Evaluating the Potential for Bias with Common Amphibian Protocols

Most ecological studies assume that standard protocols and sampling methods accurately sample populations in an unbiased way (Heyer et al. 1994). Violations of these assumptions can yield biased results or invalid conclusions, which could negatively influence management or conservation efficacy (Mazerolle et al. 2007; Kroll et al. 2008; Cecala et al. 2013). Extensive studies have evaluated methodological factors that change the detection probabilities of organisms including trapping methods, time of day, and habitat characteristics (Gamble 2006; Todd et al. 2007; Connette et al. 2015). However, few have investigated whether the captured individuals of a species are representative of the studied population as a whole (e.g., Willson et al. 2008; Michelangeli et al. 2016). Furthermore, little information is available for accuracy of measurements from standard protocols (e.g. Roitberg et al. 2011). Because amphibians are declining at unprecedented rates and represent an important taxon for understanding ecological and evolutionary phenomena (Stuart et al. 2004; Adams et al. 2013; Grant et al. 2016), determining if standard methods could introduce previously undocumented bias is important for future studies of amphibians (Grant 2014; Connette et al. 2015).

Variation in capture methodology has received recent attention as researchers become aware that passive capture methods can bias samples towards individuals with particular behavioral syndromes even with random sampling of available habitats (Biro and Dingemanse 2009; Carter et al. 2012; Biro 2013). Because behavioral traits are heritable (van Oers et al. 2004), differential capture rates associated with behavioral syndromes could introduce bias in studies of any number of physiological, behavioral, or life history traits (Biro and Stamps 2008; Wolf and Weissing 2012). Wilson and colleagues (1993) discovered that behavioral syndromes could contribute to extreme sampling bias where some individuals were trapped repeatedly whereas others were never captured. For individuals more likely to take risks and explore novel objects, they may be more likely to be captured in passive traps (Biro and Dingemanse 2009; Stuber et al. 2013). Furthermore, high activity levels common to bold individuals may also result in more encounters with passive sampling techniques (e.g., traps, gill nets, etc.; Stuber et al. 2013). In harvested populations of fish, these biases led to a population of individuals that were less active, less exploratory, and less likely to take risks (Biro and Post 2008). Similarly, individuals with different behavioral types could use habitats differently such that sampling could target only a particular behavioral type (Wilson et al. 2011). For example, sampling of open-water aquatic habitats could sample bold individuals relative to shallower areas that offer refugia for shy individuals (Wilson et al. 2011). Few studies have investigated potential biases associated with active capture techniques, but a study on Delicate Skinks (Lampropholis deliciue) did not observe behavioral differences in individuals captured by hand relative to passive methods of capture (Michelangeli et al. 2016). Ultimately, population studies that do not account for bias associated with capture techniques could underestimate population sizes and lead to biased conclusions about the status of a population (Crespin et al. 2008; Pradel et al. 2010; Olivier et al. 2017).

Capture methods could also introduce biases associated with standard measurements particularly if species exhibit ontogenetic shifts or size-determined distribution patterns that could bias samples towards smaller or larger individuals of a population (Werner and Gilliam 1984; Hairston 1987; Todd and Winne 2006). Other implementations of standard protocols such as the use of anesthesia could result in higher accuracy of length measurements (Setser 2007), but impacts of anesthesia on measurements of mass are unknown. Furthermore, body length may change with environmental conditions making it critical that this variation can be attributed to environmental conditions rather than measurement error (Bendik and Gluesenkamp 2013). Mass could also be impacted by stomach contents that would result in larger mass measurements. Generally, feeding status or prey mass is unknown for wild-captured individuals, but holding individuals until digestion is complete could result in more accurate assessment of mass. For example, diet studies of Eastern Red-spotted Newts (Notophthalmus viridescens) found individuals consuming up to 55% of their body mass introducing a positive bias in morphological studies (Burton 1976; Dimmit and Ruibal 1980).

In this study, we evaluated if common practices in amphibian ecology could bias results. We determined if active versus passive capture techniques affected morphometric data or was biased towards a particular behavioral type. Once individuals were captured, we also investigated how time since capture and anesthetization impacted morphometric measurements. As a case study, we evaluated these methods on measurements of length (snout–vent length; SVL), mass, and exploratory behavior of N. viridescens.

METHODS

Adult Notophthalmus viridescens were captured from Lake Cheston in Franklin County, Tennessee, USA. We had two sampling periods from October to November 2016 and in March 2017. In the fall, we quantified the effects of anesthesia on morphometric data. In the fall and spring, we quantified the effect of capture method on morphometric data and behavioral data. We captured individuals by active dipnetting up to 1 m from the shore or by plastic minnow traps (Shaffer et al. 1994; Graeter et al. 2013). Minnow traps were set approximately 0.5 m from shore around the perimeter of the lake where emergent vegetation was absent. Traps were set at least 5 m from one another and checked every 24 h while deployed. Upon capture, newts were placed in

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a behavioral arena (described below), measured and weighed before placed in an 8 oz deli cup with lake water and a paper towel for the remainder of the experiment. Morphometric data was always collected after the behavioral assay to minimize the effects of handling stress on behavior. Individuals were kept without feeding at 11°C for five days before being released within 20 m of their capture location.

We evaluated morphometric and behavioral data from 52 individuals captured in the fall and 41 individuals captured in the spring. Effects of anesthesia were quantified from 40 additional individuals captured in the fall. We measured individual SVL to the nearest mm (to the posterior edge of the cloaca) and mass to the nearest 0.01 g (Fellers et al. 1994). Morphometric data were collected immediately upon capture, 24 h and 120 h after capture to evaluate if capture method or time since capture impacted measurements of length or mass. To determine if anesthesia impacted these same measurements, we measured length and mass before and during anesthetization. All evaluations of anesthesia were conducted within six hours of capture. Individuals were measured immediately before being placed in a 1 g Oragel L\textsuperscript{1} solution with buffered dechlorinated water (Cecala et al. 2007). Once individuals were unresponsive to a toe pinch, we removed individuals from the anesthesia bath, rinsed and measured them. Individuals were allowed to recover on a wet paper towel until their righting reflex was restored before being released at their capture location.

To determine if behavioral traits were different between individuals captured in a passive (minnow trap) or active (dipnetting) method, we evaluated individual activity levels immediately upon capture in the field and again 24 h and 120 h after capture in the lab using the same behavioral arena. The behavioral assay was performed using a 5-gal tank with a 5-cm grid drawn on the bottom filled with 5 cm of lake water refreshed for each individual. Each individual was tested independently by being placed under a dark cup in a randomly selected corner for 30 s before the cup was removed. For five minutes, we recorded the number of boxes the individual entered with all four legs.

All data were analyzed using linear mixed models using individual as a random factor using package lmekTest (Kuznetsova et al. 2014), and posthoc tests of significant main effects were evaluated using Tukey p-value adjustments. Means are presented with standard errors (SE).

**RESULTS**

*Notophthalmus viridescens* mean SVL was 40.8 ± 0.28 mm, and mass was 2.32 ± 0.04 g. We captured 42 individuals using minnow traps in the fall and 21 in the spring. Active dipnetting surveys captured 20 individuals in the fall and 20 in the spring. Statistical models had neutral residuals. Capture method did not affect length ($F_{df=1, 201} = 1.19, P = 0.278$) or mass of individuals ($F_{df=1, 201} = 0.02, P = 0.884$), but activity was 79.2 ± 33.2% higher in individuals captured in minnow traps ($F_{df=1, 201} = 5.20, P = 0.033$). Time significantly affected measurements of mass ($F_{df=2, 201} = 4.41, P = 0.039$) and activity ($F_{df=2, 201} = 4.72, P = 0.033$). Mass declined 0.079 ± 0.013 g between capture and 120 h ($Z = -3.18, P = 0.004$) but was not different between capture and 24 h ($Z = -1.41, P = 0.335$). We observed a significant interaction between capture method and time on activity ($F_{df=2, 201} = 5.20, P = 0.025$). Specifically, individuals captured using minnow traps exhibited high levels of activity at capture that returned to levels similar to individuals captured in dipnets and remained consistent between the two later time periods (Fig. 1). Anesthesia did not affect measurements of length ($F_{df=1, 38} = 0.062, P = 0.805$) but did affect measurements of mass ($F_{df=1, 36} = 6.12, P = 0.019$). Individuals weighed 0.032 ± 0.012 g less under anesthesia than they did before the process.

**DISCUSSION**

These studies documented that common amphibian capture methods of dipnetting and minnow traps capture individuals with similar characteristics. Significant effects of capture method on activity likely represented an immediate escape behavior that diminished through time (e.g., Morellet et al. 2009; Seress et al. 2017). We observed consistent negative effects of time and anesthesia on mass though these changes were less than 3% of adult mass in our study population. Finally, our study provides support for allowing comparisons of length among salamander studies that did or did not use anesthesia.

Novelty associated with introduction of passive sampling tools into a pond does not appear to introduce bias. Despite testing only a single behavior (Sih et al. 2004), we observed consistent exploratory behavior of individuals captured using passive or active techniques after the initial behavioral assay (Michelangeli et al. 2016). These results are counter to observations that passive techniques tend to capture bolder or more active individuals than shy, less active, or neophobic individuals (Biro and Dingemanse 2009; Stuber et al. 2013), but we recommend future evaluation of individuals from different habitat types (Wilson et al. 2011). Although individuals in our experiment might have demonstrated different behavioral traits in another assay, tendencies towards exploration or activity are frequently used traits to characterize behavioral syndromes (e.g., Sih et al. 2004; Dingemanse et al. 2007; Minderman et al. 2009). Furthermore, consistency between the two post-capture time intervals (24 and 120 h) provides additional support that these are indicative of individual tendencies (Sih et al. 2004). Results from this behavioral survey suggest that researchers carefully consider capture methods and timing until the first
test of behavior. Individuals captured using active techniques exhibit immediate behavioral differences by exploring a novel enclosure more than individuals captured using passive traps or if individuals were contained for 24 h prior to testing. Capture can induce an acute stress response, and this initial exploratory behavior may be associated with capture stress and search for escape (Morton et al. 1995; Romero and Reed 2005). Therefore, we recommend that behavioral studies with individuals captured using passive methods refrain from initiating studies until 24 h after capture.

Small, but consistent, declines in mass measurements were observed through time and with anesthesia. Despite small changes, these could amplify if measurements are extrapolated for biomass estimates (e.g., Burton and Likens 1975; Semlitsch et al. 2014; Milanovich and Peterman 2016). We recommend that researchers maintain consistency in measurement protocols among samples taking into account time since capture and the use of anesthesia. Digestion and absence of feeding even over short temporal intervals were sufficient to change mass measurements. We are unaware of other studies documenting declines in mass associated with anesthesia, but suggest that it could be an osmotic response to the presence of a dissolved anesthetic or inhibition of physiological processes (Feder and Burggren 1992; Hillman et al. 2009). A future study should investigate if this effect is similar or more extreme in Plethodontid salamanders with highly permeable skin (Wells 2007; Hillman et al. 2009). Another study also found that body length and mass declined following preservation indicating that live measurements with or without anesthesia should not be compared to preserved specimens (Shu et al. 2017).

Methodological variation has been suggested as one alternative explanation for observations of declining salamander body size through time (Caruso et al. 2014; Grant 2014; Connette et al. 2015). No variation in length was linked to time or use of anesthesia in our study. Two additional variations of methodology should be tested. First, precision among personnel should be evaluated because confusion can exist between whether researchers measure to the anterior or posterior end of the cloaca, and experience with anesthesia should not be compared to preserved specimens (Shu et al. 2007).

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