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COMPARISON OF THREE WIDELY USED MARKING TECHNIQUES FOR ADULT ANURAN SPECIES *LITORIA VERREAUXII ALPINA*

LAURA A. BRANNELLY[1, 2], LEE BERGER[1], LEE F. SKERRATT[1]

[1]*One Health Research Group, College of Public Health, Medical and Veterinary Science, James Cook University, Townsville, Queensland, Australia.*
[2]*Corresponding author, e-mail: laura.brannelly@my.jcu.edu.au*

***Abstract*.—Effective marking techniques are required for both laboratory and field studies of adult amphibians, especially when individuals cannot be identified based on color or pattern. We compared the efficacy of four marking techniques: toe clipping, visual implant elastomer (VIE) tags, and passive integrative transponder (PIT) tags injected into two locations (the body cavity and subcutaneously) in the endangered Alpine Tree Frog, *Litoria verreauxii alpina*. The most effective marking method was toe clipping, with 96.1% correct identifications. The second best marking method was PIT tags injected subcutaneously, where tags were retained in 73.3% of animals after six weeks, but tag retention might decrease over time due to tag expulsion. PIT tags injected into the body cavity were poorly retained (33.3%). The least successful marking method was VIE tags, as individuals were correctly identified only 18.4% of the time. We conclude that toe clipping may remain the most effective marking method for some amphibian species, where modern tagging techniques are unreliable due to low tag retention and high tag movement. Researchers should conduct marking trials before implementing large scale marking schemes in unstudied species, and they should publish negative results as well as desired outcomes.**

Key Words.—marking methods; passive integrative transponder tags; toe clip; visual implant elastomer tags.

INTRODUCTION

Identifying individual amphibians is essential for management (e.g., captive breeding plans) and for field and laboratory based research. Research includes capture-mark-recapture studies that track individual frog behavior and survival, and are important for conservation, population dynamics, and ecological studies (McCarthy and Parris 2004). Marking is necessary when individuals cannot be distinguished based on physical features such as color or pattern (Donnelly et al. 1994). Currently, three marking techniques are commonly used for amphibians: toe clips, passive integrated transponder (PIT) tags, and visible implant elastomer (VIE) tags. All three of these tagging methods have been successful in a variety of taxa (see Davis and Ovaska 2001; Woods and James 2003; Curtis 2006; Waudby and Petit 2011; Hamel et al. 2012), but their effectiveness on anuran species needs to be further assessed in a greater variety of species (Funk et al. 2005; Phillott et al. 2007).

Recently, many animal ethics committees have become skeptical of toe clipping as a valid and ethical method for marking amphibians, and some have refused to approve toe clipping for marking (B. Scheele pers. comm.; L. Brannelly pers. obs.; see Perry et al. 2011; Correa 2013). Although the primary purpose of ethics boards is to restrict the pain and discomfort of research animals, the perception of pain and suffering of amphibians during toe clipping is predominantly an anthropomorphized intuition (Langkilde and Shine 2006; Fisher et al. 2013), particularly after May (2004) called the practice barbaric. Even so, toe clipping remains the most widely used marking method for anurans, and has been defended by many as simple, cost-effective, having relatively minor health impacts, and the operationally best method for particular species (e.g., Phillott et al. 2007, 2008, 2010, 2011; Perry et al. 2011; Correa 2013). Moreover, it does not increase measurable distress above that experienced from handling alone (Kinkhead et al. 2006; Fisher et al. 2013).

Alternative marking methods for amphibians, such as VIE tags and PIT tags, are relatively new, and some of the few studies assessing their use have demonstrated marking failure (i.e., Tracy et al. 2011; Brannelly et al. 2013). To date, no study has assessed the efficacy of these three marking methods on a single species to determine the most reliable marking method to be used in capture-mark-recapture studies. However, animal ethics boards are enforcing the use of these methods over toe clipping (Funk et al. 2005; Phillott et al. 2008; Perry et al. 2011; Correa 2013).

In this study, we tested four marking techniques on the Alpine Tree Frog, *Litoria verreauxii alpina*. *Litoria v. alpina* is an endangered subspecies endemic to the alpine regions of Mt. Kosciuszko National Park in New South Wales and Victoria, Australia, where population monitoring is an important part of their conservation.

Two study trials were conducted to assess the most effective marking technique for this species before a regimen was broadly implemented. The first was a preliminary trial to determine the efficacy of PIT tags injected into the coelomic body cavity of the animals, and tag retention was monitored for four months post-injection. After these coelomic PIT tags failed and our animal ethics committee rejected our toe clipping application, we conducted a second study in which we compared the efficacy of toe clips, VIE tags, and subcutaneously injected PIT tags during a six-week trial period.

MATERIAL AND METHODS

Study species.—We raised *Litoria verreauxii alpina* in captivity for 24 mo from wild collected eggs. Animals from two different populations and four clutches were haphazardly selected for each marking regimen. We used 15 animals for each marking trial. They were housed communally (3–9 individuals per terrarium) in 36 × 21 × 21 cm terraria on a gravel substrate with a layer of moss covering half the terraria. Temperatures ranged from 18–22 °C. We watered animals with aged tap water daily and fed crickets three times weekly. Adult *L. v. alpina* are small, with snout to vent length (SVL) ranging from 26.5–38.8 mm and mass ranging from 1.7–5.5 g. We recorded body condition (SVL/mass) before each animal was marked, and each time the animal was re-examined.

Infection control.—During tagging or marking, we sterilized instruments (needles or scissors) with 95% ethanol. We handled animals with nitrile gloves that were changed frequently and always between terraria. Animal enclosures were disinfected every eight weeks with 10% bleach solution, rinsed twice, and allowed to dry for at least 24 h before reuse. We autoclaved gravel substrate before use and after enclosure changes.

Study one.—We tagged and checked animals at two days, one week, eight weeks, and 16 weeks after injection. We checked animals for tag loss and state of healing. All animals were healthy at the start of the experiment. We injected Nano-PIT tags (1 × 8 mm; Nonatec^{TM}, Rodange, Luxembourg) with a 18-gauge needle into the coelomic cavity. We injected tags into the left ventral surface with the PIT tag injected toward the anterior and we applied a veterinary adhesive (Vetbond^{TM}, 3M, St. Paul, Minnesota, USA) to the site of injection. The researchers were well-trained in this tagging method, having successfully performed this procedure on >1000 animals previously.

Study two.—We assessed the efficacy of three marking methods: PIT tag (subcutaneous injections), toe clip, and VIE tag. We checked animals two days after marking and then weekly for six weeks to assess ease of identification and state of healing. We toe-clipped animals according to the Hero (1989) scheme, in which three toes were removed at the 2nd phalange, a maximum of two from each side, and not the second digit on the foot or the thumb.

We also injected two colors of visual implant elastomer, blue and red, into the ventral thigh, subcutaneously, using a 29-gauge needle (Northwest Marine Technology, Inc., Shaw Island, Washington, USA). Between one and five tags were implanted per individual, with a maximum of three into each thigh (Fig. 1). We were supervised by S. Sapsford, who successfully used VIE tagging to mark *L. rheocola* (Sapsford et al. 2013). Each tag was 1–2 mm in diameter and we chose red and blue because they could be visualized without a UV light. We measured tag movement, obscurity, and expulsion at each examination. Tags were obscured when the tag migrated to a highly pigmented portion of the body where that tag could not be visualized, or when two tags combined. Tag expulsion occurred when the tag was expelled from the body through the skin, which we detected through an open wound and we found the expelled tag in the terrarium. Identity of individuals was based on position and order of VIE tags in the thigh. Identification was not possible when tags migrated so that the order of the tags varied from implantation. We took photographs of each animal at each time point to document tag movement and to verify the identity of each animal in combination with body size and enclosure number.

Additionally, we injected Nano-PIT tags with an 18-gauge needle into the left axillary region pointing posteriorly and gently massaged down away from the injection site. Nonatech^{TM} secures their tags in the needles using a bio-safe silicone plug, which enters the animal at point of tag injection. Under ideal circumstances, the silicone is a round and smooth bead that is intended to enter the animal and to remain implanted with the PIT tag. However, when the silicone was placed in the needle, it did not always conform to a smooth bead shape. In Study One, in instances when the PIT tags were expelled from the animal through the injection site, the PIT tag came out attached to the silicone plug, which had been inadvertently glued to the veterinary adhesive. Therefore, in Study Two, we removed the silicone plug prior to implantation and the veterinary adhesive was not used with the aim of reducing the chance of expulsion.

RESULTS

Study one.—We monitored PIT-tagged animals for four months, and during that time, 66.7% (10 of 15) of the animals lost their tags. We recovered all tags from

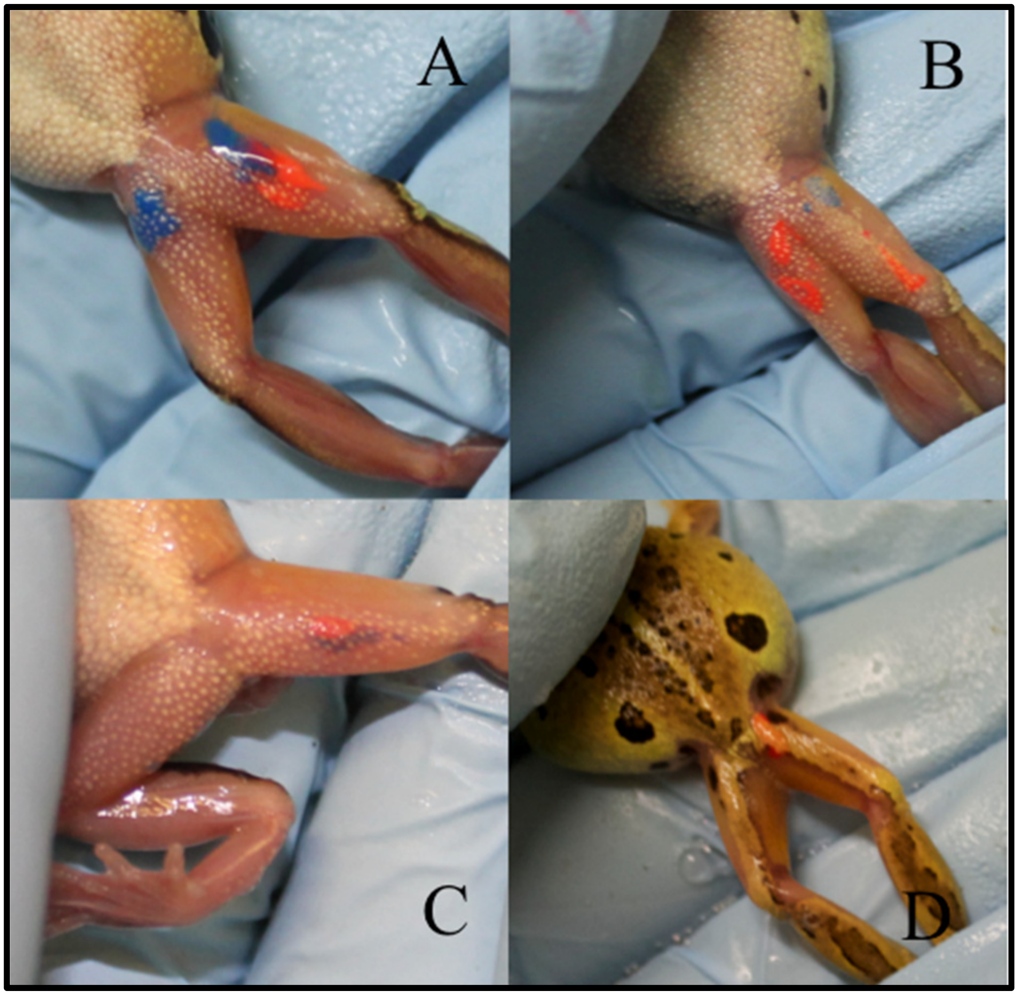

FIGURE 1. *Litoria verreauxii alpina* thighs with VIE tags. A) and B) are individual 1 and C) and D) are individual 2. A) and C) VIE tags on the day of implantation (Day-0). B) Tag recombination at Day 14. D) Tag migration from the venter to the dorsum on Day 14. (Photographed by L. Brannelly).

the animal enclosures. In the first two months after injection, 53.3% of the tags were expelled (eight of 15); 25% of those tags (two of eight) were expelled within the first two days through the site of implantation (Fig. 2). All injection site wounds had healed completely within the first week after tagging.

***Study two.*—**During the six weeks we monitored frogs, 26.7% of the PIT tags (four of 15) were expelled. The tags were expelled across the skin, not through the site of implantation. All injection site wounds had healed completely within the first week after tagging. At week three after implantation, 25% (n = 1) of the expelled tags were expelled; 25% (n = 1) were expelled at week four; and 50% (n = 2) were expelled at week six. All retained tags were successfully read.

We injected 41 VIE tags into the 15 frogs. We correctly identified 18.4% of the 103 total identity checks, and only 6.7% (one of 15) of animals were identified based on VIE tags at all identity checks. At some point during the first six weeks after implantation, 29.2% of VIE tags moved (Fig. 3A), and 63.1% of all identity checks had movement of at least one tag compared to the check before (Fig. 3B), resulting in potential inability to identify the animal based on tags alone. The most common movement was between the dorsal and ventral surfaces, followed by movement to the other leg, tag becoming obscured, and finally tag expulsion (Fig. 3A). Twenty-seven percent of animals expelled tags (eight tags from four animals). Of the tags that were obscured, 17.2% became visible at a later check. Of the tags that migrated away from the site of implantation, 5.8% of tags migrated back so that the animal could be correctly identified again.

Based on toe clips, 96.1% of identity checks resulted in frogs being correctly identified. Although clips remained consistent, in four instances a frog was incorrectly identified due to human error (i.e., removal

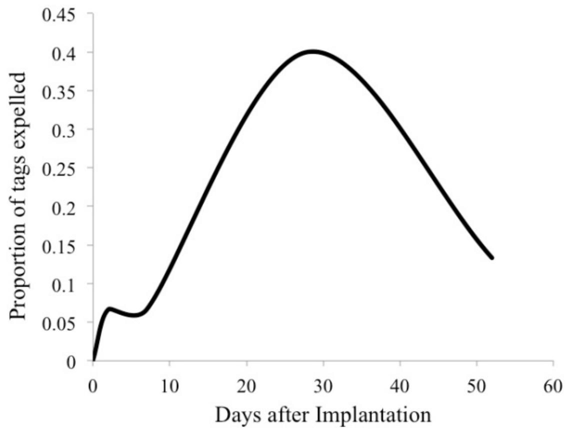

FIGURE 2. The proportion of PIT tags expelled from *Litoria verreauxii alpina* each time the animals were checked. Ten tags were expelled in the first four months (n = 15).

of the second toe on the right hand was recorded as removal of the second toe from the left hand). For most animals, it took fewer than two weeks for wounds to heal (66.7% of animals), but full healing for 20% of the animals took up to five weeks. In 13.3% of animals (two of 15), healing did not occur after six weeks and resulted in whole foot inflammation.

DISCUSSION

The purpose of this study was to assess the mark retention and reliability of three of the most widely used and discussed tagging methods for amphibians to determine the best marking method for *L. v. alpina*. Based on our data, by far the most reliable marking method for identifying *L. v. alpina* was toe clipping, which resulted in 96.1% correct identifications during the six-week second study. However, healing time was lengthy and 13.3% of the animals had infections six weeks post marking. The high infection rate observed in this species in captivity appears to be rare for anurans, and many species heal from toe clipping without complications. Although there are a few reports of high infection rates associated with toe clipping, both in captivity and in wild animals (see Golay and Durrer 1994; Lemckert 1996; Reaser and Dexter 1996; Williamson and Bull 1996), it appears to be species dependent. Toe clipping should be trialed before broadly implemented in capture-mark-recapture studies of a new species.

This study demonstrates that VIE tagging is not a viable marking method for this species as animals were correctly identified based on tags only 18.4% of the time. Because of their extensive subcutaneous lymphatic system, amphibians have little connective tissue between the dermis and the underlying muscle compared to other vertebrates (Farquhar and Palade

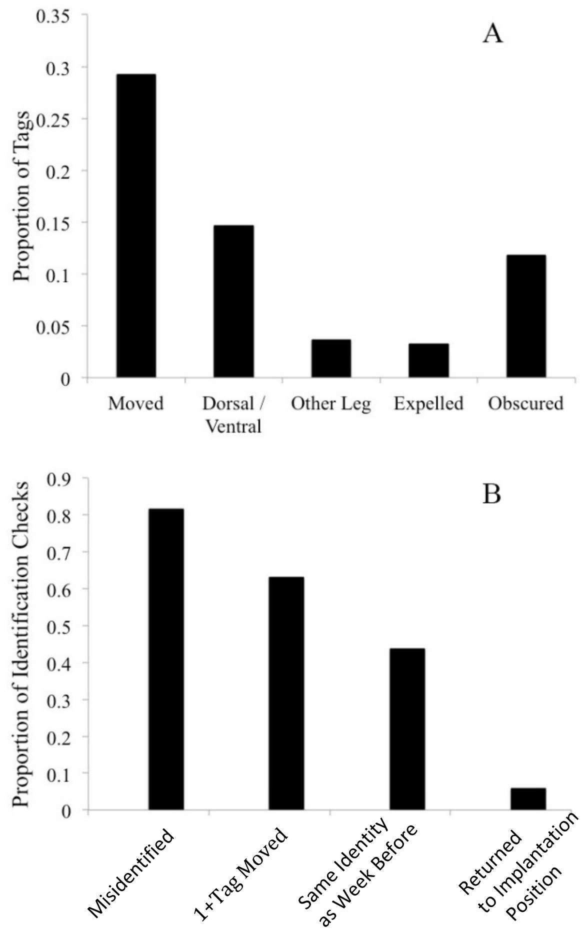

FIGURE 3. VIE tag movement, obscurity, or expulsion that occurred during the course of the experiment (n = 15). A) The proportion of VIE tags that moved during the six week study, and to where tags moved. B) The proportion of identification checks that resulted in misidentification based on VIE tags alone and quantification of tag movement.

1965), which makes subcutaneous tag movement more likely. To combat tag movement, VIE tags should be placed in a region where movement is unlikely. Although other studies suggest low tag movement in the thigh (Moosman and Moosman 2006; Sapsford et al. 2013), this region was not suitable for *L. v. alpina*. To help reduce the risk of tag movement, tagging frogs in the webbing or toes of the hind feet has been suggested (Nauwelaerts et al. 2000; Hoffman et al. 2008), although using this technique in small animals or those with minimal webbing may not be feasible. Success of VIE tags in adult anurans appears to be species dependent and highly variable.

PIT tags do not seem to be a viable option for this frog species, as there was a high expulsion rate from both implantation sites. When injected into the coelomic cavity, 66.7% of tags were expelled within four months (with 53.3% during the first eight weeks), while 26.7% of animals expelled tags within just six weeks of subcutaneous implantation. Both methods have unacceptably low success rates for use in large-scale capture-mark-recapture studies.

PIT tag retention and ideal implant location have been extensively studied in other vertebrate groups (Kaemingk et al. 2011; Hamel et al. 2012, 2013), but not in amphibians. Injection sites that have been successful in fish (Hamel et al. 2012, 2013) and salamanders (Unger et al. 2012), such as sections of muscle that are not directly involved in locomotion, are not large enough to incorporate a PIT tag in most species of frogs. Most researchers inject PIT tags subcutaneously in anurans, even though it has been suggested as a poor tagging location in fish species (Clugston 1996). Blomquist et al. (2008) tested three different dorsal subcutaneous injection sites in a large frog species and found that above the scapula had the highest retention. Multiple studies have found that tags implanted subcutaneously into the dorsum show no evidence of rejection (Brown 1997; Newell et al. 2013). In *L. v. alpina,* injections into the dorsum are impractical because the small size of adults increases risk of accidental damage to the spinal cord during injection. Although subcutaneous ventral sites of injection have been successfully used in other small anurans (Simon Clulow, pers. comm.), *L. v. alpina* seems to expel tags more readily.

PIT tags have been expelled through the digestive tract in species of snake (Roark and Dorcas 2000; Pearson and Shine 2002) and frog (Tracy et al. 2011). Possibly, tags injected into the coelomic cavity in *L. v. alpina* were engulfed and expelled by a similar mechanism resulting in the high failure rates found in this study. Interestingly in this study, the same proportion of animals marked with VIE and subcutaneously injected PIT tags expelled tags during the six weeks. None of the tags were expelled through the entry site, and all resulted in an open wound through the dermis. Because of the way the tags were expelled, we believe that tags would continue to be expelled over time. Tag retention seems to be much lower in *L. v. alpina* than species used in other published studies (i.e., Brown 1997; Newell et al. 2013), but may not be the exception as data from few marking studies are accessible.

Our incorrect identifications of the toe-clipped individuals were based on human recording error, not a failed marking technique. Human recording error is a potential concern for all marking techniques. The error experienced in this study is less than what others have reported for toe clipping (e.g., Kenyon et al. 2009). In this study, full healing occurred within two weeks of the marking procedure, although in toe-clipped individuals the healing time was longer and risk of infection was higher. Although we sterilized instruments prior to toe clipping according to standard protocol, treating animals with topical antibiotics after the procedure could be considered in future experiments to prevent infections.

Laboratory studies of marking efficacy can be adequately translated into the field as long as the mark is retained, and behavior and survival is minimally affected. Mark retention is unlikely to alter between laboratory and field settings but impact on behavior and survival is a concern (Donnelly et al. 1994) as marking can predispose to infection or increase predation (Schmidt and Schwarzkopf 2010). With invasive marking techniques, as long as the animal heals properly, behavior is likely to return to normal (Lemckert 1996; Schmidt and Schwarzkopf 2010; Sapsford et al. 2014).

Conclusion.—This study was the first to assess the efficacy of three popular marking techniques, toe clipping, VIE tags, and PIT tags, in a single anuran species. Based on our results, the most reliable marking method for *Litoria verreauxii alpina* is a toe clip scheme. The least successful marking technique is VIE tags, followed by PIT tags implanted into the body cavity. The second best marking method is PIT tags injected subcutaneously, but retention during even the short term was too low to be useful for mark-recapture studies in which individuals need to be identified with high certainty. Although toe clipping remains the most effective marking method for this species, *L. v. alpina* was susceptible to infection after toe clipping. Therefore, another identification method, such as pattern recognition, is worth assessing as a safer option, although it is time intensive and changing patterns and colors would reduce accuracy (Donnelly et al. 1994; Kenyon et al. 2009).

To be appropriate, a marking method needs to be both effective (in the sense tested in our study) and morally acceptable. Based on non-scientific perceptions, some animal ethics boards are increasingly rejecting toe clipping as a legitimate marking technique (Funk et al. 2005; Phillott et al. 2008; Correa 2013), although other marking techniques have not been thoroughly tested in a wide range of species (Funk et al. 2005; Phillott et al. 2007). Choice of method should be based on evidence; therefore, we urge field researchers and captive managers to publish their successes and failures with marking techniques.

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husbandry. The study was approved by James Cook University Ethics application A1880.

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Laura A. Brannelly has spent the early years of her career in biology studying amphibian declines due to amphibian chytrid fungus. She earned her B.S., B.A., and M.S. from Tulane University in New Orleans, Louisiana, USA. At Tulane University she worked on a number of amphibian chytrid research projects including a clinical drug treatment trial using itraconazole to treat chytridiomycosis. She began her Ph.D. at James Cook University in Townsville, Queensland, Australia, in 2012 where she is studying immunity in amphibians and understanding the mechanisms through which amphibians avoid a chytrid infection. She is also studying how those mechanisms can be incorporated into reintroduction programs and amphibian conservation. (Photographed by Matthew Chatfield).

Lee Berger is a Senior Research Fellow at James Cook University, Townsville, Australia. After completing a veterinary science degree, she earned a Ph.D. on frog diseases in Australia, completed in 2002. Since then she has focused on applied research on chytridiomycosis, including projects on pathogenesis, treatment, diagnosis, mapping, and virulence. (Photographed by Rebecca Webb).

Lee F. Skerratt is a Senior Research Fellow at James Cook University, Townsville, Australia. After completing a veterinary science degree, he earned a Ph.D. on wombat mange at the University of Melbourne, completed in 2001. He has worked as a post-doctoral researcher on sea duck viruses at the National Wildlife Health Center in Wisconsin and as a senior lecturer in veterinary parasitology and epidemiology at James Cook University. In his current position, he leads the One Health Research Group and studies disease affecting biodiversity (e.g., chytridiomycosis) or human health (e.g., Hendra virus). (Photographed by Lee Berger).