit around the blister. The goal is to make the mole foam a height that is equal to or greater than the height of the blister, so that the blister will not rub and rupture. Thus, one to three pieces of mole foam may need to be stacked on top of one another, and then can be covered with a bandage. A ruptured blister or any open wound is prone to serious infection in moist forests. If familiar with your boots, pad the blister-prone areas of your feet with mole skin prior to commencing any strenuous hikes.

Team living: In many cases, the local villagers do not venture into mountainous forests; therefore, they do not have materials needed to be comfortable there. For example, when on expedition to the Wokomung Massif in Guyana [Means *et al.* 2008], warm clothes, ponchos, and headlamps were donated to each team member-- these villagers never venture into the cloud forests on their own. Pack tarps, twine, and kitchen supplies in one duffel and give to your local cook/camp guide, who will set up the common area of your team's camp as well as a designated toilet area. Provide your team with toilet paper and meals.

A team with high morale is a productive one. It is easy to be kind and to share. Let your team know what you plan to give them upon your departure, such as the warm clothes, ponchos, etc. Be grateful that local people have welcomed you to their home and show your appreciation. On your final evening in the field or at the village base, consider throwing a small celebration for a job well done. Donate as many materials as you can part with upon departure.

Porters and security: In most cases, porters are paid a daily or per-hike rate, return to their village, and reappear on a designated day to hike back out. If camping in a village in tents or other unlocked accommodations, consider hiring a night-time security person from the village. Your cook will provide security to your camp during the day, but be certain to confirm that they will serve this security role. Your security people should be selected in coordination with the village leader, and all should be well aware that they will only be paid upon successful completion of their guard jobs.

2.4. Materials

The exact materials needed depend upon your specific secretion collection goals. In preparing for integrated expeditions to study the chemistry of amphibians and arthropods [Clark *et al.* 2005, 2006] recommended supplies are listed this chapter's Appendix.

Sample containers: If you plan to analyze secretions for their chemistry, contamination and leakage of samples must be prevented. Glass vials with Teflon-lined (PTFE) screw caps are the most inert vials, but glass is heavier and easier to break than plastic. Polypropylene centrifuge tubes ranging in size from 0.5 mL to 50 mL are useful and have so far not caused any contamination issues; however, they occasionally leak even when parafilm is wrapped around the lid. Despite this downfall, polypropylene tubes are the authors' preference because they are durable, lightweight, and inert. Tubes with rubber-lined caps should never be used since the rubber linings leech small molecules into samples, resulting in contamination [Daly *et al.* 2008]. Small tubes can be organized into sample boxes with dividers. All vials and sample boxes should be labeled indelibly and clearly coordinated with field notes.

Solvents: Deionized water filtered through a millipore system can be obtained in most countries. Ethanol available at pharmacies (i.e., usually 90-95% pure) is not desirable for chemical samples, but can be used for preserving vouchers. Pure 'anhydrous' ethanol (EtOH) and methanol (MeOH) are usually unavailable in tropical host-countries, so other arrangements must be made to ensure that you have these critical solvents to make clean secretion collections. This analytical grade alcohol is available from numerous chemical suppliers, or at a discount from most Chemistry Department stockrooms in developed countries. In any case, make arrangements many months in advance to have analytical grade chemicals for your fieldwork. *Dilute* acid, such as 1% formic acid and trifluoroacetic acid (TFA) can be packed in checked baggage in small amounts— parafilm the caps of 15 mL eppendorf centrifuge tubes and triple bag them, and pad them for anticipated rough handling of luggage. Once in the field, make and label your first samples as the controls with only the solvent(s) being used.

3. COLLECTION METHODS

3.1. Record Keeping

Receipts and records. Waterproof field notebooks and pens are essential, and rite-in-the-rain[®] products are recommended for all record keeping. Keep dated records, including signatures for non-receipted expenditures, in a separate pocket notepad. Many services and products you purchase are not normally receipted (e.g., guides, porters, taxis, market produce), so you must make your own records. Retain all receipts in plastic zip-top bags. Also keep copies of your passport, VISA, and local address in each piece of your baggage.

Field notes. Clear field notes are essential for your research and for making an Excel[®] spreadsheet of all vouchers for the museum(s) that will curate your vouchers. First note the date, GPS coordinates, habitat observations/photographs, and place name (e.g., name of nearest village), then list the field tag numbers for each amphibian voucher. Each voucher entry should include species name, microhabitat, reference to photographs, snout-vent-length (SVL) and weight, sex, and any other observations such as coloration/pattern and smell/taste. If arthropods are collected, note the number of collections, taxonomic identifications, proximity to collection of frog vouchers, microhabitat, smell/taste, and other observations. Label each tube of arthropods with site number (e.g., S1, S2) and date. Field-friendly labeling machines such as the LABPAL[™] label printer may prove useful.

3.2. Avoiding Contamination

One can contaminate samples in a variety of ways. Pay attention to (1) hands/gloves and your work area, (2) collection materials and tools, and (3) team collection methods. Use only analytical grade solvents. Do not put anything (e.g., pipettes) into your alcohol or other stock bottles; rather, pour alcohol from your stock container into 50 mL polypropylene tubes and remove smaller amounts with pipettes from this 50 mL aliquot. Never return any solvent to the stock bottle.

You and your work area. If you have touched anything such as soil, a cigarette, a frog, or an insect, then your hands are contaminated. If you collect samples with dirty hands/tools, your samples would likely become contaminated as well. Even if you are wearing gloves, you can spread contamination to every sample you touch, if your gloves are not kept clean. After handling a subject, wash your work area and hands/gloved hands with water followed by a wiping with a fresh paper towel/Kimwipe[®] moist with chemical grade ethanol. Be careful not to breathe on, get spit in, or let hair get into samples, especially those slated for peptide analysis or cloning. Saliva can digest proteins and hair can contaminate protein samples with keratin. Even traces of cigarette residue can lead to contamination of your samples with nicotine. Ideally, smoking should *not* occur anywhere near the collection/study sites.

Your collection materials. After making each sample, all tools (e.g., forceps, TAS probe) used in the secretion collection must be cleaned with water and alcohol. Plastic zip-top bags should not be reused to collect additional frogs—use a fresh collection bag for each amphibian subject. If collecting insects, note that aspirators are not easily cleaned and can result in mixed insect collections and contamination with soil. Therefore, when making collections of insects for chemical analysis, entomological forceps are recommended. After making each single-species arthropod sample, rinse off forceps in a designated cleaning tube filled with alcohol, wipe off the forceps with a Kimwipe[®], and change the cleaning alcohol frequently. As mentioned above, rubber lining on tube caps can lead to chemical contamination [Daly *et al.* 2008].

Your collection methods. When capturing amphibians, use a fresh zip-top bag and do not touch your subject nor get soil into the collection bag. Simply put the clean plastic bag in front of a frog, and move a leaf or twig behind the subject to persuade it to jump into your bag. If you try to grab the subject with the bag, you are likely to get soil and litter in the bag. Do not blow air into the bag. Again, do not reuse collection bags for multiple subjects/samples as this will lead to cross-contamination of frog secretion samples.

Also avoid cross-contamination when collecting leaf-litter arthropods. Putting different species into the same tube or bag, or reusing forceps without cleaning between subjects can lead to cross-contamination, as detailed later in this chapter. The only time you don't need to clean forceps is for collections of multiple subjects in the same tube; for example, if making a single sample of multiple individuals from any one ant colony, then washing forceps after each individual ant is neither necessary nor desirable.

3.3. Nonlethal Amphibian Skin Secretion Collection

When threatened by a potential predator, amphibians ooze natural chemical defenses from skin glands. It is this defensive skin secretion that is desired for chemical analyses, not

every component of the amphibian integument. In general, collection techniques that both keep the samples cold and minimize the need for additional sample preparation are ideal.

In many classical studies of amphibian skin chemistry, hundreds or even thousands of frog specimens were sacrificed and their skins removed for extraction in methanol [see Anastasi *et al.* 1977, Myers *et al.* 1978, Daly *et al.* 1980, Erspamer 1994]. For several reasons, some ethical and others scientific, this approach is unacceptable today. Amphibians are in rapid global decline [Stuart *et al.* 2004, Lips *et al.* 2006, Pounds *et al.* 2006], thus rendering sacrifice of such substantial numbers of any given population unreasonable. With regard to the robustness of subsequent analytical data, the full skin extraction approach solubilizes components of all skin tissues, rendering an uncontrolled sample that is not representative of a glandular *secretion*. The glandular defensive secretion is desired for chemical analyses and cloning experiments, not a random sample of components of the amphibian integument. Skin secretion samples can be collected non-lethally via a variety of stimulation techniques followed by washing or wiping the amphibian's skin with de-ionized water, since water does not compromise or modify any compounds of potential interest. However, samples stored in water alone are particularly susceptible to degradation by enzymes and contamination with bacteria; therefore, such aqueous samples must be kept cold and frozen immediately.

3.3.1. Secretion Induction via Electronic Stimulation

Defensive secretions can be collected in a variety of ways by simulating an attack on the amphibian subject, via electronic or mechanical stimulation. 'Milking' methods described here have been tested on captive amphibians to reveal no complications in breeding and feeding patterns. Chemical stimulation is not recommended and therefore is mentioned only briefly.

Trancutaneous Amphibian Stimulator (TAS). A device that electrically stimulates amphibians to induce secretion was originally developed by Tyler and colleagues [Tyler *et al.* 1992], and modified in design to result in the TAS [Grant & Land 2002]. The TAS delivers an adjustable electric pulse via an inert metal probe to stimulate secretion (Fig. 3a). To find the ideal settings for a particular subject, start with the lowest settings for amplitude, rate and pulse width, and increase these settings to cause some minor twitching in your subject. If the subject straightens its legs, your settings are too harsh and could be harmful or even deadly. Small (<30 mm SVL) subjects usually respond to the lowest possible settings, whereas larger amphibians sometimes require relatively higher settings. For any given subject, the most effective settings can be estimated with just a little practice. If unsure, opt for lower settings with multiple washings. Each stimulation event should last no longer than 10 seconds, and the *amphibian's skin must remain moist* throughout the TAS process for effective and safe results. Such moistening throughout the stimulation process should use deionized water; however, after stimulation and secretion collection is complete the subject should be briefly submerged and rinsed in local water, monitored in a plastic bag or deli cup for 20 minutes, and finally returned to a hiding place near its flagged point-of-capture.

Amphibian secretions often appear as slimy and clear or as milky and white (Fig. 3b). Yet in some cases it may appear that no secretion is present at all (Fig. 3c), when in fact there actually is plenty of material sufficient for chemical analysis. For example, leaf frogs of genus *Agalychnis* do not have obviously visible secretions; however, when stimulated with a TAS and washed with water, the resulting freeze-dried sample appeared fluffy and white and

contained a wide variety of bioactive peptides in abundance [Marenah *et al.* 2004]. Therefore, whether or not secretion is visible after stimulation, the amphibian should be washed to collect whatever material it might have secreted—the solvent used to wash or wipe secretions into your sample tube will vary depending on field conditions and planned chemical analyses. In some cases (e.g., small frogs <30 mm SVL), it is more efficient to briefly (5-10 sec) place the entire subject in a clean, dry sample tube and let it move around to rub its skin against the tube (Fig. 3d), rather than to try to wipe or rinse the subject's secretion into the tube.



Figure 3. Electrical stimulation can be used to induce amphibians to secrete glandular contents. **a.** Transcutaneous Amphibian Stimulator (TAS) with arrow pointing to probe in hand, **b.** TASing a large tomato frog, *Dyscophus guineti/antongilii*, resulted in a milky white secretion, **c.** Many frogs, such as this *Epipedobates tricolor*, have little to no visible secretion upon electro-stimulation with a TAS probe, yet still yield useful samples. Only the slightest pressure should be used to hold the frog in place between two fingers, as shown. **d.** Small amphibians can be placed in tubes after stimulation to effectively collect secretions; arrow points to visible secretion. Although visible on the tube, the secretion was not easy to see on the actual frog. Photographs copyright Valerie C. Clark.

Here outlined is a general approach to collect secretions from a frog using a TAS. Samples prepared following these guidelines are usually stable at room temperature for up to two months, but should be chilled or frozen as indicated at the first possible opportunity. Use the coldest available freezer if a -80 °C freezer is unavailable. Natural compounds are highly variable in stability-- cold temperatures will not stop most but not all chemical degradation.

1. Check that the two 9V batteries are good by testing the TAS probe on your tongue.
2. Thoroughly clean your work surface, hands/gloves, tools, and TAS probe with water followed by ethanol-laced Kimwipes[®].
3. Moisten the subject with deionized/millipore water using a disposable plastic pipette.
4. TAS the subject. Slowly increase settings until there is a clear reaction from the subject. Do not overstimulate!
5. Rinse subject with solvent of choice.
 - a) Peptide sample: Rinse subject with deionized water into 15 or 50 mL tube.
 - b) Small molecules sample: Wipe subject with methanol-laced Kimwipe[®] and put into Teflon-lined glass vial or polypropylene microcentrifuge tube.
 - c) Cloning sample: Scrape secretion into 1.5 mL tube containing 100 μ L of cell lysis buffer. Aim to obtain at least 100 μ L volume of secretion (200 μ L total).
6. Repeat steps 3 through 5ab two more times. If possible, keep samples on ice.
7. Wet subject with local water and monitor for 20 minutes prior to release.
8. Finish preparing the samples according to what analysis is desired.
 - d) Peptide sample: Add 1 mL 1% acid (e.g., TFA, acetic acid, or formic acid) for every 9 mL of secretion + water. Acid inhibits growth of bacteria and peptidase activity in room temperature samples. If the sample can be immediately frozen and freeze-dried, then do not add acid—such a sample is useful for peptide analysis and cloning. Finally, close the cap and secure with parafilm.
 - e) Small molecules sample: Close the cap and secure with parafilm.
 - f) Cloning sample: Close the cap and secure with parafilm. Do not add alcohol or acid to this sample!
9. Clearly label all tubes with species, site #, date, solvent, and record these and other observations of secretion collection in your water-proof field notebook.
10. When available, keep the samples on ice and store them as follows:
 - g) Peptide samples: Store in the freezer at -80 °C. Freeze-dry using a lyophilizer and immediately store lyophilate at -80 °C.
 - h) Small molecules sample: Store in the freezer at -80 °C. If you did not add too much solvent, then this sample may be useful for direct injection onto a GC-MS. If planning NMR experiments, then Kimwipes[®] will be filtered out later.
 - i) Cloning sample: Store in a refrigerator at 4 °C.
11. Further details on preparing these samples for chemical analyses follow in section 4.

Note that samples destined to be analyzed for tetrodotoxin alkaloids can be collected by using a TAS and washing the subject to result in 70 vol% methanol containing 0.1 vol% acetic acid [Yotsu-Yamashita & Mebs 2003]. Alternatively, skins can be kept for many years in methanol and later extracted with acetic acid:H₂O (2:98) [Yotsu-Yamashita *et al.* 2004].

3.3.2. Secretion Induction via Mechanical Stimulation

Mechanical stimulation of glandular regions, such as the parotid glands of toads (Fig. 4a,b) and certain frogs (e.g., Fig. 4c), can be achieved using the TAS or by simply squeezing the glands and catching the squirted secretion on a glass plate or in a vial. This secretion can

be washed off the glass, or dried and scraped off, then stored in a freezer with or without solvent. Secretion on gloves can also be smeared onto the sides of the sample tube and gloves and subject then washed off with choice of solvent. Many frogs also have prominent glands on their tibia (e.g., bufonid toads and phyllomedusine frogs; Fig. 4a,d) that can be efficiently milked in the same manner as the parotid glands.

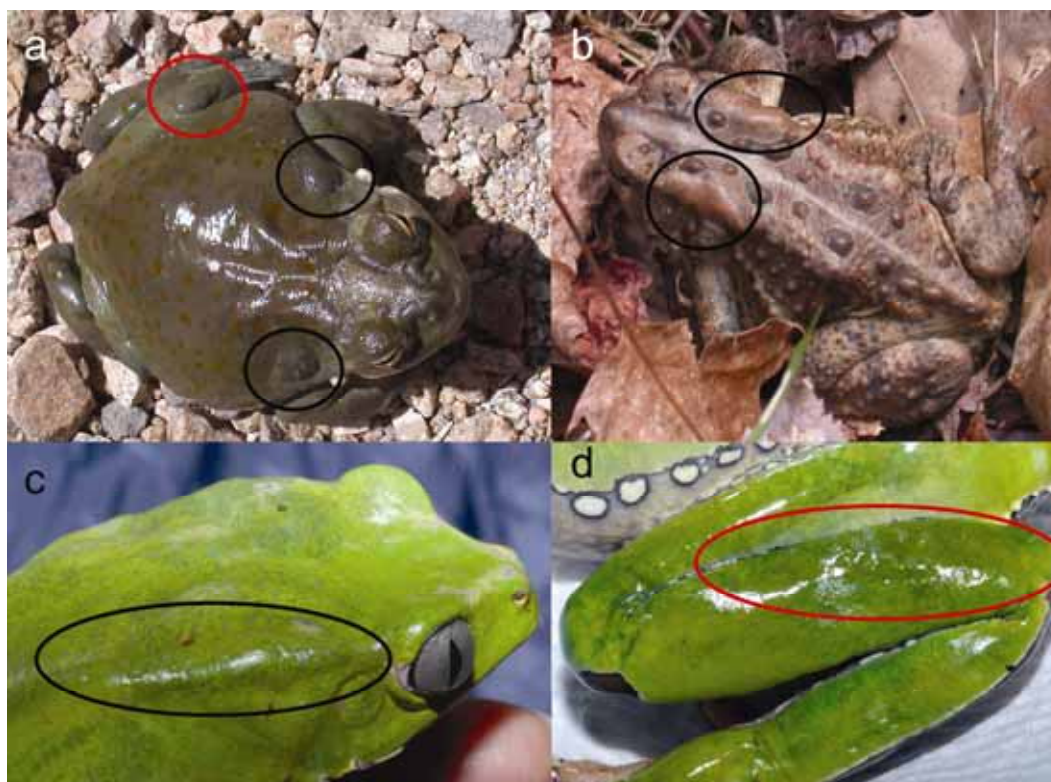


Figure 4. Prominent parotid glands (black circles) and tibial glands (red circles) can be squeezed to induce secretion. a. Parotid and tibial glands on the toad, *Bufo (Incilius) alvarius*, b. Parotid glands on *Bufo (Anaxyrus) americanus*, c. Parotid glands of *Phyllomedusa bicolor* are not as prominent as those of bufonids, d. Secretion is visible on a tibial gland of *P. bicolor* after gentle but targeted squeezing. Photographs copyright Valerie C. Clark.

The ‘hassle-bag’ technique. Another method of mechanical stimulation, the ‘hassle-bag technique,’ is effective in producing copious amounts of secretions from certain amphibians, but has not been published elsewhere. Some amphibians that do not produce much secretion when TAsed can be efficiently ‘milked’ using the hassle-bag technique. Prime candidates for this technique can be recognized in the field—if there are any bubbles or foam in a collection bag with an amphibian, then a little additional hassling will most likely produce a lot of additional secretion. In general, a foamy secretion indicates the presence of a detergent, and the most common type of detergent in amphibian secretions is probably charged peptides.

Here outlined is a general approach to collect secretions from an amphibian subject using the ‘hassle-bag technique.’ This method has proven to work well for certain but not all caecilians, salamanders and frogs (unpublished data, Fig. 5). Sometimes multiple subjects of the same species in a single bag will secrete more than if each subject is hassled in separate

bags. If it is your first look at a species, then combining multiple individuals of a species might be advantageous. In any case, try to keep samples of males and females separate.

1. Start with a subject in a clean plastic bag with no soil or debris. If the bag your subject was captured in contains any debris, then gently rinse your subject with deionized water and place subject in a new, clean plastic bag.
2. Thoroughly clean your work surface, tools, and hands/gloves with water and ethanol.
3. Gently pinch and prod your subject (through the closed bag) focusing on glandular areas near the head, dorsum, and base of the tail. Be aware that some subjects will occasionally bite a hole in the collection bag! Hassle subject for up to 5 minutes, then remove from bag and wash subject with deionized water into sample tube.
4. Using a ruler on flat surface, push all the visible secretion/foam to one corner of the plastic bag. With clean scissors, cut a small hole in the bottom of the plastic hassle-bag, and push on the outside of the bag to squeeze the secretions through the cut hole into a 15 or 50 mL polypropylene tube (for peptide analysis). For peptide cloning experiments of each species, add about 100-500 μ L of this concentrated secretion to a microcentrifuge tube containing about 100 μ L of cell lysis buffer.
5. Fold back the cut portion of the bag and hold shut manually. Add deionized water and agitate to wash the bag's contents into the polypropylene sample tube. Repeat washing the bag 2-3 times into the sample tube using 2-3 mL portions of deionized water.
6. Carefully rip the bag open along seams, and use a *clean* flat tool such as a ruler to scrape any remaining residue into the major sample tube; if small molecule analysis is desired, scrape into a fresh tube to yield 1:1 secretion: absolute methanol.
7. Finish preparing the samples, respectively.
 - a) Peptide sample: Add 1 mL 1% acid (e.g., TFA, acetic acid, or formic acid) for every 9 mL of secretion + water. If sample can be immediately frozen and freeze-dried, then do not add acid—such a sample is useful for both peptide analysis and cloning. Finally, close the cap and secure with parafilm.
 - b) Small molecules sample: Use the TAS method if possible. Alternatively, put some of the secretion with as little water as possible into absolute methanol or ethanol for later evaporation. Close the cap and secure with parafilm.
 - c) Cloning sample: Close the cap of the microcentrifuge tube and secure with parafilm. Do not add alcohol or acid to this sample!
8. Clearly label all tubes with species, site #, date, solvent, and record these and other observations of secretion collection in your field notebook.
9. When available, keep the samples on ice and store them as follows:
 - d) Peptide samples: Store in the freezer at -80°C . Freeze-dry using a lyophilizer and immediately store lyophilate at -80°C .
 - e) Small molecules sample: Evaporate solvent and store dried sample in the freezer at -80°C .
 - f) Cloning sample: Store in a refrigerator at 4°C .
10. Further details on preparing these samples for chemical analyses follow in section 4.



Figure 5. Mechanical stimulation via the hassle-bag technique. **a.** *Typhlonectes* caecilian being hassled in a bag. **b.** *Amphiuma pholeter* salamander being hassled in a bag. **c.** *Amphiuma means* salamander being hassled in a bag. **d.** Resulting *Amphiuma* secretion in the bag. **e.** Acidified *Amphiuma* secretion in sample tube. Photographs copyright Valerie C. Clark (a) and D. Bruce Means (b-e).

3.3.3. Other Techniques for Secretion Induction and Collection

Chemical stimulation. Noradrenaline/norepinephrine can be injected to chemically induce amphibians to secrete [Gibson *et al.* 1986, Rollins-Smith *et al.* 2006]. However, this method not recommended because it might lead to complicating factors.

Adaptation of secretion collection methods for the species in hand. Some amphibian secretions are exceptionally sticky and thus difficult to handle and collect. Nonetheless, slight adaptations to the methods outlined above can yield excellent samples.

For example, collecting the thick, sticky secretion from wildly wriggling tails of *Ensatina* salamanders is quite a challenge. For these salamanders, the resultant secretion collected using the hassle-bag technique appeared brown and crusty, whereas an adapted TAS method resulted in fluffy white material after freeze drying.

Briefly, the salamander subject was stimulated with a TAS on lowest setting by gently tapping it on the head and body. Too much stimulation at the base of the tail caused tail loss in one instance—this area should be avoided in autotomous salamanders [tail autotomy is reviewed in Wake & Dresner 1967]. After secretion appeared as in Fig. 6a, the subject was placed tail-first into a 15 mL polypropylene tube, and allowed to wriggle in the tube so that its secretion rubbed onto the tube's sides. The subject crawled out of the tube and deionized water was pipetted to moisten its body. The previous steps are repeated two more times to complete this method.

It is important to TAS the salamander while it is on a moist, clean paper towel/Kimwipe[®] because electrical stimulation can cause the salamander to excrete waste from its cloaca. Such waste is not desirable in the sample—that is why subjects should *not* be TAsed while in the sample tube. Finally, water was added to the tube and shaken to obtain a final volume of 9 mL of secretion and water, and then 1 mL of 1% TFA was added to obtain 0.1% acid in 10mL water. Samples obtained in this manner appeared foamy and bubbly like a detergent when shaken, and fluffy and white after freeze drying in a lyophilizer, thus indicating that peptides/proteins are likely present (Fig. 6b). Indeed, several peptides were observed in resultant 2D gels (unpublished data) and the identities of these peptides are currently under investigation with collaborators.



Figure 6. Adaptation of TAS method to collect sticky salamander secretions. **a.** Mild electrical stimulation of an *Ensatina eschscholtzii picta* resulted in a sticky white secretion on the tail and head. **b.** After secretion appeared, the salamander was placed in a tube tail first and rubbed secretion onto the tube. Photographs copyright Valerie C. Clark.

3.3.4. Genetic Material

Collection of skin secretions suitable for cloning experiments was described above; however, there are alternative methods to secure material suitable for constructing cDNA libraries from skin secretions. Secretion can be stored in the cell lysis buffer that comes with PCR kits—this is the first solvent that secretions are placed in to construct cDNA libraries from skin secretions. Note that secretions stored in cell lysis buffer should NOT be frozen, but rather kept at 4 °C. Alternatively, the secretion can be washed into a sample tube with millipore water and kept on ice until being frozen. The sample can be frozen solid in a -80 °C freezer or by pouring liquid nitrogen around but not in the tube, and then should be freeze-dried in a lyophilizer and the lyophilate immediately stored at -80 °C. Alternatively, the

secretion can be collected without solvent if it is immediately frozen—such a sample can be used for many purposes.

Toe clipping is recommended to obtain muscle needed for genetic studies to probe phylogenies. If a voucher is taken (see below), then the muscle from the inner thigh can be cut out to make samples for frozen genetic collections.

3.4. Frog Voucher Collection

If a suspected new species is discovered, a series of five voucher specimens should be photographed live, and then curated for permanent deposition at a major museum. To the extent possible, include equal numbers of males and females in any type series.

3.4.1. Euthanization and Skinning

Specimen sacrifice. If you plan to sacrifice any vertebrate animals, then write a detailed protocol for approval by the agencies governing animal welfare in your country. In the United States, such animal-use protocols are submitted for approval by your University's Institutional Animal Care and Use Committee (IACUC) only after you take an online training course and examination. This approval process can take several months or more.

Although pithing has been used by numerous other investigators to kill frogs, it is not recommended because it seems inhumane and also can lead to contamination of skin samples with other tissues. Ideally, put the subject in a clean plastic bag or deli cup in a freezer for 10 minutes—this approach will slow down your subject to a sleep and then peaceful death. However, freezers are not usually available in the field, in which case one can use an eyedropper or plastic pipette to insert into the subject's mouth a drop of absolute alcohol (e.g., analytical grade 'anhydrous' ethanol or methanol). This usually kills the subject within 3 seconds, but sometimes additional drops of pure alcohol are needed, especially for larger subjects.

If you have already finished collecting secretions but must take a voucher of a new species, simply put the amphibian into an airtight container (e.g., plastic box with an o-ring seal) containing paper towels doused with at least 95% ethanol. Many researchers use chlorotone, and *if* you have already collected secretions, this will of course have no effect.

Skimming amphibians is not recommended, but if you must, first be certain that the subject is completely dead and take care to keep the sample concentrated and free from contaminants. Do not cull in alcohol as the compounds of interest will be immediately and partially extracted into these solvents. Many amphibians can appear to be dead when they are merely sedated, so take care to be ethical.

Skimming dead anurans. Once the frog is dead, then the skin can be removed by using fine tip scissors (e.g., manicure/cuticle scissors) to cut two slits in a 'V' shape at the rear of the frog's back. Make only a very shallow snip to ensure that only the skin is barely cut, thus leaving the underlying tissues intact. If the cut is too deep, then blood may contaminate the sample, which is a problem if you plan to work with peptides, but probably does not affect the final result of alkaloid extraction and fractionation. Once the 'V' slit is cut, then gently maneuver the scissors just under the skin, and cut around the waist. Using serrated fine-point forceps, the skin can be pulled off the legs, much akin to taking off pants, and the remaining

skin removed like a shirt, sleeves and all. Sometimes the skin on the head is more difficult to remove and if avoiding blood, it may be best to leave it on the voucher.

Skins are best preserved in pure methanol if planning to analyze for alkaloids. If planning to analyze for peptides or construct a cDNA library for cloning peptide precursors, then dry out skins on a clean surface in sunlight.

Voucher preparation. Once dead, the specimen will begin to stiffen. Thus, move quickly to tie a field tag to the knee of the hind limb, and then position the voucher in an airtight plastic box for fixing. Adjust the limbs and spread out all toes per museum guidelines. Layer ethanol-soaked paper towels both below and above the fixed vouchers, and secure the airtight lid. If you are taking vouchers, try to preserve them within 30 minutes of capture to preserve stomach contents (see below).

3.4.2. Stomach Contents

If collecting voucher specimens, preserve them within 30 minutes of capture to maximize preservation of stomach contents. Larvae are digested quickly leading to results likely biased toward prey items with indigestible exoskeletons if the vouchers are not preserved quickly. Once the vouchers are preserved in alcohol, then stomach contents are also preserved. Try to maximize your ‘dietary snapshot window’ by collecting amphibians only after their major period of feeding activity. Foraging activities vary considerably among amphibians so the best time to sample is determined on a case by case basis. Stomachs of 21 small frogs (i.e., <30 mm SVL *Mantella*) were sampled to reveal a range of 7 to 66 prey items in each frog’s stomach [Clark *et al.* 2005].

The actual stomach dissection and removal of prey items is best performed with the subject in a Petri dish under a microscope and a very steady hand. Scalpels with replaceable No. 11 blades have a sharp enough tip to make precise cuts—such blades can be purchased alone or as disposable scalpels (e.g., Fisherbrand single-use scalpels with No. 11 blades = Cat. No. 08-927-5B). Precision will decrease dramatically as the blades become dull. Ergo tweezers with super fine pointed tips (Aven tools, Fisher Scientific) can pick up the tiniest of prey. Wash your tweezers with alcohol into the stomach content sample tube, and wipe tweezers with a Kimwipe® before using on your next specimen since tiny items might stick to the tweezers.

To dissect the frog, make a very light incision on the left side of the body, so as to only cut the skin of the frog. Use fine tweezers to break through the thin, lateral abdominal wall muscles and gently dislodge the stomach. Isolate and cut the stomach just distal to the pylorus. Make only a very slight incision along the stomach, taking care not to cut the contents within. Then remove all contents and return the stomach to its original position.

Stomach flushing. A nonlethal alternative to curating amphibians and dissecting out their stomach contents is stomach flushing [Patto 1998]. It is best to flush stomachs only after initial secretion collection using the TAS or hassle-bag technique, although additional secretions may be collected after stomach flushing. Micro bore PVC tubing can be fitted around needles that are screwed into syringes filled with water. The tubing used for stomach flushing can be obtained from Small Parts Inc.- for example, 0.010” inner diameter/0.030” outer diameter tubing (part # TGY-010) can be fitted to a needle with an outer diameter of 0.012” (part # NE-301PL-10). The tubing is stretched around the blunt needle for a tight fit, and this needle is screwed into a syringe. These items can be challenging to find and fit, and thus should be obtained many months in advance of your planned expedition.

Once the stomach flushing apparatus is assembled, gently insert the micro-tubing into a frog's mouth only a few mm until it barely seems to touch the pylorus. Slowly plunge water into the frog's stomach, which should result in water and contents to come out of the frog's mouth. Catch the contents on clean, moist gauze. Use entomological forceps (to minimize damage to antennae, legs, etc.) to place recovered prey items into a well-labeled tube containing ethanol. Using a hand lens, check the gauze carefully to ensure that *all* tiny items (e.g., nearly microscopic mites) and arthropod body parts are collected. If you plan on making vouchers for museum collections, then practice this stomach-flushing method on a dead voucher first and dissect the voucher to see how effective you were at recovering all stomach contents. After stomach-flushing, monitor the subject for 20 minutes prior to release.

3.4.3. Genetic Material

To save time in the field, preserve vouchers in 95% alcohol, and upon return to a laboratory where frozen collections can be curated, perform leg muscle dissections. To do this, cut a shallow slit along the inner thigh and peel back the skin, followed by a deeper incision to the bone to cut out a section of leg muscle. Put the muscle in ethanol with a barcode-labeled tube and store in a curated cryo-collection.

If liver tissue is preferred, then carefully remove/avoid the gall bladder in the field to ensure that its contents do not touch your tissue sample. Using a clean (i.e., wiped off with ethanol) scalpel or fine scissors, cut a section of the liver that has not touched gall bladder and store this sample in ethanol for later genetic analysis. Liver tissues contaminated with gall bladder contents may not be desirable for PCR reactions. A number of bile acids have been reported from gall bladders of various anurans and their tadpoles [e.g., Kuramoto *et al.* 1973, Anderson *et al.* 1979, Noma *et al.* 1998].

Although skin secretions collected using the TAS are ideal for peptide cloning experiments, dried skins removed from freshly dead frogs can also be used. This is especially useful if a dead frog is found in the field or laboratory, as the skin can simply be removed (as outlined above) and dried out in the sun on a clean, dry surface [Chen *et al.* 2006b, Zhou *et al.* 2006c].

3.5. Arthropod Chemicals

Despite the even greater variety of small molecules and peptides reported from arthropods relative to amphibians, this chapter will only touch on a few guiding principles for arthropod secretion and venom collection, and refers the reader to more detailed reviews and research reports on arthropods. In particular, the scholarly works of Thomas Eisner and Jerrold Meinwald provide excellent examples of detailed collection methods, structure elucidations and syntheses, and behavioral assays [Eisner *et al.* 1978, 2007, Eisner 2003].

All the guidelines mentioned for amphibians regarding cleaning of tools, surfaces, gloves, etc. and selection of appropriate materials such as vials of course apply here as well. Also, be sure to keep your subjects and thus samples free of debris including specks of soil, leaves, etc. Although there are several approaches to collecting pheromones and/or defensive chemicals from arthropods, only a few methods are briefly mentioned here. In general, one can 1) make extracts of an entire arthropod in alcohol, 2) dissect out, homogenize, and extract with solvent of choice specific glands from an arthropod, 3) selectively milk an arthropod to collect sprays

or droplets from hairs/spines/glands, or 4) use solid phase microextraction (SPME) to collect headspace samples of very volatile compounds. The SPME technique uses a special fiber to collect odiferous volatiles, is widely used in arthropod pheromone and plant volatile studies, and is outlined in detail elsewhere [Arthur & Pawliszyn 1990, Augusto & Valente 2002].

3.5.1. Whole Animal Extracts

Mixed collections of arthropods were collected with forceps and extracted in methanol to reveal that alkaloids known from frogs can be detected by GC-MS in such mixed arthropod samples [Daly *et al.* 2002]. After making extracts of individual arthropods in minimal amounts (i.e., just enough to barely cover the specimen in a microcentrifuge tube), Clark and colleagues pinpointed some of the exact arthropod prey species that share the same alkaloids with frog predators in Madagascar [Clark *et al.* 2005].

The key to the forceps-mediated arthropod collection method is using only a minimal amount of ‘anhydrous’ methanol or ethanol for each sample. The author prefers methanol. Also, ensure that your entomological forceps are cleaned in methanol and wiped off with a clean Kimwipe[®] before collecting each different sample, but not cleaned for each specimen contained in a single sample. For example, collecting more than five individuals of the same ant species into a single tube will make a better sample than just one ant, but do not (with exception of visible debris) clean off forceps after collecting each individual for a tube full of the same ant species. The ant may have secreted chemicals onto forceps used to pick it up, and therefore you want to retain those chemicals in the sample of that ant species. However, if you want to collect a different arthropod species into a different sample tube, then to avoid cross-contamination it is critical to use forceps cleaned in alcohol and wiped free of debris.

To collect leaf litter arthropods, start by raking a fresh pile of half a liter of leaf litter and put it on a white entomological tray (e.g., macroinvertebrate sampling tray from Forestry Suppliers, Inc.). Use a clean ruler to move the litter around while searching. Methodically go through the litter and pick out arthropods with clean forceps and put into tubes containing a few microliters of methanol. Alternatively, beat around bushes, trees, and rotten logs to find insect colonies. Colonies can be collected into one tube, but if possible, keep castes in separate tubes.

Materials preparation. Prepare collecting materials prior to hiking to your sites. Prepare several 0.6 mL or 1.5 mL microcentrifuge tubes by adding only a single or half drop (i.e., from glass eyedropper or disposable pipette, about 10-50 μ L) of methanol and closing the top tight. Entomological forceps can be kept in a 50 mL polypropylene tube with 10 mL of methanol, and this methanol should be changed after a few cleaning uses. Finally, prepare and label as ‘wash’ or ‘junk’ about three 1.5 mL tubes full of methanol to be used for cleaning forceps prior to returning them to the 50 mL soaking/cleaning tube of methanol. Cleaning the forceps twice, first by dipping in the 1.5 mL tube of methanol and then by soaking in the 50 mL tube, will decrease your chances of cross-contamination and will eliminate most debris. You can organize these tubes in boxes with dividers (e.g., Thermo Scientific Revco Freezer Fiberboard storage boxes and dividers). Explain and demonstrate to your team the importance of this cleaning/collecting protocol.

Concentrated methanolic extracts. Note that using too much solvent may limit your success in detecting alkaloids. If the solvent is evaporated off the sample, then loss of low molecular weight volatile compounds increases. For example, in one study a nitrogen stream was used to blow down samples of a millipede in methanol [John Daly, pers. comm.] to

reveal that *two* alkaloids were present in this species [Saporito *et al.* 2004]. Yet, in another study a total of *five* different spiropyrrrolizidine alkaloids were detected in the same millipede species [Clark *et al.* 2005]. In this study a single millipede was collected in a minimal amount of methanol and the resulting extract was injected directly onto a GC-MS with no sample manipulation [Clark *et al.* 2005]. Other factors may have led to this difference (e.g., age/development/sex of millipede, timing of collection, environmental factors), but in any case, samples of single arthropods should be *collected/extracted in minimal amounts of methanol* in order to both *maximize sample concentration* and *minimize sample manipulation*.

3.5.2. Specialized Arthropod Secretion And Venom Collection

Many arthropod groups have specialized glands that can be selectively milked for venoms and sprays [reviewed in Eisner 2003, Eisner *et al.* 2007]. Focus on maximizing the amount of secretion/venom/spray collected into a sample vial while minimizing contact with any possible contaminants.

Millipedes. Since the most recent review of millipedes [Eisner *et al.* 1978], many of the same compounds (e.g., hydrogen cyanide (HCN) and other nitrile compounds, numerous benzoquinones, various alkaloids) have been reported from additional millipede species. It seems likely that millipede chemical defenses evolved prior to their radiations, since even complex small molecules occur in species with distant distributions. For example, some of the same spiropyrrrolizidine alkaloids have been reported in polyzoniid millipedes from both tropical (*Rhintous purpureus* sampled in both Madagascar and Panama) and temperate (*Petaserpes cryptocephalus* sampled in Ithaca, New York, USA) forests [Clark *et al.* 2005, Meinwald *et al.* 1975, Saporito *et al.* 2003]. The author's preferred approach for rapidly assessing the occurrence of (usually known) small molecules in millipedes is to simply collect the entire animal into a minimal amount of methanol and inject this extract directly onto a GC-MS, as outlined above for arthropod chemical surveys.

Stick insects. Most walking stick insects (order Phasmatodea) have thoracic glands just behind their head that spray defensive compounds; therefore, wear safety glasses prior to handling. Such defensive sprays from phasmids contain a variety of small molecules including but not limited to glucose, monoterpenes, aldehydes, quinolines and other alkaloids [Meinwald *et al.* 1962, Eisner *et al.* 1997, Dossey *et al.* 2006, 2007, 2008]. Phasmids can be kept in captivity and milked repeatedly by simply using a clean glass vial to apply firm but gentle pressure to the thoracic gland at the base of the head (i.e., just enough pressure to restrict movement for up to 10 seconds) to induce the insect to spray into the vial. If a new species is being sampled for the first time, then combine several milkings into a single sample. However, a few milkings (e.g., about one microliter of spray) from a single insect should be enough for analysis by recently advanced capillary NMR techniques [Dossey *et al.* 2006].

Beetles and their pupae. When irritated, droplets of defensive secretions will often appear on the hairs of beetle pupae and are best observed with magnification. These droplets can be collected in a number of ways, including washing with solvent such as methanol or dichloromethane [Schroeder *et al.* 1998], using capillaries to suck the oily secretions off the hairs, or by full body extraction as described above. Although adult beetles can be extracted in their entirety, dissection of the intact glands [Eisner *et al.* 1968] followed by extraction might yield more material of higher purity than a full body extract.

Spiders and Scorpions. Both spiders and scorpions (Class Arachnida) have venom delivery systems. Glandular areas can be stimulated with a TAS with the collection vial placed for example, over the stinger of a scorpion so that venom is collected directly into the sample vial upon stimulation. A detailed apparatus to use in conjunction with the TAS has been described for the collection of scorpion venoms [du Plessis 2002]. Spiders can also be stimulated with a TAS while holding a glass capillary next to the venom glands to take up the venom as it is secreted [Schroeder *et al.* 2008]. Some scorpion, spider, and other venoms can be obtained from commercial sources as well.

4. CHEMICAL ANALYSIS—OVERVIEW FOR BIOLOGISTS AS IS RELEVANT TO COLLECTION TECHNIQUES

Herein is provided only a general overview of how field samples are prepared and analyzed in the laboratory to identify the chemical components of secretions and venoms. Please see reviews referenced in this section for more details.

4.1. Purification and Fractionation

Secretions collected using the TAS and methanol-laced Kimwipes[®] can be injected directly into a Gas Chromatography-Mass Spectrometer (GC-MS). Unlike the lethal full skin extraction/alkaloid fractionation method [Daly *et al.* 1994], no further workup is needed for TAS collections except for filtering off Kimwipes[®] for NMR as outlined below.

TAS secretion collection analyses. One of the great advantages of the TAS method is that no further sample preparation is needed to screen for small molecules (e.g., alkaloids) in amphibian skin secretion samples. That is, if the amphibian secretion was collected into a small volume of methanol, then a few microliters of this methanolic wiping can be injected directly into a GC-MS to yield results comparable to full skin alkaloid fractionation [Clark *et al.* 2005, 2006]. Avoid injecting aqueous (i.e., water-containing) samples onto GC-MS-- use organic solvents instead to maintain columns in good working order. If samples are to be analyzed by NMR, then all debris including Kimwipes[®] must first be filtered away.

Filtering out Kimwipes[®] for NMR spectroscopic analysis. Samples made by TASing and wiping subjects can also be prepared for NMR without separation or fractionation, but do need to be filtered. Here presented is a simple but detailed protocol to prepare skin wiping samples for NMR analysis. To make each filtered sample the following are needed: laboratory notebook, glass Pasteur pipettes, pipette bulbs, cotton, PTFE screw cap and corresponding glass vial, analytical grade methylene chloride (CH₂Cl₂) and absolute methanol (MeOH).

First, prepare glass Pasteur pipettes with cotton filters for each sample that requires filtering, and dispose of each '*filtering apparatus*' after use with a single sample. Use the tip of Pasteur pipette #1 to lodge a small piece of cotton into the bottom of Pasteur pipette #2 (i.e., the filtering apparatus).

Next, clean the cotton of factory residue by washing it with organic solvents. A Pasteur pipette fitted with a pipette bulb can be used to push three 2 mL portions of CH₂Cl₂ through the cotton filter. Finally, use another fresh pipette to push three 2 mL portions of absolute

alcohol (e.g., 100% MeOH or whatever solvent the sample was collected and stored in) through the cotton filter. These filters can be stored, but prior to use must be freshly 'wet' with the solvent that the original sample was collected in, which is methanol for this example sample.

The sample can be filtered through the cotton now, since the cotton filter is well washed and 'wet' with methanol. Put the filtering apparatus (pipette #2) into a fresh glass vial labeled with all information from the original sample tube *and* the date of filtering (i.e., date and method of filter preparation and filtering should be recorded in laboratory notebook).

Use Pasteur pipette tip #3 to vigorously mash up the Kimwipe[®] in the original collection tube or vial. Using Pasteur pipette #3, pipette the original sample with mashed-up-Kimwipe[®] into the top of the wet cotton filtering apparatus (pipette #2), and allow to drain into the labeled vial. After filtering all material into the labeled vial, then rinse out the original sample tube with about 2 mL methanol and filter this through the cotton as well—repeat this washing of the original sample tube with methanol two more times. Finally, squeeze the pipette bulb to force air through the filtering apparatus to remove any remaining alcohol. At this stage, the majority of any small molecules contained in your original collection tube should now be in the fresh, labeled glass vial with a Teflon-lined screw cap.

Finally, this vial's contents are dried in a rotary evaporator with a 28 °C bath or by blowing an inert gaseous stream of N₂ over the vial. To avoid bumping your solvent, do not fill the vial more than halfway. Deuterated solvent is added to transfer the dry vial's contents into a clean, dry NMR tube.

Frog skin alkaloid fractionation. If frog skins were collected, then they need to be prepared into alkaloid fractions for analysis by GC-MS. Alkaloid fractionation takes about three hours per sample and generates chemical waste that must be disposed of according to local and national laws. Any other sample components such as possible peptides would be removed during the alkaloid fractionation process. Instead of discarding the non-alkaloid-containing fractions, they can be stored at low temperature if there is interest in investigating other components such as peptides.

Daly and colleagues have outlined a frog skin alkaloid fractionation protocol for multi-skin collections [Daly *et al.* 1994] and for single-skin collections [Saporito *et al.* 2006]. Other fractionation methods have been reported with varying results and preparation times [Daly *et al.* 2008]. A summary of the primary method used by the Daly group follows, adapted for a single amphibian skin using a 28 °C bath instead of a 35 °C bath with the rotary evaporator.

Here outlined are steps to work up an individual frog skin into an alkaloid fraction:

1. Start by recording the wet weight of skin (i.e., on tared weighing paper that is then washed with methanol).
2. Mince the skin with scissors and mash it up using a glass mortar and pestle in 5 mL of methanol. Add 5 mL of deionized water and pour off liquid into a 125 mL separatory funnel. Repeat this step two more times to obtain a final volume of 30 mL methanol:water (1:1).
3. Add 15 mL of chloroform to the separatory funnel, and carefully rock back and forth. Keep your finger on the stopper at all times and release pressure often. Do not shake vigorously during this particular step because chloroform and methanol will mix to result in an emulsion that is difficult to break up (the addition of water to methanol helps to avoid this mixing). Gently tap the side of the funnel but do not add salt to break any possible emulsion.

4. Let any minor emulsions separate, and then drain off the chloroform (bottom layer) into a clean round bottom flask.
5. Repeat steps 3 and 4 twice more. The aqueous top layer can be discarded if only interested in alkaloids.
6. Evaporate the chloroform to a volume of about 3-5 mL (NOT dryness) using a rotary evaporator with 28 °C bath.
7. Add 8 mL of *n*-hexane to the concentrated chloroform extract, swirl, and pour into a clean separatory funnel. Repeat this step two more times.
8. Add 15 mL of 0.1 N HCl to the chloroform/*n*-hexane solution, and shake vigorously. Collect the upper layer into a clean beaker and return the lower layer to the funnel. Now repeat this step two more times to obtain three 15 mL upper layers. Discard the thrice extracted lower layer.
9. Add 2 N aqueous ammonia drop-wise to bring the solution to pH 9.0 (as indicated by litmus paper). Pour this basic solution into a clean separatory funnel.
10. Add 15 mL of chloroform to this basic solution, shake separatory funnel vigorously with venting, and drain bottom layer into a clean beaker. Repeat this step two more times to collect a total of 45 mL chloroform extract into the beaker.
11. Add anhydrous Na₂SO₄ to the combined chloroform extract to remove any residual water.
12. Pour 15 mL of the 'dry' chloroform extract into a small pear-shaped flask and evaporate to dryness (3x).
13. Add methanol to the pear-shaped flask to obtain a volume of 1 uL per 1 mg wet weight skin. To maximize material removed from the flask while still achieving this standard concentration, add half the total amount of desired methanol, briefly vortex, and then use a glass pipette to transfer the methanolic fraction to a well-labeled Teflon-lined screw-cap glass vial (leave the pipette in the vial). Add the remaining methanol to the pear-shaped flask and briefly vortex. Using the pipette left in the final sample vial, transfer the remaining methanolic fraction from the pear-shaped flask into the glass vial. Immediately store the sample in a freezer (e.g., -80 °C).

4.2. Spectroscopic Methods

The configuration and thus sensitivity of analytical instrumentation has been markedly enhanced in recent years. Further, the invention of PCR [Saiki *et al.* 1988] has opened up the field of protein study not only to the peptides themselves, but of their molecular organization as determined from cloning the full prepropeptides. Less natural material is needed to get better results than were possible with equipment from just 20 years ago, and the technologies are continuing to improve, most recently with the advent of Orbitrap technology [Perry *et al.* 2008].

Once a sample is fractionated, filtered, or otherwise prepared, then its chemical composition can be determined using a combination of analytical methods suitable for the type and amount of compound(s) of interest. In many cases, fractionation/separation occurs with a column (e.g., GC, LC) that is connected to a spectroscopic detector (e.g., UV, IR, MS, NMR). The detection of ions (e.g., total ion current via MS) or certain wavelengths (e.g., 280 nm absorbance via UV) generates a chromatogram with time as the X-axis and abundance or

absorbance as the Y-axis (Fig. 7). The chromatogram indicates the relative amounts and number of sample components; however, component detection is biased towards those compounds that can be ionized or can absorb light of chosen wavelengths.

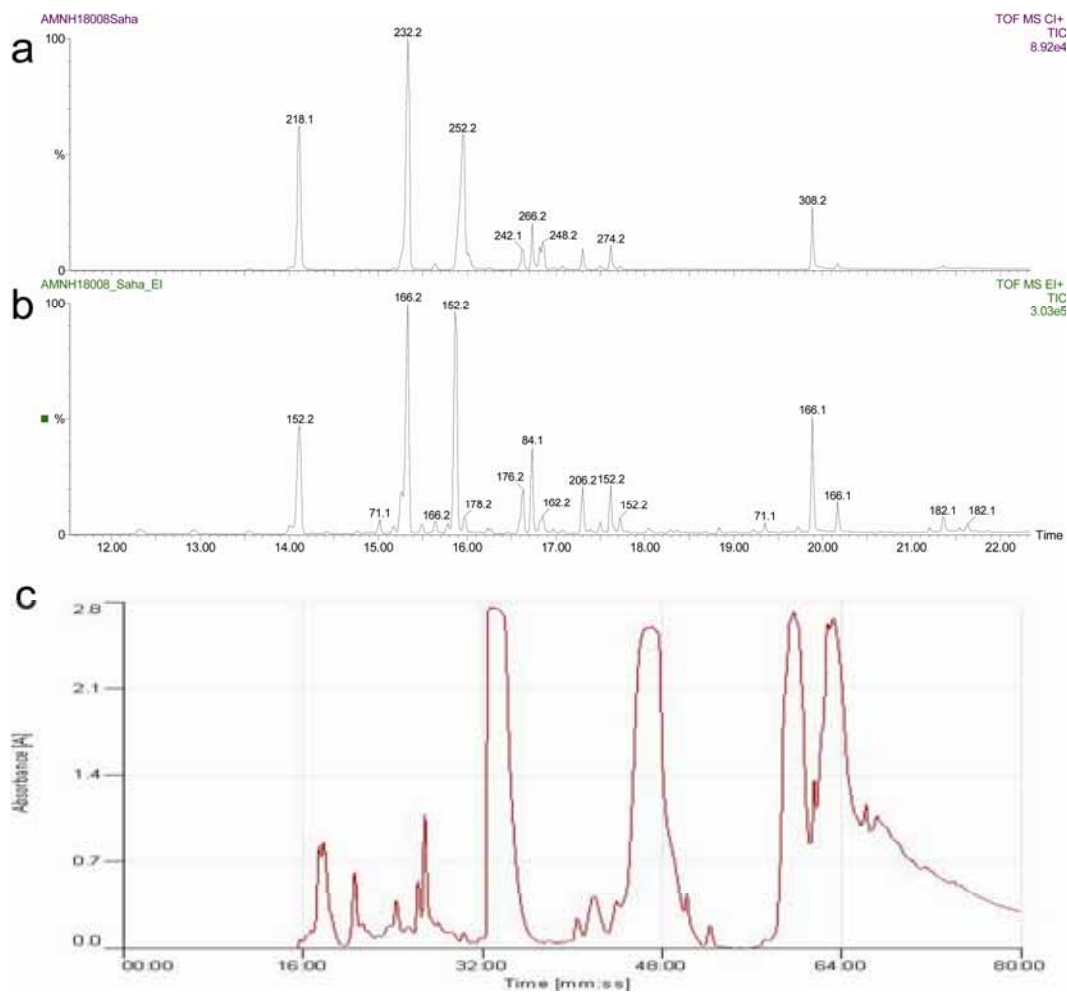


Figure 7. GC chromatograms show peaks corresponding to the abundance of ions (i.e., the Total Ion Current/Count) as detected by the mass spectrometer **a.** in chemical ionization (CI) mode with ammonia, and **b.** in electron impact (EI) mode. The most abundant ion of each peak is labeled, corresponding to alkaloid mass + 1 in CI, and to most abundant fragment ion (i.e., ‘base peak’) in EI. Data is from GC-TOF-MS runs of an alkaloid fraction of a single *Mantella baroni* poison frog from Ranomafana, Madagascar [Clark *et al.* 2005, 2006]. **c.** HPLC chromatogram resulting from 5 mg injection of *Typhlonectes compressicauda* lyophilized secretion shows peaks absorbing at 214 nm as detected by UV [Clark, Shaw, *et al.* unpublished data].

High Performance Liquid Chromatography (HPLC) is routinely used to separate small molecule and/or peptide samples into fractions using a gradient of solvents, or solvent ramp. The sample is pumped (e.g., at rate of 1 mL/min) through a column containing a solid material that binds sample components. The bound sample components are eluted off of this column (e.g., diphenyl, C-18, C-5, etc.) as the mobile phase (i.e., the solvent ramp) outcompetes the stationary phase (i.e., the solid material in the column). The presence of

compounds eluting from the column is monitored using ultra violet (UV) detection that results in a chromatogram showing peaks where compounds absorb light of a chosen wavelength (e.g., usually set at 214 nm and/or 280 nm). Fractions can be collected automatically at one minute intervals, but the UV chromatogram trace can also be watched in real time if seeking a particular peak of known retention time (e.g., for purification of a synthesized peptide). HPLC-UV is used for separation, but gives little clue as to the chemical composition of the sample. However, it does provide some measure (i.e., biased toward compounds that absorb programmed wavelengths) of the number of compounds in the sample via the number of peaks on the chromatogram.

Nuclear magnetic Resonance (NMR) Spectroscopy. The information gained from a combination of one dimensional and two dimensional NMR spectroscopic experiments is sufficient to elucidate new structures of small molecules [Dossey *et al.* 2007, Hutchinson *et al.* 2007, Clark *et al.* unpublished data] and configurations of peptides [reviewed in Pukala *et al.* 2006]. It is common to first do some kind of chromatographic separation and then dry down these purified fractions for NMR analysis [e.g., Clark *et al.* 2008]. However, certain 2D NMR experiments (e.g., NOESY) show how neighboring hydrogens of a compound interact/correlate with one another. This renders possible the analysis of unfractionated mixtures since different components in the mixture do not correlate with one another. Indeed, many unfractionated samples have been analyzed by 2D NMR to reveal new chemical structures [Schroeder *et al.* 1998, Ward *et al.* 2007]. However, after initial screening of a mixture by 2D NMR, the compound(s) of interest should be confirmed by MS, and if possible, chromatographically isolated as a pure fraction for NMR spectroscopic analysis.

To acquire data on limited amounts of natural samples, special NMR tubes with reduced volumes keep samples more concentrated. Whereas standard 5 mm NMR tubes require 0.75 mL of sample, Shigemi NMR tubes require a volume of only 0.3 mL and thus increase sample concentration. Shigemi tubes have a material in them that mimics the deuterated solvent and are available for different brands of spectrometer (e.g., Bruker, Varian) and solvent types. Capillary NMR tubes simply reduce the volume by using a specialized probe. Even if using reduced volume NMR tubes such as these, a far greater amount of material is needed for NMR experiments relative to MS experiments. However, after NMR data acquisition the sample remains intact and thus can be used for other spectroscopic analyses.

Mass Spectrometry (MS). Mass spectral (MS) analysis of any kind (e.g., GC-MS, LC-MS, FTMS, MALDI, etc.) is far more sensitive than any type of NMR spectroscopy and thus requires only picomoles or less of the compound of interest for detection. Gas Chromatography (GC) and Liquid Chromatography (LC) are used to separate sample components through a column, and upon elution from the column the separated sample components enter the MS detector. A great variety of compounds can be separated on LC columns with a solvent gradient/ramp as described above, whereas small molecules are separated on GC columns using a temperature ramp, usually from about 40 °C to 280 °C over 30-80 minutes. Samples can also be injected directly into a MS without first going through a column, for example, using electrospray ionization [ESI; Fenn *et al.* 1989].

There are several types of MS detectors/analyzers including classic quadrupole, time of flight (TOF), and Fourier Transform-Ion Cyclon Resonance (FT-ICR; Marshall *et al.* 1998]. Most recently Orbitrap FT technology has emerged as the mass analyzer with the as-yet greatest sensitivity and mass accuracy [Perry *et al.* 2008]. This technology can be used with a wide variety of injection methods (e.g., ESI, infusion, LC, etc.) suited for the sample in hand.

If studying peptides, then a Matrix-Assisted Laser Desorption Ionization (MALDI) spectrometer is typically used to analyze fractions obtained from an HPLC run. Example specifications for an investigation of peptide constitution in a lyophilized secretion sample are as follows. With a flow rate of 1 mL/min, peptides were eluted with a ramp of solvent from 0.5/99.5 (v/v) TFA/water to 0.5/19.5/80.0 (v/v/v) TFA/water/acetonitrile over 80 minutes, resulting in 80 fractions collected automatically at one minute intervals. To determine the molecular masses of peptides, 1 μ L of each fraction was mixed with 1 μ L of alpha-cyano-4-hydroxycinnamic acid matrix on a plate (Applied Biosystems) and analyzed in positive ion detection mode by Matrix-Assisted Laser Desorption/Ionization, Time-Of-Flight Mass Spectrometry (MALDI-TOF-MS) on a Voyager DE Mass Spectrometer (PerSeptive Biosystems, MA, USA). After this initial mass analysis, peptides that appear relatively pure as indicated by MALDI can be sequenced using automated Edman degradation. Such peptide investigations are currently underway using caecilian secretions [Clark *et al.* unpublished data].

The mass and fragmentation data gained from MS is best used to identify compounds that have been previously characterized. Further, any material subjected to MS detection is consumed and thus is not recoverable. Therefore, it is advisable to conduct MS analysis as the last step in solving any new structures, especially if there is a limited amount of sample.

InfraRed (IR) Spectroscopy. When coupled to a GC, IR can provide some clues about the structural configuration of volatile compounds such as low-molecular weight alkaloids. For example, the cis versus trans configuration of quinolizidines can be determined based on characteristic IR stretching frequencies known as ‘Bohlmann bands’ [Wolfe *et al.* 1978, Daly *et al.* 2005]. Experimental details and instrument specifications have been reported to use this method in the gas phase [Attygalle *et al.* 1994, Daly *et al.* 2005]. The advantage of this method is that very little material is consumed, whereas such determination of cis/trans configuration by NMR requires substantially more material.

4.3. Contamination— An Example from the Literature

Contamination. General guidelines to avoid contamination were outlined above. Here presented is a study wherein the experimental design potentially led to cross contamination, in which case that studies’ conclusions would require additional support for validation.

Certain mites from temperate habitats (e.g., Japan) have been clearly documented to contain some of the exact same alkaloids known to occur in skin of tropical poison frogs [Takeda *et al.* 2005]. However, the *extent* to which frog alkaloids are sequestered from mites remains unclear. Mixed arthropod collections were surveyed for alkaloids to reveal that certain mites and other arthropods (e.g., ants, beetle, a millipede) in a mixed collection shared common alkaloids [Saporito *et al.* 2007b]. The possibility that the various arthropods secreted chemicals on one another while together in a plastic bag for \approx 12 hours with no solvent or substrate was not suggested in that paper; rather, it was suggested that, for example, millipede spiropyrrrolizidines also occur in certain mites [See Table 3 in Saporito *et al.* 2007b]. The mixed arthropods contained in a single bag undoubtedly touched and possibly secreted their defensive chemicals onto one another. This would be expected to lead to cross-contamination, which could not be accounted for when the mixed arthropods were finally separated to species using forceps. Due to the potential for cross-contamination, this experimental design is not recommended since it could possibly lead to erroneous conclusions. Certain arthropods

could possibly have mite symbionts, which could also account for the pattern in alkaloids shared between mites and various arthropods. In any case, it does appear that mites are a dietary source of defensive alkaloids found in frog skin secretions and further studies on this topic are merited.

4.4. Artifacts

Artifacts can result from choice of solvent or tubes, and controls alone cannot always account for artifacts, since sometimes there is a reaction between sample components and solvent. For example, carboxylic acids may be converted to the corresponding methyl esters (-OMe) in the presence of methanol.

Peptide artifacts. An example is the peptide Glu(OMe)²-Litorin—in the original report [Anastasi *et al.* 1977] it was suggested that the addition of a methyl group to a glutamic acid residue could possibly be an artifact of peptide extraction in 80% methanol. Similar artifacts have been reported for peptides extracted into ethanol, for example Glu(OEt)¹⁸-caerin was detected in an ethanol based skin secretion sample of a *Litoria caerulea* treefrog [Chris Shaw, pers. comm.]. Additionally, peptides that are *O*-sulfated have been shown to desulfate after four weeks if stored in 0.1% trifluoroacetic acid [Chen & Shaw 2003]. Thus, the collection of peptides in deionized water as described above appears to yield results with the fewest artifacts.

Alkaloid artifacts. Alkaloids are best stored in absolute methanol to ease analysis by GC-MS—the more that alkaloid samples are handled and evaporated, the more severe the loss of low molecular weight volatile alkaloids and neutral compounds such as hydroquinone. Methyl or ethyl groups can add to acids to make esters, and thus may possibly represent an artifact of solvent choice.

Several artifacts in frog skin alkaloid fractions have been reported by Daly and his colleagues. Silicone polymers and dibutyl phthalate were detected in poison frog skin samples by GC-MS, and appeared to be the result of poor choice of collection tubes-- silicone rubber O-rings or any rubber should be avoided when choosing collection tubes [Daly *et al.* 2008]. Fatty acid methyl esters (FAME) were documented in many samples throughout Daly's 50 years of frog alkaloid research [Daly *et al.* 2005, 2008]. Other possible artifacts include degradation artifacts [e.g., **257B** and **285D** in Myers & Daly 1980] and the *O*-methyl esters [e.g., pumiliotoxin **321A** in Daly *et al.* 2005].

5. CONCLUSION

To understand the chemical ecology of an organism or ecological system, integrated techniques ranging from careful sample collection and analyses to behavioral and pharmacological assays are needed. Herein were outlined several protocols to obtain uncontaminated secretion samples that are resistant to degradation. In general, compounds involved in chemical defense and communication can be obtained by targeted milking of glands, and thus do not require specimen sacrifice. In particular, the effectiveness of the TAS and/or hassle-bag technique for collecting amphibian secretions has been demonstrated. By establishing mutually beneficial, long-term collaborations with scientists and local communities in tropical countries, the goal of habitat and biodiversity conservation becomes more tractable.

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8. APPENDIX: EXAMPLE PACKING LIST FOR TROPICAL FIELD WORK

Personal Supplies for Carry-on

Passport, faculty/student ID, VISA, plane tickets	Yellow vaccinations card
CASH (split up storage locations)	Credit card (Visa, MC)
Letter from collaborators	Pocket knife (packed baggage!)
Hair ties	Bandana
Camera: batteries, charger, memory cards, desiccant in hose, outlet adapters, accessories	
Computer and charger (netbook only)	CD or DVD of PDFs for team
USB memory chip with presentation, PDFs, etc.	Electrical converter set
Addresses for postcards	Medications, personal

Camping

Personal

Toothbrush, toothpaste, and floss	Hairbrush, comb
Biodegradable shampoo and conditioner	Dr. Bronner's
Hennessey Hammock, ropes and/or Tent	Thermarest+ Fleece Bag
Compact pillow	Dry bags (2 personal)
Rubber boots with inserts (clean with bleach!)	Sandals/flip-flops
Wool socks	3 Zip-off nylon pants
2 long sleeve nylon shirts	Nice shirt for city meetings
2 short sleeve/tanks	2 sportsbras, 2 regular
Fleece shirt and pants for night	14+ pairs underwear
Quick-dry towel, large	swimsuits
Tarp-poncho	Rainsuit
Water bottle	Headlamp, bulbs, batteries
Field and personal Rite-in-the-Rain [®] Level Notebooks	Pens

Medical

Malarone and another antimalarial	Codeine
Ciprofloxacin and another antibiotic	ibuprofen-Advil
First aid kit(s) with bandages	Feminine products x 3 types
Insect repellents	anti-fungals (pill and creams)
Syringes (they are sometimes reused in remote areas!)	Baby powder, rash cream
Clean wipes (for days with no bathing!)	Moleskin, molefoam, and tape
Sunblock	Antibiotic cream (Neosporin)

Team Supplies (Camping, Food, Clothes, Etc)

Hard plastic box for organizing things	Zip-top bags, many sizes
BOOKS for donation (take as carry-on)	Extra hammock with mesh
Dry bags	5 warm long sleeve shirts (donate)
5 ponchos, one rainsuit (donate)	Tarps x 3 (might buy in-country)
10 plates, cups, and fork/knife sets (lightweight)	Power bars
NUTS x10 portions	Gatorade
mini-shovel	Biodegradable toilet paper
Water bag (NUNC) for team supply after boiling	2 fleece blankets
2+ lightweight quick-dry towels for kitchen	biodegradable camp soap (dishes)
2 duffel bags for porters, team gear	large backpack for sci equipment

Scientific Supplies

General

GPS, batteries and accessories	White tray for sorting arthropods
Mini-Winklers and litter-sifter	Whirl sacs
10 pairs entomological forceps	White sheet
Thermometer/hygrometer	Machete & rake
Microscale (hanging)	blue ruler
Headlamps, spare bulbs	AA, AAA, 9V batteries
Zip-top bags	Hand lens
LABPAL™ label printer	Field photos of frog targets
Field level notebooks for team	Pens for team

Chemical

Ethanol, methanol, chloroform, etc. to be shipped	Dilute acid (e.g., 1% TFA)
Nalgene containers with millipore filtered water	Eppendorf tubes (15 and 50 mL)
0.6 mL and 1.5 mL microcentrifuge tubes	Glass vials with Teflon-lined caps
Boxes for organizing tubes	Dividers for boxes
Glass eye droppers, disposable plastic pipettes	Permanent markers
Tweezers and small scissors	Kimwipes®
Transcutaneous Amphibian Stimulator, tools, case	Parafilm