Effectiveness and Reliability of Photographic Identification Methods For Identifying Individuals of a Cryptically Patterned Toad

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Abstract.—**Various marking methods are used for amphibian population studies, some of which have been debated for their invasive nature. Although non-invasive methods exist, they are often employed on vibrant species with distinct coloration and patterning. Here, we evaluate the Photographic Identification Method (PIM) as a marking method for identifying individuals of the Schneider's Toad (*Rhinella diptycha*), which has cryptic dorsal patterning, and compare two PIM techniques: visual and computer assisted. We surveyed toads in Pilar, Paraguay, photographed dorsal patterns of each captured toad *in situ* and marked individuals with Visible Implant Elastomer (VIE) between their inter-digital webbing for cross-validation of recaptured individuals. Of 109 captured toads, we used 37 recaptures to test the accuracy of PIM and VIE methods. Volunteers matched photographs of recaptured toads with a set of all first-capture photographs to test visual PIM, and we recorded their accuracy and the time to reach a match decision. To test computer-assisted PIM, we used the program Wild-ID, which matched recapture and first-capture photographs. We cross-referenced suggested match pairs using photograph codes corresponding to individual specimens to test accuracy and recorded the time to reach a match decision. Computer-assisted PIM was the most accurate (100%) and fastest method (on average, 11.5 × faster than visual PIM), but visual PIM (86.7%) and VIE (89.2%) were also highly accurate for identifying individual toads. Despite the cryptic pattern of these toads, our results suggest that non-invasive and cost-effective methods such as PIM can be used for population studies of dull-patterned taxa.**

Key Words.—*Rhinella diptycha*; Visible Implant Elastomer; visual matching; Wild-ID

Introduction

Various methods can be used to mark individual amphibians during ecological surveys, such as Passive Integrated Transponder (PIT) tagging, toe clipping, and Visible Implant Elastomer (VIE). These methods are vital as they can aid in greater accuracy of demographic parameters, an important component for understanding the population dynamics of both common species (Edmonds et al. 2019) and species in decline (Houlahan et al. 2000; Stuart et al. 2004). Some of these marking techniques, however, are often considered controversial and outdated. In particular, toe clipping has been the cause of many ethical debates (Perry et al. 2011), especially regarding the inner-most digits of the forefeet, which allow the male to grip onto females during amplexus (Clarke 1972), and this method can affect survivability of some amphibian species (Davis and Ovaska 2001). Despite potential adverse side effects to amphibians, toe clipping is in some cases still considered a reliable and inexpensive marking technique (Guimaraes et al. 2014). Although toe regeneration can occur (e.g., Ursprung et al. 2011), toe clipping typically provides a permanent and distinguishable mark on an individual and removed toe segments can be used as tissue samples for DNA analysis and insight into skeletochronology.

A marking method now more favored is the Photographic Identification Method (PIM; Hagström 1973). This is a non-invasive technique in which a photograph (photo) is taken of an animal *in situ* and used to later re-identify the individual based on unique natural patterns (e.g., spots, stripes, or blotches). This method can be applied to mark-recapture studies examining population size and dynamics (Arntzen et al. 2003) and has been employed for a wide range of species from Tigers (*Panthera tigris*) to Whale Sharks (*Rhincodon typus*; e.g., Arzoumanian et al. 2005; Hastings et al. 2008; Hiby et al. 2009; Sherley et al. 2010). One application of PIM, visual matching, only requires the observer to compare photographs of individuals to successfully identify a specimen (e.g., Forcada and Aguilar 2000), and can be easily applied to species with simple but vibrant patterns. Computer-assisted PIM requiring pattern recognition software may then be necessary for large datasets (e.g., Arzoumanian et al. 2005; Hiby et al. 2009) and/or if a pattern is too complex for the naked eye, as these factors may increase error rates. Furthermore, error variation in situations with more than one observer will decrease with the use of computer-assisted PIM as there will be no variable biases in identifications (Cruickshank and Schmidt 2017). For example, a study found computer-assisted

PIM to have 100% accuracy in identifying individuals of the Alpine Newt (*Ichthyosaura alpestris*) and the Smooth Newt (*Lissotriton vulgaris*) in a database consisting of almost 4,000 images (Mettouris et al. 2016).

The Photographic Identification Method has been used on amphibians as early as the 1970s (Hagström 1973) but has been more widely used on salamanders than other amphibians (Carafa and Biondi 2004; Gamble et al. 2008; Bendik et al. 2013) as a substitute for toe clipping because of their regenerative abilities (Heatwole 1961; Ott and Scott 1999; Davis and Ovaska 2001). Although it is typically thought that anurans lack this ability, it has been found in other studies (e.g., Ovaska and Hunte 1992; Jungfer and Weygoldt 1999; Richter and Seigel 2002), with one study observing consistent regeneration for individuals of the Brilliant-Thighed Poison Frog *Allobates femoralis* (Anura: Dendrobatidae)*,* over a three year period (Ursprung et al. 2011). These findings highlight the need for alternative methods such as PIM to improve the accuracy of mark-recapture studies for taxa with regenerative abilities. Despite availability of digital technology and photo-matching software that can be used to identify individual amphibians (e.g., Elgue et al. 2014; Sannolo et al. 2016; Renet et al. 2019), there are few examples of PIM being used as a mark-recapture method to study amphibian populations (see Edmonds et al. 2019). First, determining validity of this method on a target species is necessary if previous experimentation has not yet been done, which is why many studies may still opt for more invasive mark-recapture methods (e.g., PIT-tagging) to study amphibian populations.

Due to its widespread distribution across South America and because individuals of this common species are readily available, the Schneider's Toad, *Rhinella diptycha* (Anura: Bufonidae), is an ideal candidate to further assess the effectiveness of PIM. Additionally, the reliability of PIM is especially put to the test whenever a target species, such as *R. diptycha*, is cryptic and/or drab in coloration. This coloration might suggest visual or computer-assisted photo matching would not be reliable for this species, but PIM has been shown to work on anurans similar in color (Smith et al. 2018; Edmonds et al. 2019) and pattern (Schoen et al. 2015) to *Rhinella* species. Herein, we evaluated whether PIM is an accurate marking method for identifying individuals of *R. diptycha* populations. We also compared the accuracy of visual and computer-assisted PIM for this species, and we applied and evaluated injected fluorescent tags (VIE) in the interdigital webbing skin of hindfeet as a secondary method of marking in the field to aid in accurate identification of individuals throughout these comparative analyses.

Materials and Methods

Study site.—We collected data from October 2018 to March 2019 in southwest Paraguay within the city of Pilar (Ñeembucú Department). Pilar and the surrounding area of Ñeembucú are part of the Humid Chaco ecoregion (Sato et al. 2015) with various habitats throughout, including Gallery Forest, Humid Chaco Savannah, Humid Chaco Thicket, and a vast and diverse wetland complex. Observations took place along a 1-km stretch of raised pedestrian walkway known as the Costanera (26°51'17.8"S, 58°18'03.9"W, 60 m above sea level), which has a mean width of 8.6 m and runs along a section of the Arroyo Ñeembucú, a tributary to the Paraguay River. For the purpose of data collection, we divided the Costanera into four 250-m transects.

Data collection.—We captured *R. diptycha* during 35 sampling nights. Using a headlamp, we inspected the Costanera, various structures (e.g., drainage pipes, flowerpots, waste bins), and cracks between uneven concrete blocks. Additionally, we searched areas of vegetation within 5 m of the edge of the Costanera and inspected toad burrows non-invasively. Upon capture, we paused the survey for data collection and released the toad where it was originally found before continuing the survey. We surveyed one transect each evening during hours of high toad activity (typically between 2030 and 2330), with surveys lasting 2–3 h.

To avoid potential ontogenic changes in patterning, we used a caliper to measure the snout-vent length (SVL) of captured toads. For many *Rhinella* species in the *Rhinella marinus* species group, the average minimum SVL of sexually mature females is 100 mm (Zug and Zug 1979; Echeverria and Filipello 1990). We did not include any toads < 100 mm SVL in further analysis. We prepared toads for photography by removing any debris that may obscure dorsal markings and drying wet skin with a towel to reduce the risk of flash reflection. We photographed toads in a natural stance, at least five times, against a plain white plastic tray, using a Digital Single-Lens Reflex camera (Canon EOS 1100D; Ōta, Tokyo, Japan) with a fixed focal distance of 50 mm (shutter speed of 1/60 s, aperture of f/4.5, and ISO 100), mounted onto a tripod at a fixed height, and saved all images at the full 12.2 megapixel size. We checked all images for clear focus and optimal exposure and selected one per toad per capture event for use in visual and computer-assisted PIM testing. We cropped selected images to focus on the dorsum without indication of relative toad size.

The first time we captured a given toad with an SVL ≥ 100 mm, we tagged the individual using a Visible Implant Elastomer (VIE) tag developed by Northwest Marine Technology, Inc. (Anacortes, Washington, USA). The tags consisted of two components, a colored dye and a curing agent (both non-toxic), which we previously mixed in a laboratory setting at a 10:1 ratio and inserted into a 0.3-cc injection syringe. We kept the injection syringes frozen to preserve them in a liquid
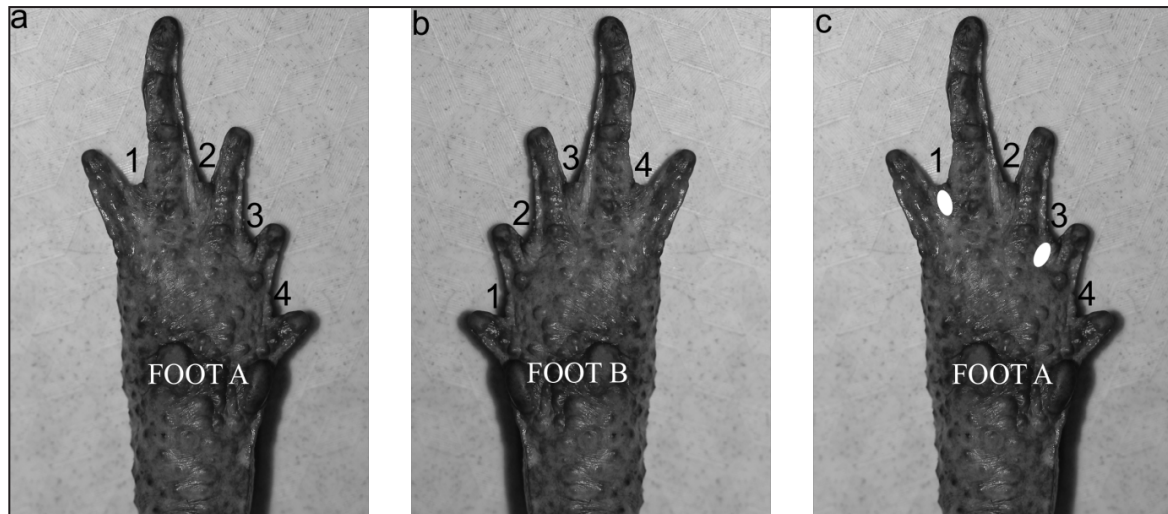
FIGURE 1. Inter-digital webbing locations used for alphanumeric tagging sequences on (a) the left and (b) the right hindlimbs of Schneider's Toad (*Rhinella diptycha*), and (c) an example of a Visible Implant Elastomer (VIE) tag with the alphanumeric code A1W3W (Foot A with white tags on inter-digital webbing 1 and 3). (Photographed by Harry-Pym Davis).

state as the curing agent allows the tag to solidify under warm temperatures (e.g., when injected into a living organism). We injected tags, which were detected using a Flashlight VI Light (405 nm, 82 mW; Northwest Marine Technology, Inc.), under the translucent webbing between the toes of the hindlimbs of the toads according to an alphanumeric sequence (Fig. 1a and b) adapted from Donnelly et al. (1994), whereby we labeled the hindlimbs A (left) and B (right), the inter-digital webbing from 1 to 4 (reading from left to right), followed by the first letter of the tag color arranged alphabetically (blue, green, orange, yellow). For example, the code A1W3W indicates the toad has a white (W) tag on the left foot (A), in webbing sections 1 and 3 (Fig. 1c).

Because VIE has been shown to reliably identify individuals without toe clipping (Hoffmann et al. 2008), we used VIE to cross-validate and visually determine whether an individual had been previously captured and match recaptured individuals with first capture data. We anticipated some level of inaccuracy with this method, which is why we also evaluated the reliability of this method in our analysis. For each captured toad, we collected the following information: digital photograph numbers, time of capture, sex, SVL, geographic coordinates (using a global positioning systems unit: Garmin GPSmap 64, Olathe, Kansas, USA), and VIE alphanumeric code (if previously tagged). If a captured toad was not a recapture, we marked the individual according to the next available alphanumeric code in the sequence. We did not implant any additional VIE tags on recaptured individuals.

Evaluating identification methods.—To assess the effectiveness of visual PIM, 12 volunteer participants completed image matching tests. Each test contained five images (labelled A to E) of randomly selected

(using a random-number generator) recapture images of toads to be identified using the complete collection of first-capture images (representing all 72 toads labeled with their original camera-allocated image number that could be cross referenced to their VIE tag sequence). Participants conducted all tests on a laptop computer (1366 × 768 screen resolution; Pavilion model; Hewlett-Packard, Palo Alto, California, USA) using the split screen action so that first capture images and photos of toads to be identified could be viewed simultaneously. We recorded the time taken for a participant to decide which first-capture photo best matched a recaptured toad.

We tested computer-assisted PIM using the Java program Wild-ID 1.0 (Bolger et al. 2012), which assigns a goodness-of-fit score for potential matching images (ranging from 0 to 1, with higher values indicating better matches). To evaluate the reliability of this method, we confirmed match suggestions by cross referencing the photo codes, which corresponded to the VIE alphanumeric sequence of a toad, the date of capture, and the image number assigned by the camera (see Fig. 2 for example image codes and how Wild-ID performed for *R. diptycha*). To determine the total time to reach a positive photo match using computer-assisted PIM, we added the time to search through our photo code database to confirm a given match pair with the time Wild-ID took to generate that match suggestion. We completed statistical analyses using the software MATLAB v. R2018a (The MathWorks, Inc., Natick, Massachusetts, USA). We used a Fisher's Exact Test to evaluate if one method was more accurate than another. We log-transformed times to determine a match pair for each method and used a two-sample *t*-test to compare differences in time to determine matches between computer-assisted PIM and visual PIM. For each test, $\alpha = 0.05$.

Figure 2. An example of successful image pair matching in Wild-ID is shown here for a Schneider's Toad (*Rhinella diptycha*) individual first captured 9 November 2018 (lower right photograph) and recaptured 13 November 2018 (lower left photograph). The goodness-of-fit score for this pair of images was 0.406 and the top suggested matching image was the correct match. The top row of images includes the current image being processed (13 November 2018) to the far left, with the remaining images being the suggested matches ranked on goodness-of-fit scores from highest to lowest (left to right). (Photographed by Harry-Pym Davis).

RESULTS

During our survey period, we captured 72 individual toads, 22 of which we recaptured once and 15 of which we recaptured two or more times (totaling 37 recaptures and 109 total captures). Across all recaptures, the time between first and subsequent captures for individuals ranged from 1 to 70 d. For all captures, the mean SVL was $120 \pm$ (standard deviation) 14 mm (n = 109; range, 100–176 mm), 21 individuals were females (mean SVL = 124 ± 12 mm; range, 106–146 mm) and 51 individuals were males (mean SVL = 118 ± 16 mm; range, 100–176 mm).

We found all three identification methods (VIE and visual and computer-assisted PIM) to have high levels of accuracy. Across all visual PIM tests, participants correctly matched 52 out of 60 recapture images (86.7%) with their respective first-capture images. For computer-assisted PIM, Wild-ID correctly matched all 37 recapture images with their respective first capture photos with a mean goodness-of-fit value of 0.42 ± 0.18. The VIE method was accurate in matching 33 of the 37 recaptures (89.2%). There were two instances of VIE-tag rejection that we identified upon recapture of the two individuals by the VIE-tag residue that had solidified around the injection site on the interdigital webbing. We re-injected the two individuals with their original colored VIE tag for further use in the study. Additionally, we misidentified two more recaptured toads as previously uncaptured toads, which we did

not discover until cross-validation with computer-assisted PIM. Computer-assisted PIM was significantly more accurate than visual PIM (Fisher's Exact Test, $P = 0.022$), but there was no significant difference in accuracy between VIE and either visual (Fisher's Exact Test, $P > 0.999$) or computer-assisted PIM ($P = 0.115$).

The average time to identify one recaptured toad during visual tests was 200 ± 256 s (range, 17–1,466 s), and the average for computer-assisted PIM was 10 ± 8 s (range, 2–113 s) with a total time of 1,131 seconds when including cross-validation. Computer-assisted PIM was significantly faster than visual PIM ($t = 13.2$, df = 95, $P < 0.001$). Because VIE tagging occurred alongside photographing toads in the field for PIM testing, we did not consider a comparison between field-time efficiency for VIE tagging and PIM for this study. We did not time how long it took to identify a toad in the field by searching for its VIE tag but, in some cases, it took up to 10 min.

DISCUSSION

We found all three methods to be highly accurate for identifying individual specimens, which confirms that the seemingly dull dorsal patterns of *R. diptycha* is unique to the individual. Additionally, we found computer-assisted PIM to be the fastest method and more accurate than visual PIM, correctly identifying all matching image pairs. Although pattern changes over time have been observed in anurans (e.g., Kenyon et al.

2010), the high level of accuracy for Wild-ID suggests that any pattern changes of specimens in this study were not substantial enough to be detected, but this should be more rigorously tested with a longer study period.

The high accuracy of the PIM technique is especially beneficial to cryptically patterned species, which typically require invasive marking methods in mark-recapture studies. Although PIM has not been previously evaluated on *R. diptycha*, it has been shown to be similarly accurate for other species of bufonids, including one study that found both PIM techniques to be highly accurate (> 90%) in identifying individuals of the Southern Red-Bellied Toad (*Melanophryniscus cambaraensis*), with visual PIM as the most accurate (Caorsi et al. 2012), but see Morrison et al. (2016) for an example of wild-ID performing poorly at image matching on the Wyoming Toad (*Anaxyrus baxteri*). Our finding that computer-assisted PIM is more accurate than visual PIM is likely because, unlike the strikingly colorful and contrasted focal section of *M. cambaraensis*, the dorsal pattern of *R. diptycha* is comparatively drab, sometimes showing large uniform dark spots, and may be more difficult to differentiate to the naked eye. In another study, visual PIM was found to have less accuracy (62%) than toe clipping (92%) for identifying individuals of the Green-Eyed Treefrog (*Litoria genimaculata*), which, like *R. diptycha*, also has relatively dull dorsal patterning (Kenyon et al. 2009). Their finding that toe clipping was more accurate than PIM at identifying individuals could be explained because computer-assisted PIM was not included in their analysis. Not only does this illustrate the importance that different marking methods and consequential analyses will always be species dependent, but it also suggests that using computer-generated algorithms in place of visual PIM could increase the accuracy in identifying individuals of a species, as highlighted by Cruickshank and Schmidt (2017).

Our results indicate that computer-assisted PIM is a highly accurate and relatively fast method for identifying individual *R. diptycha*, which suggests that PIM is a suitable method for identifying individuals of similarly cryptically patterned species, especially species within the *Rhinella* genus. Furthermore, computer-assisted PIM matched two individuals we misidentified from VIE tags, showing how photo matching software can help reduce human error. Regardless of the slight inaccuracy with this method, VIE was instrumental in confirming recapture matches both in the field and during our photo matching process. Despite misidentification errors with VIE tagging, there was no significant difference between the accuracy of VIE and visual or computer-assisted PIM in identification of specimens. Although the time to identify individuals using VIE *in situ* was not recorded, the time to search the interdigital webbing for a VIE tag varied considerably and, at times, we cross-referenced tags through photograph comparison with previously tagged individuals. The amount of time it takes to mark an individual in the field is important to consider when conducting population observations (Arnzten et al. 2003), and further work should evaluate processing time and the cost-effectiveness of this method.

We found PIM to be highly accurate in identifying adult toads based on their distinct dorsal patterns, but we believe that future population analyses of *R. diptycha* using PIM methods should also include juvenile individuals. The reliability of PIM on juveniles of *R. diptycha* is yet to be evaluated, so we suggest conducting similar research to that of Kenyon et al. (2010) that observed no significant pattern changes over time on juvenile *L. genimaculata* specimens. Being able to easily mark and identify juveniles could provide valuable data on population demography and juvenile survival estimates. Additionally, we acknowledge that our study took place over a relatively short period of time (about 4 mo) and recommend a longer study period (> 1 y) for future work evaluating the reliability of PIM on juveniles of *R. diptycha*.

Overall, we have demonstrated that computer-assisted PIM can be a reliable method of marking and identifying *R. diptycha* and believe this method may have similar accuracy for other species of *Rhinella* and similar cryptically patterned species. We encourage future PIM based mark-recapture population studies on *Rhinella* species to employ PIM methodology not only because its non-invasive and cost-effective nature and identification accuracy, but also because ecological information on relatively wide-spread, common species is of great importance in understanding the structure and function of ecosystems (Edmonds et al. 2019). As a final remark, we believe that PIM could allow for long-term amphibian monitoring projects to geographically expand on their data collection range by incorporating citizen science as a tool. Not only can photographs provide an accurate source for species and individual identification, but the method also allows for minimal disturbance during an encounter, which can decrease animal stress and the risk of spreading harmful diseases. This latter aspect is incredibly relevant when considering the global spread of *Batrachochytrium dendrobatidis* (Scheele et al. 2019).

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