Placement of Intracoelomic Radiotransmitters and Silicone Passive Sampling Devices in Northern Leopard Frogs (*Lithobates pipiens*)

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Abstract: Historically, wetland toxin exposure studies have relied on single time point samples from stationary sampling devices. Development of passive sampling devices (PSDs) that can be attached to individual animals within wetland habitats has greatly improved in recent years, presenting an innovative sampling technology that can potentially yield individual-specific, quantifiable data about chemical exposure. In this study, silicone based PSDs were attached to the ventral skin of 20 northern leopard frogs (*Lithobates pipiens*) with polypropylene sutures after radiotransmitters had been surgically implanted into the coleomic cavity. After a short recovery period, frogs were released back into the wetland habitat where they were acquired. The animals were located daily using radiotelemetry to assess how long PSDs would remain attached in the frogs' natural habitat. After one week, PSDs remained on 18 of the original 20 frogs. At two weeks, 17 frogs were recovered and no PSDs remained attached. Although valuable data can be obtained over a short time period, more research will be necessary to demonstrate the effectiveness of externally attaching silicone PSDs to northern leopard frogs for time periods longer than 1–2 weeks.

Key Words: Northern leopard frog, *Lithobates pipiens*, passive sampling, pesticides, radio transmitter, silicone.

Introduction

Amphibians are excellent sentinels for contaminant exposure in prairie wetlands because of the unique characteristics of their integument and distinctive aquatic and terrestrial life cycles (Bernanke and Köhler, 2009; Papoulias *et al*., 2013). Historically, research on the exposure of amphibians to contaminants focused on the acute lethality of pesticides (Hall and Henry, 1992). Recent research has largely focused on the sublethal effects of agricultural/industrial chemicals on amphibian growth, development, immune function, reproduction, and behavior (Carey and Bryant, 1995; Davidson, 2004; Fellers *et al*., 2004; Mann *et al*., 2009). However, chemicals into which anurans and other amphibians are coming directly into contact have not been extensively investigated. Chemical exposure inferred from single time points or stationary samplers may not provide the level of accuracy that could be provided from sampling methods incorporating lengthier exposure history of individual animals (Sriyaraj and Shutes, 2001; Eimers *et al*., 2008; Smalling *et al*., 2013, 2015).

External attachment of sampling devices is a potentially useful approach for measuring exposure to environmental contaminants, but there are numerous challenges associated with both the effectiveness of attachment and potential effects on behavior and health of the animal. Previous amphibian studies have tested harnesses for external attachment of radiotransmitters, but these studies reported mixed success, and cutaneous ulcerative lesions were common (Bartelt and Peterson, 2000; Goldberg *et al*., 2002; Muths, 2003; Weick *et al*., 2005). Passive sampling devices (PSDs) are materials that sequester organic molecules through passive diffusion (Kot *et al*., 2000). PSD technology has greatly improved in recent years, and analysis of small pieces of PSD can be assessed for hundreds of different chemical exposures (Namieśnik *et al*., 2005). Materials used for PSDs vary widely (Tommasino, 1998; Namieśnik *et al*., 2005; Bohlin *et al*., 2010). One such material is silicone, which has been shown to absorb a wide range of compounds in the field. Because of its light weight and flexibility, it has potential to be attached to amphibian skin (Seethapathy and Górecki, 2012; Allan *et al*., 2013; O'Connell *et al*., 2014).

We hypothesized that silicone PSDs could be attached externally to the ventral skin of northern leopard frogs (*Lithobates pipiens*) via polypropylene sutures and use intracoelomic radiotransmitters to track animals to recover the PSDs. Our hope was that the PSDs would stay attached to the animals for up to four weeks, making sample collection financially feasible for chemical exposure analysis. To test this hypothesis, we aseptically implanted intracoelomic radiotransmitters and externally

attached silicone PSDs to 20 northern leopard frogs. If the silicone PSD stayed attached to the frogs for greater than four weeks, then our group would assess the PSDs for chemical exposure in their native habitat.

Materials and Methods

Animals: Adult northern leopard frogs ($N = 20$) were collected using dip nets from two constructed wetlands surrounded by agricultural land in north central Iowa. Body weights prior to surgery ranged from 20 to 47 grams (average = 29.3 grams) and snout to vent lengths ranged from 55.8 to 81.2 mm (average = 64.6 mm). Animals were collected over a two week period. Frogs were housed in 41.9 × 55.9 × 33.1 cm clear plastic enclosures with moist coconut fiber substrate (Eco Earth® Coconut Fiber Substrate, Zoo Med Laboratories Inc., San Luis Obispo, CA) and screen netting tops until the surgical procedures could be completed. Enclosures were kept at 21.7°C (71.1°F) and were misted with deionized water twice daily. Frogs were fed earthworms ad libitum. All frogs were determined to be healthy prior to surgery based upon a complete physical examination. Body condition scores ranged from 2–3/5. The frogs were weighed on the initial capture date, postsurgery, and when retrieved from the field at one and two weeks post release. This study was approved by the Iowa State University IACUC committee (protocol 3-15-7989-D) and the U.S. Geological Survey (FORT IACUC 2016-07).

Anesthesia: Frogs were fasted for 2 hours prior to being anesthetized with 1 g/L tricaine methanesulfonate (Finquel® or MS-222, Argent Chemical Laboratories, Redmond, WA) in 1 liter of lactated Ringer's solution (Lactated Ringer's Irrigation, Baxter Healthcare Corporation, Deerfield, IL) maintained at 21–23°C (69.8–73.4°F). The mixture was buffered to a pH of 7.3–7.4 using sodium bicarbonate (Sodium bicarbonate, Major Pharmaceuticals, Livonia, MI). The anesthetic solution was aerated with a portable 2 speed aerator and oxygen stone (Two Speed Aerator, Promar Nets, Gardena, CA). A surgical plane of anesthesia was determined by the loss of the righting and corneal reflexes and cessation of gular movement (Gentz, 2007). Pain response was assessed and monitored by deep pain response to a toe pinch with mosquito hemostats covered in white surgical tape prior to surgery as well as response (i.e., movement, elevations in heart rate or respiratory rate) to the surgical procedure (Gentz, 2007). Cardiac impulses were monitored using a Doppler Flow Detector (Parks Medical Electronics Inc., Aloha OR). Frogs under anesthesia were monitored via Doppler and pulse oximetry (OxiMax N-65, Nellcor Puritan Bennett, Minneapolis, MN) throughout the procedure. After the surgical procedures were completed, frogs were recovered in clean and aerated lactated Ringer's solution maintained at 23–24°C (73.2–75.4°F). Frogs were returned to their home enclosures after gular movement and righting reflexes were observed.

Radiotransmitter placement: Radiotransmitters (17 × 8.5 × 5.5 mm, 1.8g; Holohil BD-2H, Holohil Systems Ltd., Carp, Ontario, Canada) were first sterilized by immersion in 70% ethanol for 3 min, dried with sterile Kimwipes (Kimwipes, Kimtech-Kimberly Clark Professional, Roswell GA), and were rinsed with sterile saline. Frogs were positioned in dorsal recumbency on two sponges soaked with lactated Ringer's solution once a surgical level of anesthesia was obtained. A surgical preparation was made with 0.75% chlorhexidine solution applied with cotton tip applicators and the solution was rinsed with sterile saline (Gentz, 2007). Sterile powder-free surgical gloves (DermAssist™, Innovative Healthcare Corporation, Sand Springs, OK) were first wetted with sterile saline to prevent desiccation of handled tissue. A 1 cm paramedian incision was made 5 mm right lateral of midline through the skin and then through the muscle layers and coelomic membrane using a number 15 blade. Once the coelomic cavity was visible, a sterile radio transmitter was inserted (Fig. 1). Hemorrhage was considered minimal, and pressure with cotton tip applicators provided sufficient hemostasis. The muscle layer was closed using 5-0 polydioxanone (PDS II, Ethicon, Somerville, NJ) in a simple interrupted suture pattern. The cutaneous layer was closed using 4-0 polypropylene (Prolene, Ethicon, Somerville, NJ) in a simple continuous suture pattern.

Figure 1. Placement of a radiotransmitter into the coelomic cavity of a northern leopard frog (*Lithobates pipiens*) (Photo courtesy Lindsey Yaw).

Silicone passive sampling device (PSD) attachment: Triangular shapes, approximately 1.5 cm in length by 0.1 cm thick, were cut from thin silicone sheets (NSF 51 Compliant Silicone Rubber, Rubber Sheet Roll, Shippensburg, PA) for use as PSDs. PSDs were cleaned via exchanges in ethyl acetate/hexane and ethyl acetate/methanol as described by O'Connell (2014) two days prior to surgery. PSDs were allowed to dry and were rinsed in sterile saline on the day of surgery prior to attachment to frogs. Immediately after radiotransmitters had been surgically implanted in the coelomic cavity, PSDs were sutured to the ventral skin using 4-0 polypropylene placed as four simple interrupted sutures in an inverted central triangle with the two cranial corners located at the caudal aspect of the axilla (Fig. 2). PSD weight (less than 0.2 grams) plus the weight of the radiotransmitter (1.8 grams) were determined to be less than the recommended 10% of the frog's body weight (Richards *et al*., 1994).

Recovery, release, and monitoring: Meloxicam (Metacam, 0.5 mg/ml [diluted to 0.15 mg/mL], Boehringer Ingelheim Vetmedica, St. Joseph, MO) was administered at 0.4 mg/kg orally every 24 h for two treatments postsurgery. Frogs were held for 48 h and were visually inspected every 12 h before being released back into their native wetland. After release, frogs were located daily by radiotelemetry, and visual examinations with binoculars were performed when possible (Fig. 3). All frogs were to be captured at 7, 14, 21, and 28 days to visually

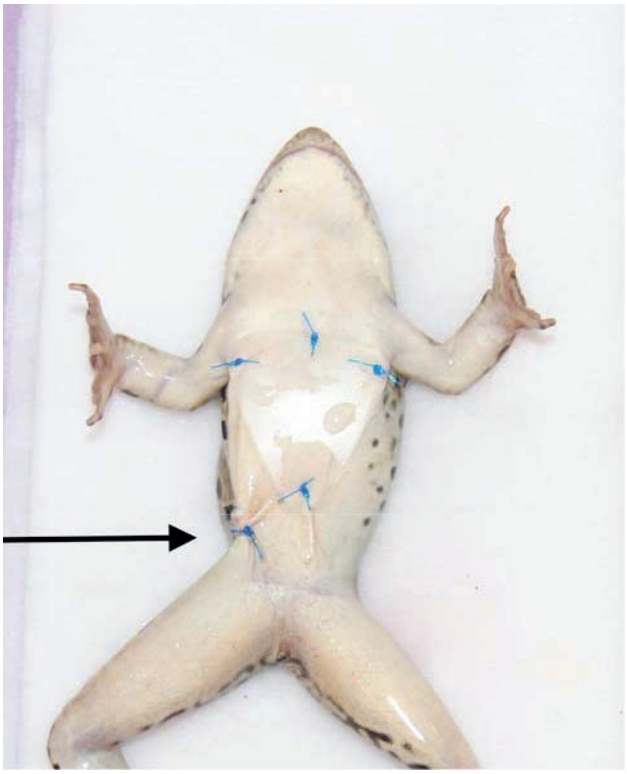

Figure 2. Location of attachment of a silicone passive sampling device (PSD) to the ventral aspect of a northern leopard frog (*Lithobates pipiens*). Note small paramedian surgical site for placement of intracoelomic radiotransmitter (arrow) (Photo Courtesy of Lindsey Yaw).

assess the sites of their transmitter implantations and PSD attachment. At the end of four weeks, the frogs were to be recaptured for collection of the PSDs for sample analysis, and euthanasia was to follow to collect the radiotransmitters. Full necropsies were to be performed to assess cutaneous lesions associated with PSD placement.

Results

There were no surgical complications encountered during this project. Time to appropriate anesthesia was highly variable between individual frogs but ranged between 10 and 25 min (mean of 15 ± 4 min). Surgical times for transmitter placement and PSD attachment ranged from 7–10 min. All frogs recovered uneventfully, but recovery times varied from 20–40 min (mean of 32 ± 7 min). Each frog was observed eating earthworms within 24 h of surgery, displayed behavior consistent with pre-surgical behaviors, had no signs of lameness, and had baseline parameters (heart rate and respiratory rate) that were consistent with presurgical levels.

No adverse effects or changes in behavior were observed during the 48 h after surgical placement of the radiotransmitters and PSDs. Incision sites for implantation of radiotransmitters appeared to be healing appropriately. A moderate amount of coconut fiber fragments accumulated between the PSDs and the ventral skin of the frogs in the enclosures, but it was easily washed away with deionized water before release. At the time of release, all sutures were intact.

Because of vegetation and location of some frogs underwater, not all frogs were observed every day. Further, during the second weekly collection, one frog was not located with radiotracking. The animal search commenced for an 8 h period but was discontinued at that time. It is unknown whether this animal's radiotransmitter failed to work or whether the transmitter was destroyed.

One frog (5%) was found dead on the third day after release; suture failure at the transmitter implantation incision was the probable cause. One week after release, all 19 remaining frogs were recaptured for visual assessment of surgical wounds, sutures, and PSDs. PSDs were attached to all 19 frogs (Fig. 4), and surgical sites appeared to be healing well other than a minor amount of erythema around the incision sites. The PSD on one

Figure 3. Attempts were made to visualize frogs daily with the use of radiotelemetry (Photo courtesy of Clay Pierce).

Figure 4. Northern leopard frog (*Lithobates pipiens*) observed seven days after release following placement of intracoelomic radiotransmitter and external PSD. Radiotransmitter implantation incision and PSD are clearly visible and sutures all appear intact (Photo courtesy of Clay Pierce).

Figure 5. Northern leopard frog (*Lithobates pipiens*) observed 14 days after release following placement of intracoelomic radiotransmitter and external PSD. Radiotransmitter implantation incision is clearly visible and appears to be healing well. PSD is no longer attached (Photo courtesy of Clay Pierce).

frog had several single suture failures of unknown cause and were found dangling from the ventral skin of the frog.

Two weeks after initial release, 17 of the remaining 19 frogs (89%) were located alive. One animal (5%) was found dead with its PSD attached. One frog could not be located with radiotelemetry. The dead frog was too autolyzed to conduct a necropsy or evaluate the status of the sutures. None of the recaptured frogs had PSDs attached at two weeks, and it appeared that all the sutures had gone through complete marsupialization or had torn out at that time (Fig. 5). All of the frogs had gained weight since the surgery and appeared to be in good condition with no major cutaneous wounds. At five weeks, a subset of the remaining frogs were recaptured and were humanely euthanized (N = 8). Whole bodies were submitted for analysis for chemical exposure. Necropsies were not performed at that time for assessment of potential cutaneous reactions to the PSDs.

Discussion

There were no visual cutaneous lesions or ulcerations observed in the 19 of 20 (95%) frogs that retained their PSDs during the first week of this study. This suggests an advantage of sutures for external attachment of PSDs over harnesses, at least for short periods of 1–2 weeks. Ulcerations and erythema are commonly associated with external harnesses and attributed to the difficulty of determining how tight to attach the harness (Goldberg *et al*., 2002; Weick *et al*., 2005). Harnesses attached loosely allow frogs to shed them, but if the harness is too tight, it can restrict the frog's mobility, causing blood pooling in extremities (Bartelt and Peterson, 2000). One clear advantage the harness apparatus has over suturing of PSD devices is the ability to stay on the animal for longer periods of time. Previous studies have reported harnesses staying on various amphibian species between 2 and 9 weeks (Goldberg *et al*., 2002; Bartelt and Peterson, 2000; Weick *et al*., 2005). Future development of silicone based harnesses that can serve as PSDs is warranted.

One animal was found dead three days after release, and the cause of death was determined to be suture failure. The animal's coelomic entry site was open to the environment, and there was no suture present in the skin. Because of severe autolysis, it was difficult to determine whether the suture had been torn from the skin or whether the suture had fallen out (suggestive of failed knot security). Study site restrictions did not allow for the animals to be held for multiple days to heal from the coeliotomy in the current study. An alternative protocol would allow a period of recovery after transmitter implantation during which wound healing could be monitored. This alternative approach should be considered in future studies. However, if PSDs are attached with suture, a second anesthetic event would be required to attach the PSD closer to the release date to maximize attachment time in the environment.

Polypropylene sutures did not maintain attachment of PSDs for as long as hypothesized. Frogs have an extensive subcutaneous lymphatic system and relatively little connective tissue between the muscle and dermis layers, both of which make suture movement and expulsion more likely. No studies involving suture reaction have been conducted in amphibian species. However, all suture materials can cause some degree of tissue reaction. Suture materials chosen for this study included polydioxanone for closure of the coelomic musculature and polypropylene for skin closure and for attachment of the PSDs. Polydioxanone was shown to cause minimal tissue response in rock doves (*Columba livia*) (Bennett *et al*., 1997). Polypropylene was chosen for attaching PDSs because of its documented minimal tissue reaction in other applications and high knot security (Fossum, 2007). Future studies comparing other suture patterns, sizes, and types of suture in amphibian species are warranted. Necropsies were not performed on any of these animals because of significant autolysis. Animals found dead and animals that were euthanized were submitted for chemical analysis because of limited PSD exposure to the environment. In the future, necropsies of the animals with failed suture lines to compare to those with successful sutures would be beneficial to improve technique.

Two of 20 frogs (10%) died, and one animal was not recovered at the two week mark. The mortality rate for this project was far less than that recorded by other similar studies involving implanted transmitters (Lamoureux and Madison, 1999; Pember *et al.,* 2002). Although the mortality rate in the current study was less than similar projects, the potential to diminish mortalities may have been reduced further if animals had been housed for a longer period of time prior to release, allowing for longer incision healing time.

Our study demonstrated a procedure for external attachment of PSDs that was successful for at least seven days but by two weeks had failed. Successful development of procedures to deploy PSDs on frogs and other species has the potential to yield contaminant exposure histories of individual animals. Coupled with radiotelemetry, these contaminant exposure histories could be directly linked to occupied habitats. Successfully linking exposure histories and habitats would greatly improve the ability of managers and decision makers to evaluate various types of wetlands, associated habitats, and wetland management and restoration alternatives for amphibian conservation.

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