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An effective way to mark cohorts of juvenile terrestrial direct-developing frogs

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Abstract. Marking juveniles of terrestrial direct-developing frogs is challenging because of their small size (< 18 mm) and fragility. This difficulty has limited studies on demography or population dynamics where empirical data on the survivorship of juveniles or their recruitment to adulthood are missing. In a controlled laboratory experiment, we tested the survivorship of wild-caught juvenile *Eleutherodactylus coqui* Thomas, 1966 to marking with a single colour visual internal elastomer (VIE) in the thigh, with and without additional ventral skin-swabbing for disease or microbiome monitoring. Results revealed 100% survival in all groups, and all juveniles remained unharmed, moved freely, and fed actively during three days after treatment, suggesting that this type of manipulation does not cause direct mortality. After 17 months of the experiment, we have recaptured 11% of the marked juveniles as adults, indicating that they can survive to recruitment age. We propose the use of a single VIE colour as a method to mark and follow date-specific cohorts of juvenile direct-developing frogs or young metamorphs until they reach older and larger age classes. This marking method can be used safely together with skin swabbing and provide valuable information for studies on population biology and age-specific response to environmental or disease stressors.

Keywords. Amphibians, direct developers, juveniles, cohorts, marking, VIE

Amphibians are difficult to mark permanently because their skin is thin and permeable, making them vulnerable to intoxication (by paints or polishes that may work well for reptiles) or skin rupture when using external tags (Donnelly et al., 1994). A variety of techniques have proven effective for long-term marking and identification of amphibian adults, such as toe-clipping (e.g., Woolbright, 1996; McCarthy and Parris, 2004; Phillot et al., 2007; Longo and Burrowes, 2010; Burrowes et al., 2011; Grafe et al., 2011), pattern mapping or photography (e.g., Arntzen et al., 2004; Carafa and Biondi, 2004; Del Lama et al., 2011; Šukalo et al., 2013), passive integrated transponder (PIT) tagging (e.g., Camper and Dixon, 1988; Pope et al., 2001; Arntzen et al., 2004; Schulte et al., 2007), genotyping with microsatellites (Ringler et al., 2015), and visual internal elastomers (VIE), a UV-fluorescent polymer injected subcutaneously (e.g., Fogarty and Vilella, 2002; Moosman and Moosman, 2006; Campbell et al., 2009). The latter method has also proven successful when marking anuran larvae (Donnelly et al., 1994), and yielded high survivorship of *Bufo bufo* Linnaeus, 1758 tadpoles, especially when marked in in the upper tail (Iannella et al., 2017).

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Marking young metamorphs or juveniles of direct-developing frogs is especially challenging due to their small body size. The biology and ecology of *Eleutherodactylus coqui* Thomas, 1966, a terrestrial direct-developing frog native to Puerto Rico, is well known (reviewed by Joglar, 1998), and although several studies have addressed population densities of this species (e.g., Stewart and Woolbright, 1996; Woolbright, 1996; Fogarty and Vilella, 2002; Burrowes et al., 2004; Woolbright et al., 2006), there are no studies on demography, nor models on its population dynamics based on empirical data on juvenile survivorship and recruitment. Perhaps one of the reasons for this is the difficulty encountered when trying to mark the small juveniles. In the highlands, young *E. coqui* froglets range in body size from 4–18 mm, and lowland specimens may be even smaller (Joglar, 1998). While adults and subadults of *E. coqui* have responded well to mark-and-recapture studies using a variety of toe-clipping codes (Stewart and Woolbright, 1996; Woolbright, 1996; Burrowes et al., 2004, 2011), we found it extremely difficult to toe-clip juveniles because the manipulation necessary may result in damage to individuals and possibly death. As far as using a unique code consisting of a VIE colour combination to mark *E. coqui* frogs is concerned, our experience was similar to that of Fogarty and Vilella (2002). While we were successful at recapturing adult and subadults, we failed to recapture juveniles. Thus, to our knowledge, an effective method to mark juveniles of *E. coqui* is currently unavailable.

We here present the results of an experiment to test the viability of a single colour VIE injection as a method to mark juveniles of terrestrial direct-developing frogs. The purpose of this method is not to identify individuals, but rather to recognise and follow young cohorts until they reach an older, reproductive age class. Thus, even if the elastomer moves between body parts, as has been reported for adults marked with multiple colour codes (Moosman and Moosman, 2006; Bainbridge et al., 2015), a single colour VIE would still allow for the identification of a cohort marked during a particular dated period. We hypothesised that survivorship would be compromised by the degree of manipulation, and that larger (older) juveniles would fare better than the smaller (younger) individuals.

An effective technique to safely mark immature froglets that allows for their identification as they survive to reproductive age classes is necessary to inform studies on the population dynamics of amphibians (Skelly and Richardson, 2010; Phillot et al., 2013). In current times, global amphibian declines due to disease (Skerrat et al., 2007; Scheele et al., 2019) and/or other factors associated with global climate change phenomena (Stewart, 1995; Cushman, 2006; Burrowes, 2009; Becker et al., 2010) are evident. Thus, baseline population biology studies including recruitment information are critical to understanding the mechanisms by which diverse factors (anthropogenic, biotic, or abiotic) may contribute to the decline of populations and potentially lead to the loss of biodiversity.

Materials and Methods

We collected juvenile *E. coqui* (also referred to as froglets) at El Yunque National Forest in Puerto Rico (18.3016°N, 65.7854°W) at an elevation of 661 m, where a population has been censused and studied for the incidence of the pathogenic chytrid fungus *Batrachochytrium dendrobatidis* Longcore et al., 1999, for over 20 years (Burrowes et al., 2004, 2017; Longo and Burrowes, 2010). Froglets of snout–vent lengths (SVL) ranging from 9–18 mm, were captured in the wild and brought to the laboratory (Fig. 1A). Because collecting many juveniles of *E. coqui* at one time can be challenging, especially after the devastation to the forest caused by Hurricane Maria (Burrowes et al., 2021), we worked with smaller sample sizes but conducted three independent trials of this experiment in June 2018, November 2018, and August 2019. In each trial a total of 18 juveniles were divided into three treatment groups, each including six juveniles within the full range of body sizes: two of SVL 9–12 mm, two of SVL 12.1–15 mm, and two of SVL 15.1–18 mm. Treatments consisted of (1) VIE only, with juveniles marked using a single VIE in yellow colour injected subcutaneously on the left thigh using a small, 29-gauge syringe (Fig. 1B); (2) VIE + Swab, with frogs marked as above and also gently swabbed four times in the ventral area using cotton swabs (Medical Wire); and (3) control, with frogs not marked or swabbed. We followed company instructions (Northwest Marine Technology Inc.) for preparation of VIEs for injection, a procedure that requires mixing the coloured polymer with a fixative, and we used a fresh pair of gloves for each individual throughout the experiment. Swabbing was included in the experimental design as an additional manipulation, because we are especially inter-ested in monitoring the prevalence and infection intensity of *B. dendrobatidis* in hosts, which is done by obtaining a sample of epidermal tissue on cotton swabs (Hyatt et al., 2007). It is important to highlight that swabbing of *E. coqui* juveniles should be done gently and not exceed five strokes to prevent rupture of the skin (P. Burrowes, unpubl. data). Skin swabs can also be used to sample for other pathogens, as well as the general microbiome (e.g., Longo and Zamudio, 2017; García-Recinos et al., 2019). Thus, determining if simultaneous VIE marking and swabbing was safe for froglets would be useful when considering these methods for future studies.

We kept froglets overnight in the plastic bag, in which they were collected in the field, and treatments were applied the next morning in the laboratory. Afterwards, each juvenile was housed separately in a 15 x 10 cm plastic container previously cleaned and rinsed with a 10% chlorine bleach solution. We perforated six holes into the lid of the containers to allow for adequate air circulation, and we lined the container bottoms with clean white paper towels sprayed with aged tap water to maintain humidity. The side of each container was labelled with the treatment type and the froglet's SVL. Containers were then placed in our animal care unit at room temperature (25°C) with 12-hour light/dark regime for three days. Every morning on these three consecutive days, each froglet was checked for survivorship, stimulated with a thin glass rod to examine its ability to respond to stimuli and test for lethargy or limping, sprayed with aged tap water, and fed with approximately ten fruit flies. Faeces and unconsumed flies were removed daily from the containers. On the fourth day after the treatment, froglets were returned to the site where they had originally been collected. In subsequent monthly amphibian monitoring efforts at the same site, we looked for marked juveniles, subadults, or adults with single yellow VIE markings and, when these were found, we noted the position of the VIE in the body.

Results

Treatments resulted in 100% survivorship of a total of 54 juveniles during the three independent trials of the experiment. We did not encounter any problems marking or swabbing the froglets. Regardless of the treatment received, all individuals responded to stimuli by moving away and without any indication of lethargy or limping that would suggest a selective disadvantage in the wild. While larger individuals consumed all their prey (with only two exceptions when two flies were left uneaten), the mid-sized and smallest juveniles left on average 5 and 3 flies, respectively, every time they were fed. We found no relation between treatment and food consumption that would suggest that marking and/or swabbing affected the appetite of juvenile frogs.

Contrary to our hypothesis, we did not find an effect associated with body size or degree of manipulation. Because juvenile *E. coqui* are fragile and typically difficult to maintain in the laboratory (Colon-Piñeiro et al., 2017), we assumed that if they survived for three days after treatment, unharmed and with no signs of lethargy or limping, the treatments could not be considered a direct cause of mortality. Seventeen months after the last experiment, between August 2019 and January 2021, we had recaptured six of the 54 VIE-marked juveniles (now as adults) at our study site in Puerto Rico. In three of the six recaptured individuals, the yellow marking had moved from the thigh to the flank area (on the same side in two of the individuals, and to the opposite side in one), indicating that in *E. coqui* VIE markings do move around. Thus, the use of multiple VIE colour codes in specific body parts to identify individuals can lead to confusion as has been reported for other frogs (Moosman and Moosman, 2006; Bainbridge et al., 2015).

Discussion

We showed that marking juveniles of terrestrial direct-development frogs with a single VIE colour did not kill or cause any damage to *E. coqui* froglets. The fact that they remained healthy and consumed fruit flies readily for three consecutive days suggests that the VIE marking method, as well as the manipulation required for marking in this manner and even simultaneously swabbing their venter, does not cause mortality. However, juveniles of *E. coqui* are prey to many invertebrates (Stewart and Woolbright, 1996; Joglar, 1998) and studies have shown that this age class is the most vulnerable to chytridiomycosis (Longo and Burrowes, 2010; Burrowes et al., 2017). Thus, if field studies yield low recapture rates of juveniles marked with a single VIE colour and/or swabbed, it may be the effect of death from natural causes rather than due to the marking technique. Nonetheless, 17 months after the experiments in the lab, we obtained an 11% recapture rate for single-VIE marked juveniles, suggesting that this kind of cohort marking can inform recruitment into adulthood. A study on *Allobates femoralis* in French Guyana, revealed a much lower survival rate (3.72%) from tadpoles to adults, as identified via microsatellite genotyping (Ringler et al., 2015). Even though our recapture data are still preliminary, they suggest that juveniles marked with a single VIE may readily survive to adulthood.

Marking juveniles of terrestrial direct-development frogs with a single VIE colour allows for the identification of young cohorts in a population. For example, in a particular study, one can choose to mark all juveniles within a dated period with a yellow VIE while subadults are marked with blue, and use different colours for the next year. By distinguishing the years, or the seasons when juveniles occur in a population, we can then use data from capture-recapture methods to estimate survivorship from one age class to the next and make predictions about the reproductive growth of the population based on empirical data.

Since both subadults (SVL 18–28 mm) and adults (> 28 mm) of *E. coqui* can be marked and effectively identified when recaptured using toe-clipping, or even more accurately with mini (1.4 x 8 mm) passive integrated transponders (Burrowes and Aleman, unpubl. data), we would need to rely on VIE marking only for cohorts of juveniles. With individuals of all age classes effectively and safely marked, we would be able to learn about growth rates at different stages, identify age classes that are more susceptible to stressors (e.g., disease, hurricanes, droughts, forest fragmentation, etc.), and ask a variety of ecological and evolutionary questions informed by data on population dynamics. From a conservation standpoint, these studies could advise management decisions that may result in the protection of species before they fall into critical numbers that merit listing them as vulnerable, threatened, or worse by the International Union for the Conservation of Nature. In conclusion, based on the positive results of our experiment, we recommend the use of a single colour injection of VIE as an effective way to mark (and recapture) date-specific cohorts of juveniles of direct-developing frogs. This method can be used in conjunction with swabbing the ventral area of individuals and may be applied to young metamorphs of other species of anurans.

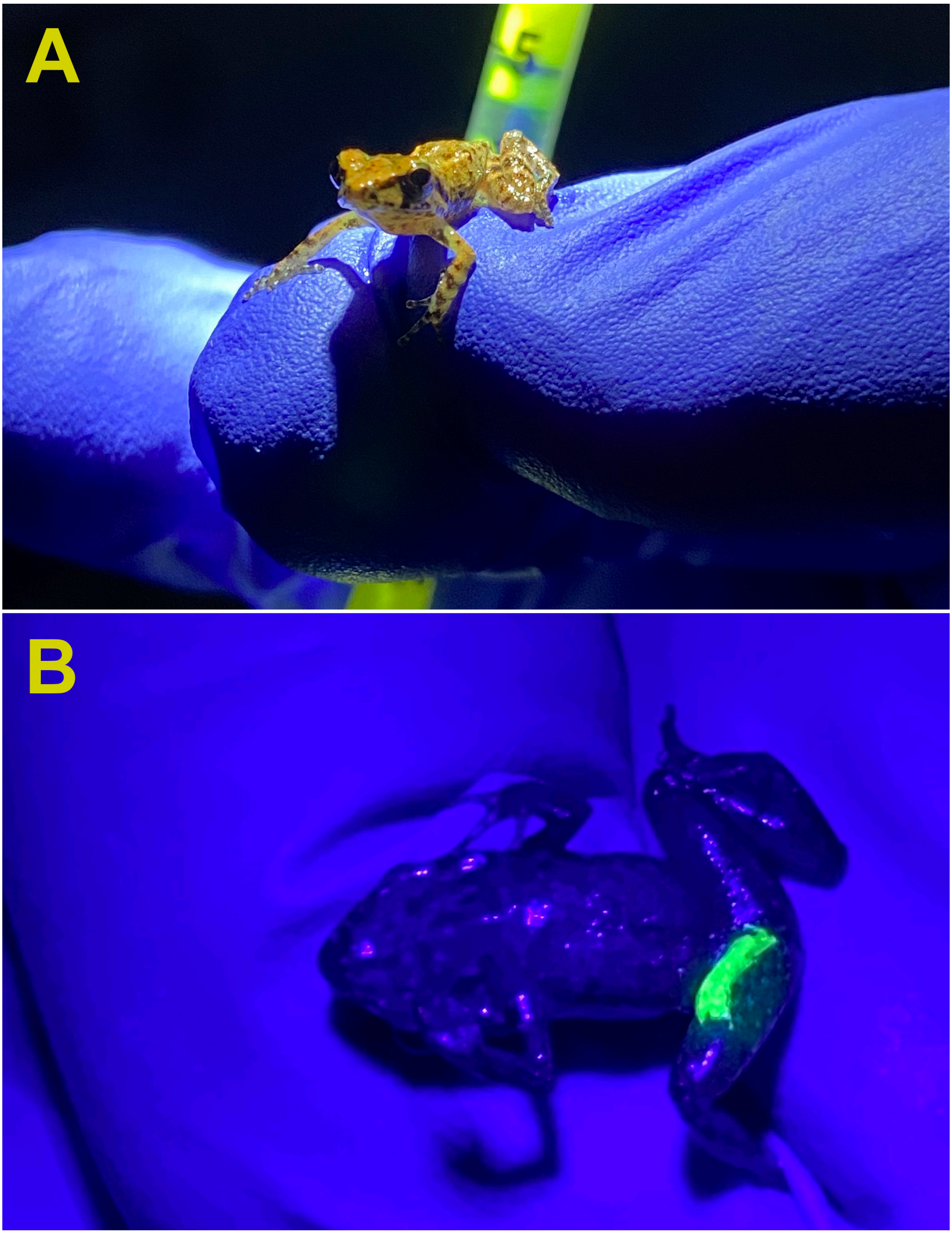

Figure 1. (A) Juvenile of *Eleutherodactylus coqui* before receiving one of the treatments in the laboratory, showing its small size. (B) View of a juvenile *E. coqui* under UV light, showing the fluorescence of its single yellow VIE marking in the thigh.

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