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Evaluating a Method for Non-destructively Obtaining Small Volumes of Blood from Gilled Amphibians

In many herpetological research projects, researchers incorporate measures of immunity, stress or other aspects of physiology that necessitate obtaining blood samples from their amphibian subjects. There are a number of proven techniques for sampling blood from amphibians (Baranowski-Smith and Smith 1983; Gentz 2007; Tapley et al. 2011; Thrall 2004), although some may only be useful with large species. For the smaller species (which have limited blood volume), the animals sometimes must be sacrificed. There are certain physiological measures though, that only require minor amounts of blood (less than 10 µl), such as investigations involving blood smear analysis, or molecular assays. For such projects, it would be beneficial to have a method for non-destructively obtaining blood so that the animals could be returned to their natal environment following the study, or so that more than one sample could be obtained from individual amphibians.

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If larval or paedogenic salamanders are the subjects under study, it might be possible to obtain blood samples from their external gills, where blood flows continuously as part of normal respiration. In fact, such methods are already established for small fish (Watson et al. 1989); by making a minor incision in the gill filaments on one side of the animal, small amounts of blood can be siphoned with a microcapillary tube. The goal of the current project was to modify this same procedure for use with gilled amphibians, and evaluate its effectiveness for herpetological research. Here we report the results of an experiment where a collection of gilled salamanders (a paedogenic species) were sampled in the above manner, with and without anesthesia, to determine if the procedure affects survival in the days following the sampling.

Salamander collection and housing.—On 3 March 2011, we captured 34 aquatic, paedogenic Mole Salamanders (*Ambystoma talpoideum*), with dipnets from an impounded pond near the University of Georgia campus (Athens, Georgia, USA). This species is routinely used for studies in our lab (Davis and Maerz 2008a, b; Davis and Maerz 2010) and they are common

in northeast Georgia, especially in permanent water bodies. All salamanders were transported to the laboratory in a cooler of pond water, where they were placed individually into 1.9-L plastic containers filled with dechlorinated tap water and with one to two leaves (also collected from the source pond) for refugia. All containers were placed in an environment chamber that was set to a 12 h day length and at a constant temperature of 15°C. Salamanders were left in the chamber for 24 h before undergoing the blood sampling procedure (below).

Blood sampling.—Salamanders were randomly divided into three groups. Salamanders in Group 1 (N = 13) were first anesthetized via immersion in a solution of Orajel (20% benzocaine) following Cecala et al. (2007). Once immobile, each was blotted dry and weighed with an electronic balance, then lightly wrapped in a paper towel (to aid in handling), leaving the head and gills exposed. Then, using a sterilized pair of scissors, the distal end of one gill frond (Fig. 1) was cut off, and a heparinized microcapillary tube used to siphon the blood that welled from the severed gill. Salamanders in Group 2 (N = 13) underwent the same procedures (weighing, blood sampling), only without anesthesia. For salamanders in groups 1 and 2 we also noted the volume of blood obtained in the microcapillary tubes. The remaining salamanders (N = 8) were weighed and were subjected to the same degree of handling, but were not sampled. These salamanders served as a sham, or control group. All salamanders were returned to their respective containers (and placed in the environment chamber) following the processing and were monitored daily thereafter for four days. After four days, the salamanders were re-weighed, then released into their original pond.

Data analysis.—We first examined whether the volume of blood obtained with this procedure differed between anesthetized and non-anesthetized salamanders, using a Student's t-test. Next, we examined the degree of weight change for salamanders in each group using paired t-tests. Finally, we had intended to test for differences in survival rates across the three treatment groups, although there was no mortality in any group (below). Tests were performed using Statistica 6.1 software package (Statistica 2003).

Effects of the procedure.—No salamanders died in the experimental or the control groups during the four-day monitoring period. We also did not observe any atypical behavior in the salamanders that had been bled, during the post-procedure monitoring. The severed gills of experimental salamanders appeared to have healed by day 4, but we saw no evidence of regeneration. When we examined the change in weight from the day of bleeding to the last day, we found no significant weight change in either the Group 1 ($t = 1.22$, $df = 12$, $p = 0.243$) or Group 2 salamanders ($t = 0.311$, $df = 12$, $p = 0.760$). Collectively, these results all indicate that this blood-sampling procedure has minimal outward effects on salamanders.

Effectiveness of the procedure.—The average volume of blood obtained in both bleeding groups was 3.2 µl (standard deviation = 3.1 µl). Interestingly, we obtained significantly more blood from anesthetized salamanders than from non-anesthetized individuals ($t = -2.14$, $df = 24$, $p = 0.043$); the average for anesthetized salamanders was 4.4 µl while for non-anesthetized salamanders it was 1.9 µl. Part of this difference may stem from our inability to draw blood from one non-anesthetized salamander that thrashed during the procedure and made it difficult to place and hold the capillary tube on the gills. In fact, all non-anesthetized salamanders wiggled somewhat during the procedure, making it challenging (but not impossible) to siphon the blood. On the other hand, we cannot rule out the alternative possibility that more blood flowed from the anesthetized individuals because of some "relaxation" effect of the anesthesia on the animals' blood vessels.

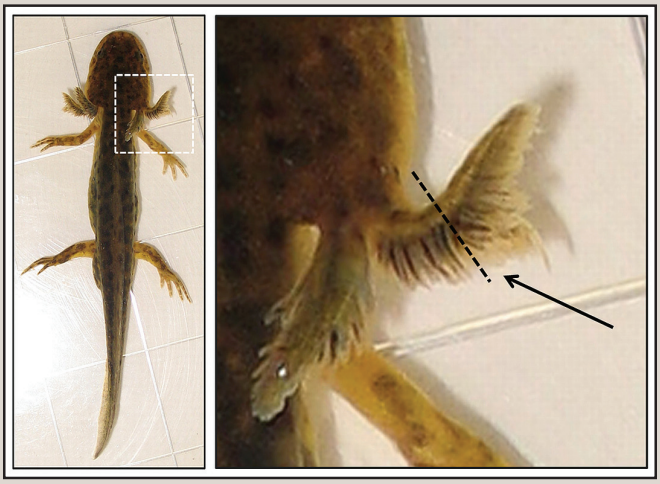

FIG. 1. Paedogenic *Ambystoma talpoideum* with close-up of gill filaments where blood was obtained. The distal tip of one filament (arrow) was severed with sterile scissors. Blood that welled from the cut was siphoned off with a heparinized microcapillary tube.

It is important to point out that the overall volume of blood yielded by this procedure (~5 µl or 0.005 g) would constitute less than 1% of the body weight of *A. talpoideum* (the average weight of *A. talpoideum* in this study was 3.0 g ± 0.6 g SD) and of other similarly-sized salamanders, which is a criterion recommended by Wright and Whitaker (2001). Similarly-sized species (with gills) would include paedogenic newts (family Salamandridae), paedogenic Tiger Salamanders (*A. tigrinum*), and Axolotls (*A. mexicanum*). Late-stage, gilled larvae of other species could also be used if they weighed more than 0.5 g.

The small amount of blood obtained may also limit the suitability of this technique for research purposes (because much larger volumes are needed for many clinical tests). Nevertheless, this volume would indeed be sufficient to conduct certain hematological investigations where only blood smears are needed (e.g., Davis and Maerz 2009; Davis and Maerz 2011; Davis and Milanovich 2010). With practice, it is possible to make (small) blood smears with this amount of blood (AKD, pers. obs.), which can be used to examine blood cells or search for intra- or extra-cellular parasites (e.g., Davis and Cecala 2010; Desser 2001; McAllister et al. 1993). Therefore, although the volume of blood obtained is small, this procedure appears to be useful for non-destructively collecting blood from gilled amphibians, and should serve a variety of purposes to researchers and herpetologists.

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The Use of Photo-Identification as a Means of Identifying Western Painted Turtles (*Chrysemys picta bellii*) in Long-Term Demographic Studies

Critical to any successful long-term demographic study is the ability to accurately identify subjects during recapture events. Previous mark-recapture studies involving freshwater turtles have employed a number of various mutilation techniques such as toe clipping, notching of the shells, or the use of PIT tags. However, the use of mutilation techniques may present a potential risk to the turtles as notching or filing of the shell can cause fracture or lead to potential infections, particularly in hatchling and juvenile turtles (Cagle 1939). Additionally, there are ethical concerns when marking rare or endangered species. While the use of PIT tags can reduce the risk of injury, PIT tags cannot be used on hatchling or small juvenile turtles due to body size limitations (Buhlmann and Tuberville 1998).

The use of photo-identification to identify individuals offers several advantages over other methods in that it is less invasive, eliminates the risk of injury or infection, and can be used to accurately identify hatchlings and juveniles on subsequent recaptures. Further, a photographic data base could have long-term applications that could be shared among researchers. However, in order for photo-identification to be reliable, the meristic character used for identification must be unique to each individual and must remain relatively unchanged over time. For example, plastron pigmentation in Midland Painted Turtles (*Chysemys picta marginata*) has been shown to change in response to environmental variables such as substrate color (Rowe et al. 2006a, 2006b, 2009). Juvenile pigmentation patterns may change as individuals mature, as in the case of plastron markings in juvenile Wood Turtles *Glyptemys insculpta* (Cowin and Cebek 2006). However, photo-identification has been successfully used to identify individuals in populations of Spotted Salamanders (*Ambystoma maculatum*, Loafman 1991), Daruma Pond Frogs (*Rana porosa brevipoda*, Kurashina et al. 2003), Leatherback Turtles (*Dermochelys coriacea*, McDonald et al. 1996), and Loggerhead Sea Turtles (*Caretta caretta*, Schofield et al. 2008). The plastron markings of some species of turtles are unique to individuals and as such may provide an accurate means of identifying individuals during demographic studies. Janzen et al. (2000a; 2000b) used plastron marking to successfully identify hatchling and juvenile Red-eared Sliders (*Trachemys scripta elegans*). More recently, Tichy and Kintrova (2010) demonstrated that plastron morphology could be used to accurately identify individuals in populations of European Tortoises *Testudo graeca ibera*. To date, the use of photo-identification to identify individuals has not been employed in any long-term demographic studies on Painted Turtles (*Chrysemys picta*). The Western Painted Turtle (*C. p. bellii*) possesses expansive and distinct plastron markings that make it an ideal candidate for the evaluation of photo-identification as an individual recognition technique. The goal of the present study was to determine if the use of plastron markings on *C. p. bellii* could provide a non-invasive method for accurately identifying

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