wetlands where roofing had been in use showed that they did not contain any petroleum products.

Surface sheens are common in many wetlands where high inputs of detrital carbon generate surface-active compounds that accumulate as a thin film at the air-water interface. Humic substances, lipids, and proteins originating from higher plants and microorganisms have been shown to be components of the film (Mills et al. 1996).

In conclusion, roofing material has several logistical advantages for use in field studies in which coverboards are suitable. The finding that no chemical hazard is created for the animals that use roofing material for shelter or that are present in surrounding waters adds value to its use as a monitoring technique in some situations.

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Transcutaneous Amphibian Stimulator (TAS): A Device for the Collection of Amphibian Skin Secretions

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Amphibians, despite their vulnerable appearance, possess a veritable armamentarium of integumental defenses against both vertebrate and invertebrate predators. Chief among these are skin secretions, the major components of which have long intrigued biologists, chemists, and pharmacists alike. Researchers have employed a variety of methods to harvest skin secretions, some more invasive than others. Among the more invasive techniques are the injection of neurotransmitters (Barthalmus 1994; Bols et al. 1986) and whole skin extraction (Daly 1998; Das et al. 2000; Morikawa et al. 1992; Roseghini et al. 1976; Roseghini et al. 1986; Roseghini et al. 1988; Zasloff 1987). Whole-skin extraction (the removal and maceration of an entire amphibian skin) has two major disadvantages: introduction of contaminants to the sample and fatal outcome for the amphibian. Contaminants introduced by macerating whole skins can complicate sample cleanup, while the current worldwide decline in amphibian populations demands that they be treated as non-renewable resources. Fortunately, amphibian skin has an anatomy that permits simple harvest of its secretory products without injury to the animal itself.

Amphibian skin contains both mucous and granular glands (Pough et al. 2001). Granular glands produce the defensive compounds of interest. Each granular gland is encircled by a muscle responsive to electrical stimuli. Such stimulation results in ejection of the glandular contents onto the skin's surface, where they are readily collected. Researchers have used this technique to advantage and claim to have obtained results comparable to those of the whole-skin technique (Tyler et al. 1992).

Although others have used this electro-stimulation technique, they have relied upon commercially available but expensive devices (Tyler et al. 1992), or have not specified what equipment was used (Barteczko and Kuziemski 1970; Dockray and Hopkins 1975; Flucher et al. 1986; Lábler et al. 1968). We present here for the first time instructions for the assembly of a Transcutaneous Amphibian Stimulator (TAS), an affordable alternative to commercially available acupuncture units. It is a small handheld unit that is easily adapted for an individual researcher's needs in the field or in the laboratory. Powered by two 9-volt batteries, the TAS is an excellent device for collecting amphibian epidermal granular gland products without causing harm to the amphibian.

The TAS stimulation parameters were based upon those of the modified acupuncture unit used by Tyler et al. (1992). Pulsewidth of the stimulus is set at 2.5 or 5 msec, with a single-pole/double-

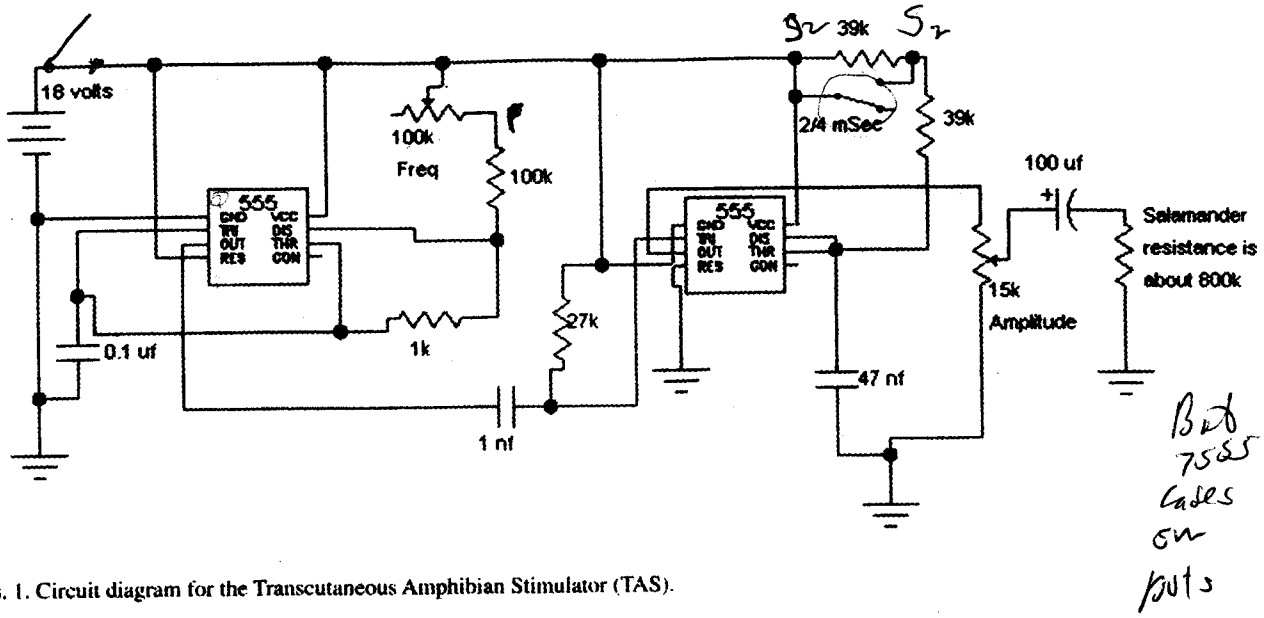

FIG. 1. Circuit diagram for the Transcutaneous Amphibian Stimulator (TAS).

throw switch, in conjunction with an ICM7555 timer in the monostable operation mode. A 15 kOhm potentiometer permits adjustment of the voltage from 0 to 18 volts. Frequency is adjusted within the range of 60 to 130 Hertz by use of a 100 kOhm potentiometer combined with an ICM7555 timer in the astable operation mode. The stimulus is harmlessly delivered by silver-silver chloride disk-electrodes (4.0 mm or 8.0 mm diameter; Warner Instrument Corporation, Hamden, Connecticut, USA). This type of electrode is ideal because of its lack of toxicity and non-abrasive surface. A less expensive alternative is the bare silver wire electrode, but it entails the risk, if of too narrow gauge, of perforating the amphibian's skin. Standard binding posts connect either type of electrode to the circuit. The TAS circuit can be built for approximately US $100 with commercially available parts (Mouser Electronics, Inc., Ottawa, Canada) by following the circuit diagram provided (Fig. 1). We housed our completed circuit in an 80 mm x 160 mm x 60 mm ROLEC watertight polycarbonate transparent Techno Case (Hammond Manufacturing, available through Mouser Electronics, Inc., Ottawa, Canada). Watertight cases with rubber O-ring-sealed fittings are recommended for fieldwork, but cheaper, non-watertight cases may be substituted for laboratory purposes.

Extraction of skin secretions is accomplished by first lightly moistening the animal with deionized water then gently massaging its skin with the activated TAS electrodes for 10–20 seconds (Fig. 2A). For our research we adapted the technique for the animals at hand, massaging anurans (with the exception of *Bufo americanus*) laterally and dorsally from neck to thigh; *Bufo americanus* directly on parotoid glands (Fig. 2B, showing production of the milky white secretion); and urodeles along base of tail. Coincident with stimulation, the animal is rinsed with deionized water. The aqueous solution is collected in a vessel held beneath the animal. Preparation of the secretion at this point varies with the intended purpose of the extraction.

The technique has any number of applications: the determination of evolutionary trends within different populations of a single species (Steinborner et al. 1996a; Steinborner et al. 1996b), differentiation of morphologically indistinguishable species (Daly 1998), in biochemical taxonomy (Cei et al. 1967; Cei et al. 1968; Cei et al. 1972), in pharmacology (Roseghini et al. 1986), in medi-

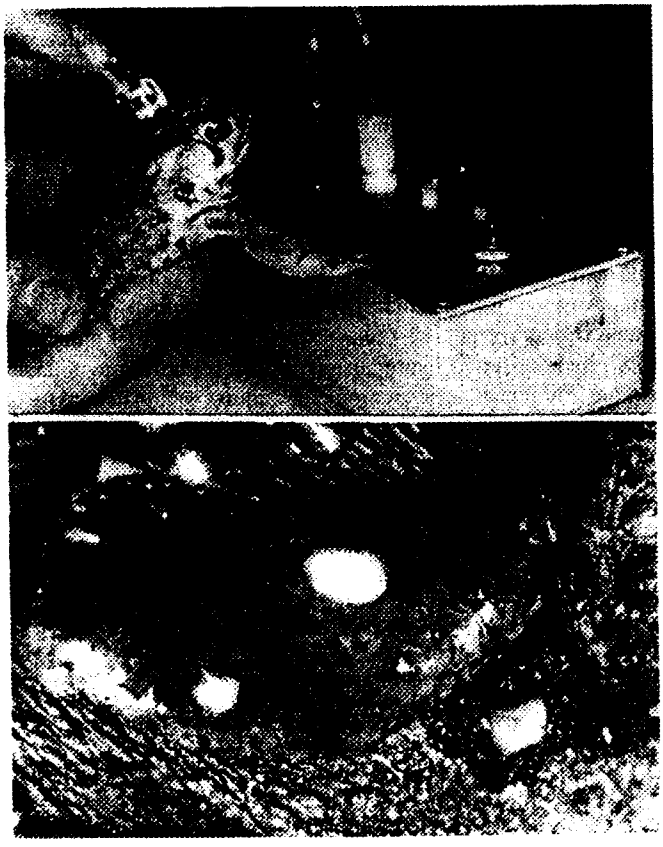

FIG. 2. A. The Transcutaneous Amphibian Stimulator (TAS) and a study subject (*Bufo americanus*). B. TAS-elicited defensive secretion (milky-white spots) from the parotoid gland of *B. americanus*. Photos by Tom Eisner.

cine (Barteczko and Kuziemski 1970; Bradford et al. 1996; Zasloff 1987), in chemistry and chemical ecology (Clarke 1997; Daly et al. 1987), and in behavioral studies including pheromonal (Wabnitz et al. 2000) and predator-prey interactions (Barthalmus and Zielinski 1988; Barthalmus 1994, Brodie 1983; Brodie and Gibson 1969). The TAS has also been used in our lab to study the production and chemical composition of amphibian-produced volatile compounds (unpublished data).

In our current studies of amphibian chemical ecology, we have used the TAS to collect secretions from the following species: *Ambystoma maculatum, A. tigrinum, A. jeffersonianum* complex, *B. americanus, Hyla chrysoscelis, H. gratiosa, Osteopilus septentrionalis, Plethodon cinereus, P. glutinosus, Rana catesbeiana, R. clamitans clamitans, R. c. melanota, R. pipiens, R. palustris, R. septentrionalis, R. sylvatica,* and *Scaphiopus holbrookii*. We have found that smaller amphibians require <10 V, <75 Hz, and 2.5 msec pulsewidths for induction of glandular emission, while larger animals require >10 V, >60 Hz, and 5 msec pulsewidths. No species required the use of >15 V to elicit granular gland discharge. Despite their size, ambystomatids require minimal settings (<10 V, 60 Hz, 2.5 msec) to elicit appropriate granular gland response. Among the species under study in this lab, secretory yields ranged from 10 to 100 mg per animal with the exception of the ambystomatids, which produced copious amounts (in excess of 200 mg per animal).

In conclusion, the TAS is a portable and inexpensive device for "milking" amphibians of their skin secretions. By being minimally harmful to the test organism, it is a distinct improvement over alternative techniques relying on whole-skin analysis or injection of epinephrine or acetylcholine. It is an ideal technique in chemical studies because the samples collected are relatively free of impurities and therefore in a condition requiring minimal preparation (filtration) prior to chromatographic and spectral analysis.

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HERPETOLOGICAL HUSBANDRY

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Minimizing Fungal Invasion During the Artificial Incubation of Sea Turtle Eggs

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The artificial incubation of sea turtle eggs has become increasingly common for research purposes. It usually involves the collection of eggs, transport to a laboratory (potentially long distance), then incubation within a container and/or incubator on sand or an artificial substrate such as vermiculite. During incubation it may be necessary to inspect eggs to monitor development and mortality, and maintain moisture conditions.

At all stages during these procedures, eggs are exposed to infectants which have the potential to kill a proportion, or all, of the eggs. To minimize egg mortality, precautions can be taken to reduce egg exposure to microbiota.

Collection.—Collecting eggs directly from the ovipositor minimizes their exposure to fungal spores dispersed during the turtle's body-pitting and egg-chambering. Eggs may be caught by a gloved hand placed in the rear of the egg chamber (part of the chamber may need to be widened). Though some species are more tolerant than others, care must be taken not to disturb the turtle by use of excessive light, or by touching the ovipositor and hind flippers. If eggs cannot be collected during oviposition, they may be excavated after laying has concluded, but this method increases contact with soil microbiota. Some workers have attempted to place a collection bag in the egg chamber during oviposition. This often disturbs the turtle, resulting in nest abandonment, collapse of the egg chamber during bag insertion, and difficulty removing it when full. This method may be more successful with species that do not dig a deep body pit and/or on beaches with relatively moist sand, where the rear of the chamber may be excavated completely for easier insertion and retrieval.

Eggs should be placed directly into sterile bags; autoclave disposal bags (e.g., Sarstedt®) are recommended because of their strength. Placing the bag inside a bucket helps support the weight of the eggs and prevents weakening and tearing of the bag under stress. The neck of the bag should be twisted and folded over before securing. Prior to transport or incubation, eggs can be washed in sterile distilled water or a solution such as Aricide® (Hibberd 1996) to remove microbes from the egg exterior. However, washing removes the cloacal mucous and its potential anti-fungal properties (Phillott, unpubl.). After washing, the exterior of the egg should be patted dry using a clean disposable cloth to remove excess water and prevent ice-crystal formation and disruption of the shell structure during low-temperature transport.

Transport.—Eggs may be transported long distances by following the procedures of Harry and Limpus (1989). Eggs depressed to 7–10°C (within 2 h of oviposition) may be held for 48 h, allowing collection of multiple clutches over several nights and subsequent transport. It is recommended that eggs remain in the collection bags during this time to minimize exposure to microbes. The bags should be arranged so that they are stable, and air spaces filled with clean, expanded polystyrene pellets for support and insulation.

Handling.—To minimize movement induced mortality, egg orientation should be maintained (Limpus et al. 1979; Parmenter 1980). Single-use sterile gloves should be worn, and eggs handled in a room or area with minimal air flow or disturbance. If the area is to be used permanently for incubation purposes, a dual door system with an intermediate isolation area minimizes air disturbance during entry and exit. Workers must wait in the isolation area until the first door has completely closed before opening the second.

Incubation.—Prior to egg collection, incubators should be cleaned with a 5% sodium hypochlorite bleach solution, then rinsed with a 5% solution of disinfectant. Incubation containers (which will hold the eggs) may be sterilized by autoclaving at 121°C for 15 mins with the mouth covered in aluminium foil, or with a 5% bleach solution followed by a sterile water rinse.

If incubating on sand, it should be collected from areas relatively free of organic material. Sand can be sterilized by autoclaving in small lots. Thermal sentinels (e.g., Thermalog® S strips) should be used to ensure effective heat sterilization at the core. These checks can be ceased when the performance of particular autoclaves, sand types, etc., is quantified.

Labels on cardboard or other biodegradable material should not be placed in or on the substrate during incubation as they provide nutrient sources for mycobiota. Instead, the exterior of the container should be labeled. The required moisture conditions are maintained by adding sterile water either from clean spray bottles, or by use of a sterile water, sub-surface trickle irrigator (as described by Phillott and Parmenter 2001). If the plastic tubing from the latter is to be re-used, it should be first be cleaned with a commercial algaecide (used in cleaning swimming pools) then rinsed with sterile water.

When removing eggs (to weigh, measure, candle, etc.) wear sterile, single-use gloves and ensure the equipment is clean and that air flow around the area is minimal. If eggs are to be weighed, sand can be removed using a soft brush that will not damage the eggshell. Using a cloth to remove sand has the potential to drag sand across the egg surface, disrupting structural integrity of the eggshell. Eggs that fail to develop a white spot, that show signs of yellowing, or have fungal growth on the exterior should be re-