A comparison of short-term marking methods for small frogs using a model species, the striped marsh frog (*Limnodynastes peronii*)

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Abstract. We compared three methods of marking individual small frogs for identification in short-term (several days) research using a model species, *Limnodynastes peronii* (the striped marsh frog). We performed a manipulative experiment under laboratory conditions to compare retention times of gentian violet, mercurochrome and powdered fluorescent pigment. Gentian violet produced the most durable marks with retention times between two and four days. Mercurochrome was retained for at least one day by all treated frogs. Fluorescent pigment was either not retained at all or for one day at most which suggests that this marking method may not be reliable for short-term studies where identification is required. No adverse reactions to any of the marking methods were detected in our study. Our findings indicate that gentian violet represents a promising alternative as a minimally-invasive marking technique for studies of small frogs requiring only short-term retention of identification marks.

Keywords

Amphibians, small frogs, minimally-invasive, marking, short-term studies.

Marking of individuals for identification and tracking of movement is critical in population studies as a means of avoiding pseudoreplication and biased estimates of abundance (Corn, 1994; Mellor et al., 2004). For amphibians, commonly used long-term (months to years) marking techniques include toe clipping, branding and tattooing (Donnelly et al., 1994; Halliday, 2006; Ferner 2007). Some studies have employed fluorescent dyes for marking through the use of heat (Ireland, 1973), compressed air (Nishikawa and Service, 1988; Brown, 1997), or abrasion (Ireland, 1991) to allow dyes to penetrate. Other studies have used acrylic polymers, visible implant elastomers (VIE), visible implant alphanumeric (VIA) tags or passive integrated transponder (PIT) tags for marking, all of which involve subcutaneous injection (Woolley, 1973; Davis and Ovaska, 2001; Ferner, 2007; Heard et al, 2008). Visible implant elastomers have also been combined with toe clipping (VIE-C) to improve the reliability of identification (Hoffman et al., 2008; Campbell et al., 2009)

While all of these long-term marking techniques are valuable for amphibian research in that they can produce marks that last for months or years, one disadvantage is that their invasiveness can lead potentially to an increased risk of infection, pain, injury, reduced locomotor performance, behavioural alterations or mortality in frogs (Clarke, 1972; Golay and Durrer, 1994; Davis and Ovaska, 2001; Schmidt & Schwarzkopf, 2010). Furthermore, techniques requiring the use of compressed air may not be suitable for use on very small or fragile frogs (Nishikawa and Service, 1988; Nishikawa, 1990) while PIT tags may also be unsuitable for some frogs smaller than 40mm SVL (Johnson, 2009). In addition, for studies requiring only short-term marking of frogs (i.e. over one to three days), the costs associated with long-term marking techniques are unwarranted. Thus, there is considerable need to develop minimally-invasive, low injury risk marking methods for small frogs for research where marks need only be retained for short periods. Such research needs include visual encounter or trapping studies conducted over a period of several days or nights and short-

65 term studies of animal movement and behaviour. Pattern mapping of individual markings
66 (Donnelly et al., 1994; Halliday, 2006; Ferner, 2007) offers a minimally-invasive recognition
67 method that has been used successfully in large-scale studies (see Gill, 1978; Davis &
68 Grayson, 2007), but this technique is not suitable for species that lack identifiable individual
69 markings or where temporal shifts in patterning occur (Johnson, 2009). The technique may
70 also be time consuming and difficult to use reliably on large populations (Johnson, 2009).
71 In this study, we performed a manipulative experiment under laboratory conditions to
72 compare the retention times of three short-term, minimally-invasive skin marking methods
73 for frog identification. The methods were: the application of one of two medical dyes, gentian
74 violet and mercurochrome, used for the treatment of minor injuries and infections in humans
75 and animals, or the application of fluorescent powder, all without skin abrasion, heat or
76 compressed air.
77 For the purposes of this study, we focussed on a model species representative of small
78 frogs, *Limnodynastes peronii* (the striped marsh frog), which has a body size of 46-73mm
79 (Tyler & Knight, 2009). Additionally, adults of the species display average size and life-
80 history traits common to many Australian frog species.
81 Frogs were obtained from captive bred stock produced by a licensed amphibian
82 breeder and all were transferred to a licensed amphibian keeper at the conclusion of the
83 experiment for ongoing care.
84 In the laboratory, individual frogs were each housed separately in identical plastic
85 aquaria (length 31 cm, width 18 cm, height 21 cm). The aquaria contained water and land
86 areas; leaf litter, bark and aquatic plants provided retreats and environmental enrichment.
87 Substrate for land areas consisted of moistened coconut husk fibre (Exo-Terra Plantation
88 Soil™, Exo-Terra) which allowed frogs to burrow beneath leaf litter. The frogs were fed

every 2-3 days on live crickets, dusted with vitamin and calcium supplement powder and were maintained in these conditions for 1 week prior to the beginning of the experiment.

Frogs were divided randomly into one control (unmarked) and three treatment groups with five animals in each of the four groups. Frogs in the treatment groups were marked with either 1% weight/volume (w/v) gentian violet, 2% w/v mercurochrome or yellow powdered fluorescent pigment (Glow Paint Industries, Glow in the Dark Pigment, median particle diameter: $d50 \leq 6.0 \pm 0.5$ μm) on 23 December 2009. Control group frogs were handled and weighed but not marked in order to control for the procedural technique. Marks were applied by using a cotton bud to paint a whole foot. No attempt was made to abrade the skin in order to increase penetration of dye or pigment; however, gentle pressure was used to assist in the application of fluorescent pigment. Visibility of marks was checked once daily until all marks had disappeared. Visual assessments of mark presence or absence were conducted with frogs remaining in aquaria. Fluorescent pigment marks were assessed under both ambient light and with a UV light source (Loon UV Mini-Lamp™, Loon Outdoors). All inspections were conducted by the same observer at a distance of approximately 30cm from each frog. Observations were made at the same time each day.

All frogs were observed for 60 minutes following application of marks to check for adverse reactions. Normal, resting behaviour resumed within 10 minutes of the application of marks for all animals. We visually inspected each frog twice daily from 23 December 2009 until 2 January 2010 to check for signs of ill health. Frogs were weighed immediately prior to marking and five days after marking to identify any differences in weight loss or gain between control and treatment groups. Normal, resting behaviour resumed within 10 minutes of the application of marks for all animals. No signs of pain or irritation in response to marking were observed and no signs of ill health were detected at any time over the course of the experiment.

Data for mark retention (presence or absence of marks at each inspection) and weight change were analysed using separate one-way ANOVA in SPSS v17. We used Fisher's least significant difference (LSD) post-hoc tests to determine whether there were differences in mark retention times between the experimental groups. This included an analysis of whether retention times differed significantly from the control group. This is important in determining whether marking provides any advantage in identifying individuals (e.g. recaptures) over not marking. Retention times of marks applied to frogs differed significantly among the experimental groups ($F_{3,16} = 19.93$, $P < 0.0001$). Mean retention times for each of the three treatment groups differed significantly from the control group (LSD tests: gentian violet $P < 0.0001$, mercurochrome $P < 0.05$, fluorescent pigment $P < 0.05$). Markings using gentian violet were retained for between two and four days (mean ± SE = 2.4 ± 0.4). This was significantly longer than retention times for both mercurochrome (LSD test: $P < 0.0001$) and fluorescent pigment (LSD test: $P < 0.0001$). Nevertheless, mercurochrome was retained for at least one day by all frogs (mean ± SE = 1.0 ± 0.0) while fluorescent pigment was either not retained at all or for one day at most (mean ± SE = 0.8 ± 0.2). This suggests that fluorescent pigment may not be reliable for short-term studies where identification is required. However, powdered fluorescent pigment remains a useful tool for tracking amphibian movements as this approach relies on animals shedding pigment to create a trail detectable by ultraviolet light (Windmiller, 1996; Birchfield & Deters, 2005). Detectability of gentian violet marks may have been assisted by the fact that gentian violet was observed to contrast more strongly with striped marsh frog colouration than mercurochrome. Further investigation is required to determine if this is an important factor in the choice of marking agents.

All groups of frogs gained weight during the experimental period with no significant differences among groups in weight change ($F_{3,16} = 0.449$, $P > 0.05$), which suggests none of the marking methods tested here lead to changes in animal condition. This is important

because marking methods should have minimal effects on survivorship or behaviour (Mellor et al., 2004; Ferner, 2007).

Although our experimental work was based on one model frog species, our findings indicate that skin staining with gentian violet represents a promising alternative to more invasive techniques for studies where long-term mark retention is not required. To build on this finding, we recommend both further testing with gentian violet on a range of amphibian species to assess the suitability for general amphibian use as well as testing with additional dye types to determine their potential for longer retention times of marks. Further studies should also be conducted to test for longer-term reactions to skin staining.

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