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Correspondence

Marking tadpoles with Visible Implant Elastomer (VIE) tags: methods for improving readability and decreasing mortality

Mattia Iannella[1], Lorenzo Liberatore[1] & Maurizio Biondi[1]

[1)] Department of Health, Life and Environmental Sciences, University of L'Aquila, 67100 Coppito-L'Aquila, Italy

Corresponding author: Mattia Iannella, e-mail: mattia.iannella@univaq.it

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The Global Amphibian Decline is nowadays one of the most important challenges in the animal biodiversity conservation. Understanding population dynamics in amphibians is a crucial matter for their protection (Blaustein & Wake 1995, Alford & Richards 1999, Gardner 2001). Eggs, larvae and tadpoles represent vulnerable stages of their life both because of natural causes (Crump 1984, Werner 1986) and/or anthropogenic activities (Carey & Bryant 1995, Lefcort et al. 1998, Broomhall 2002, Cushman 2006, Bernabò et al. 2008). Furthermore, toadlets are a representative age class giving important information about the structure and the reproductive potentiality of a given population (Skelly & Richardson 2010).

Although catching tadpoles may be easy, for example by funnel trapping or dip-netting in ponds, lakes and small wetlands (Scott & Woodward 1994, Skelly & Richardson 2010, Bower et al. 2013), estimating their numerical consistency usually is difficult. Different methods were proposed to assess amphibian populations numerically, but only a few are applicable to estimate the density of the tadpoles. Among them, for example, Capture-Mark-Recapture method (CMR) allows the identification of individuals or groups of individuals through time (Jung et al. 2002, Govindarajulu & Anholt 2006, Grant 2008). To perform a correct CMR study, marks must not affect the survivorship, the development phases or the normal life activities of the animals but rather be stable and readable as long as possible (Grant 2008, Ferner 2010).

Marking techniques commonly used for adult frogs and salamanders, such as toe-clipping (e.g. Phillot et al. 2007, McCarthy & Parris 2004), pattern mapping (e.g. Arntzen et al. 2004, Carafa & Biondi 2004, Šukalo et al. 2013) and passive integrated transponder (PIT) tagging (e.g. Pope et al. 2001, Arntzen et al. 2004, Schulte et al. 2007), are logistically difficult to apply to the amphibian larvae. Particularly, tail clipping in larval stages is not a recommended marking method, because it may influence negatively the locomotion and the survivorship of anuran and caudate tadpoles (Turner 1960, Guttman & Creasy 1973, Morin 1985, Arntzen et al. 1999). Other methods, such as tadpole dyeing, are not reliable for a long period (Ferner 2007); for example, Neutral Red (Herreid & Kinney 1966, Guttman & Creasy 1973, Jung et al. 2002) usually disappears after few days (Guttman & Creasy 1973) and affects the growth rate of the tadpoles (Travis 1981).

Several researchers used the VIE tags, a UV-fluorescent polymeric material, for their studies on anurans and caudates (e.g. Nauwelaerts et al. 2000, Davis & Ovaska 2001, Moosman & Moosman 2006, Heemeyer et al. 2007, Hoffmann et al. 2008, Bull 2009, Campbell et al. 2009, Márquez-García et al. 2010, MacNeil et al. 2011), but there are few contributions on the use of VIE tags in tadpoles (Anholt et al. 1998, Pfennig & Murphy 2000, Belden 2006, Grant 2008, Bainbridge et al. 2015), and even less studies about readability, tag retention and migration, or possible effects on the larval development or on the survival rate – in a word: reliability – of this technique. Anholt et al. (1998) tested VIE tags only on tails as a tag position, using different colours, both in *Pelophylax lessonae* and *Rana temporaria*. Pfennig & Murphy (2000) observed possible larval mortality during marking procedure on *Spea bombifrons* and *S. multiplicata*, while Belden (2006) marked *Rana sylvatica* tadpoles with three different colours, in dorsal position, but he did not report any observations on the VIE tags effects on the treated animals. Grant (2008) tested survival, tag loss or movement and readability of two-colours tail tag in *Lithobates sylvaticus* tadpoles and tags near-limb in larvae of *Eurycea bislineata*. Finally, Bainbridge et al. (2015) analyzed different effects of the VIE tags marking in ventral area on *Ranoidea aurea*, considering time of metamorphosis, mortality, readability and tag movements.

In this paper, we report the results of our marking experience using the Visual Implant Elastomer (VIE) tags. The aim of our study is to assess differences among different VIE injection sites in tadpoles and toadlets of *Bufo bufo* considering six different tag body positions and reporting the observed changes in the survivorship of individuals, tag migration and readability of the marking through time.

To avoid possible impacts on endemic, rare and/or protected anuran species occurring in Italy, we chose the common toad *B. bufo* (Linnaeus, 1758) for this study, which is widespread in Europe, Asia and parts of North Africa and abundant in Italy and in the Abruzzo region (Soccini & Ferri 2007). Common toad is classified 'Least Concern' by IUCN Red List Category & Criteria (Agasyan et al. 2009). In addition, its wide adaptability to different habitats, including gardens, parks and other human-modified territories, and to different altitudes, from the sea level to 3,000 m a.s.l. (Agasyan et al. 2009), makes this species an easy and ideal model for many researches.

Bufo bufo egg strings were collected in small ponds around L'Aquila city, Italy, in April 2014, and put in a raising tank filled with aged tap water (200 L) to which were added 10 liters of pond water used as a starter for bacteria and algae, filtered to avoid presence of possible predators or parasites of *B. bufo* eggs or larvae. Larvae were kept in the raising tank until the stage 24 sensu Gosner (1960), when the *B. bufo* larvae measured 9–12 mm in snout–vent length (SVL). Then the larvae were moved to five aquariums (150 × 65 × 55 cm) each filled with 300 L of aged tap water, and set up with an aerator, a neon light and a UV-B lamp (ReptiStar UV-B 26W, 5%) simulating solar light. Seven smaller plastic tanks (sub-aquaria), with holes all around for the water circulation, were put in each aquarium (Fig. 1). In addition, a floating land zone with gravel, soil and moss, with branches to connect the "terrestrial" and "aquatic" environments was built into each tank (Fig. 1) which was also provided with the same amount of dead leaves and chops of willow and poplar branches (20 g) as food for the larvae. After metamorphosis, the toadlets were fed with adults of *Drosophila melanogaster* (Insecta, Diptera).

We marked six larvae for each of the six tag positions, plus a control group, for a total of 42 tadpoles for aquarium; sub-aquaria (seven for each aquarium) were used to host the different tag groups. We replayed this scheme in five aquaria, for a total of 210 tadpoles (Fig. 1). A digital caliper (resolution 0.01 mm) was used to assess the SVL in larvae and toadlets.

Larvae were marked by one right-handed operator with Visible Implant Elastomer (VIE), a technology based on a two-part colored polymer reactive to UV light and biologically inert (Northwest Marine Technology 2000); before marking, a 500 mg/L buffered (pH = 7.00) solution of tricaine methylsulfonate (MS-222) was used for anaesthetizing the tadpoles to avoid possible movements injuring themselves and making not accurate the injection of the tag (Anholt et al. 1998, Grant 2008, Bainbridge et al. 2015). Partial anesthesia was preferred because complete anesthesia has long recovery time that may be uncomfortable, especially for large-scale experiments requiring many captures and markings in the open field; for this purpose, five seconds of immersion in the MS-222 solution resulted enough to perform the mark. Syringes gauge 29, provided by the VIE kit, were used to administer the mark.

Bufo bufo tadpoles were marked in six different body positions (Fig. 2): group A (upper tail membrane, near the tail musculature), group B (lower tail membrane, near the tail musculature), group C (dorsal area of body), group D (ventral area of body), group E (right side of body) and group F (left side of body).

These six groups were compared with an unmarked control group (G), which contained tadpoles treated similarly to the other groups, anaesthetizing them and inserting the needle but without injecting VIE. The mark for the groups A–F consisted in a continuous thin line of yellow VIE about 2 mm long, at the center of the six body positions considered; after the marking, the larvae were moved to their respective sub-aquaria and checked daily. SVL was taken when the stage of tadpoles (sensu Gosner) changed: first measure on day 10, when tadpoles started to develop toes (stage 31); second measure on day 14, when tadpoles completed the toes development (stages 36–38). On day 26 most of tadpoles completed metamorphosis; the adults were still checked for the following three weeks (days 33, 40, 47).

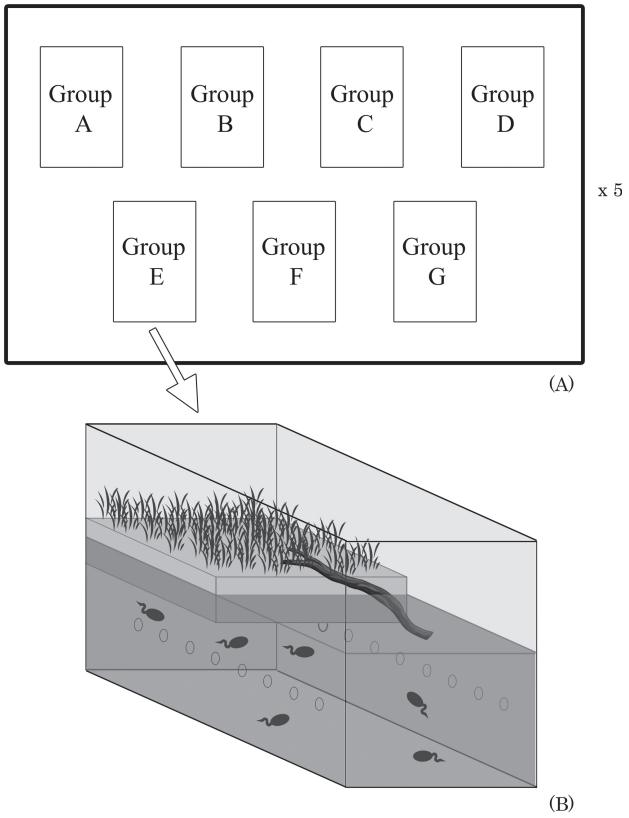

Figure 1. Experimental aquarium scheme: (A) aquarium with 7 sub-aquaria inside, one for each tag group considered; (B) detail of a single sub-aquarium.

Every time the larvae were measured, the marks were also checked in order to evaluate readability and possible anomalies in the behavior of the VIE tags; this check was made with the UV-light provided in the NMT kit. Individuals that did not change mark position during the experiment were named "cis-", while the individuals that showed migration of the tag (from one position to another) were named "trans-".

Statistical analyses were performed and graphics produced using the NCSS version 9.0.7 package for Windows (Hintze 2013). We performed Kaplan-Meier survival curves for estimating the probability of surviving for every time-check considered (day 1, 10, 14, 26, 33, 40 and 47; Rich et al. 2010) and the log-rank test within the pairs formed by each tagged group versus the control (i.e. group A vs group G, group B vs group G, …, group F vs group G), to compare the estimates of the hazard functions, considered as the conditional probability of dying at time t having survived to that time (Bewick et al. 2004). To test for differences in SVL between the seven differently treated tadpole groups at the beginning of the experiment, a one-way ANOVA with SVL as dependent and marking type (treatment) as independent variable was performed. Mann-Whitney U test was also performed to assess possible significant differences between each marked group and control for time of metamorphosis.

Tadpoles used for the different treatments (markings, control) did not differ in their SVL in the beginning of the experiment (ANOVA: DF = 34, F = 1.135, P-level = 0.294; Supplementary Fig. 1). Survival of individuals for each group of marking (A–F) versus the control group (G) was visualized using the Kaplan-Meier curves (Fig. 3). Group B showed the highest rate of mortality, with a Standardized Z-value equal to 4.481 (P-level = 0.000; Fig. 3). Overall, tags on tail (group A and B) showed very low percentage of survival between Gosner stages 36–38 and 45–46 (Fig. 3). The rates of mortality did not show significant differences in the groups C, D and E if compared to the control group G, with standardized Z-values 0.040 (P-level = 0.968), 0.273 (P-level = 0.785), 1.308 (P-level = 0.191) respectively (Fig. 3). For the groups A and F, the respective standardized Z-values were 2.076 (P-level = 0.038) and 2.199 (P-level = 0.028) (Fig. 3). Hazard Function curves (Fig. 4) confirmed the highest hazard rate for the group B, and the lowest rates for the groups C and D (log-rank test and P-level: BG = 19.087, 0.000; CG = 0.002, 0.969; DG = 0.074, 0.786).

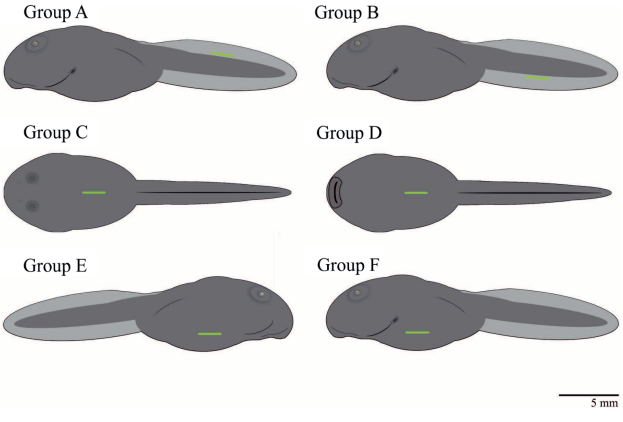

Figure 2. Tag positions: Upper tail membrane, near the tail musculature (group A); lower tail membrane, near the tail musculature (group B); dorsal area of the body (group C); ventral area of the body (group D); right side of the body (group E); left side of the body (group F).

The number of individuals for each marked and unmarked groups, divided into five sub-categories, is shown in Supplementary Figure 2. In Supplementary Table S1, a detail of the number of individuals for each tag category through time is also reported. Because we consider a "migration" as a substantial tag shift which may give a reading error (e.g. a VIE injected in C position is found some time after as it was injected in E position), the "tail" tags (groups A and B) show an obvious elevated trans-trend in toadlets because of the reabsorption of tail. In these cases, the tag was lost or migrated in different positions, preferably in cloacal area. In addition, "dorsal" tag (group C) shows a high rate of mobility also during tadpole phase (46.67%). Conversely, the "ventral" tag (group D) exhibits a lower rate of tag mobility in tadpole phase (6.67%) and a high rate of tag retention in toadlet stage (53.33%) (for further details, see Supplementary Table S2).

About the "right side" tag (group E), we observed the highest number of tag retention in toadlets (56.67% of the total individuals), while the "left side" marked individuals (group F) had a high rate of tag migration in tadpole stage (23.33%); all the percentages of the individuals retaining the same tag position through time are shown, for each group, in Supplementary Figure 3. The elastomer of VIE tags was lost, or changed in form and/or position, in several animals during the sampling period and after 40 days, when tadpoles metamorphosed, the percentage of retention was much lower than the starting day of the experiment.

In accordance to Grant (2008), no significant differences were observed in time of metamorphosis among marked and control tadpoles/toadlets (Mann-Whitney U test: groups AG, P-level = 0.082; BG = 0.060; CG = 0.254; DG = 0.225; EG = 0.410; FG = 0.860).

Our results suggest that markings with VIE tags may influence the survival in tadpoles and toadlets of *B. bufo*, particularly when the tadpoles are tagged on the tail, especially in the upper part, increasing the mortality rate during the metamorphosis. That could be due to the interference of the VIE tags during the process of the reabsorption of the tail and re-arrangement of the tissues and internal organs; future histological studies are needed to confirm this hypothesis. Our results partially agree with Grant (2008), who reported a rate of mortality equal to 30% in the tadpoles of *Rana sylvatica* before the metamorphosis, marking them with VIE tags of two different colours in position between the tail and the dorsum just above the tail musculature. Marking on left side of the body (group F) is also a position causing a high rate of mortality during metamorphosis, probably due to the proximity of the operculum with the insertion point of the VIE tag.

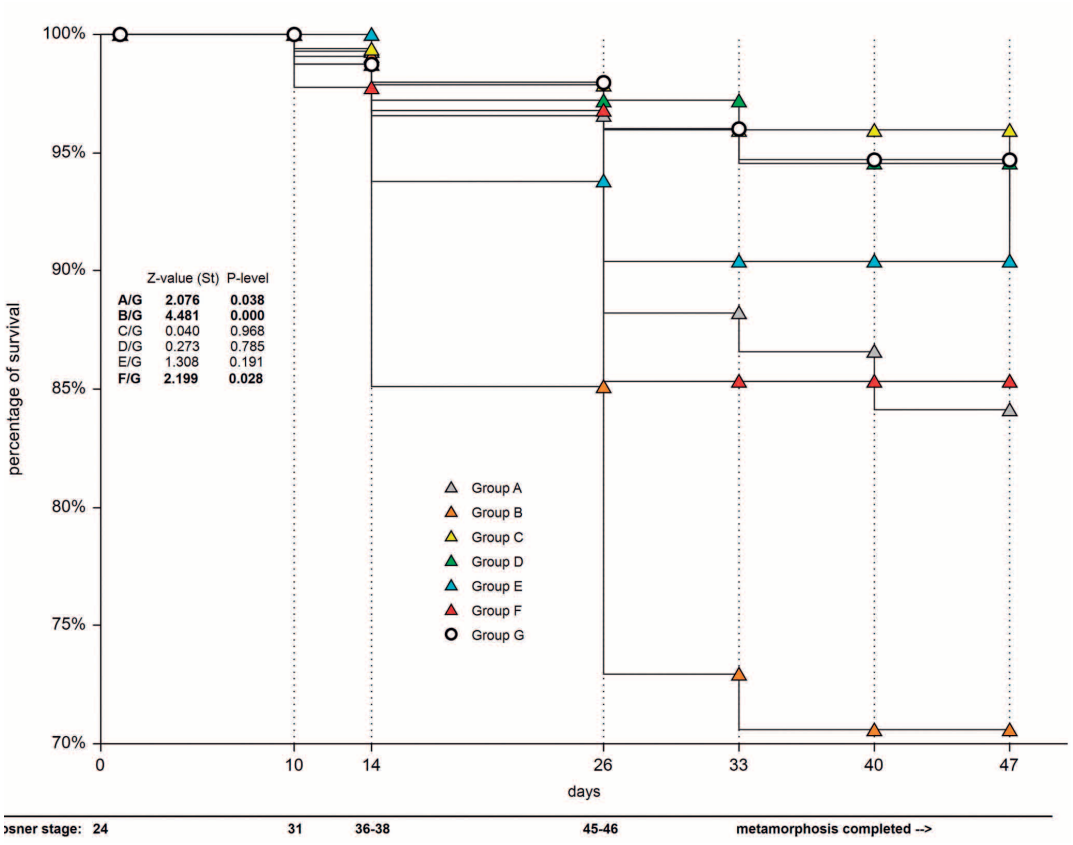

Figure 3. Kaplan-Meier curves for each tag group considered (A–F) vs. control group (G).

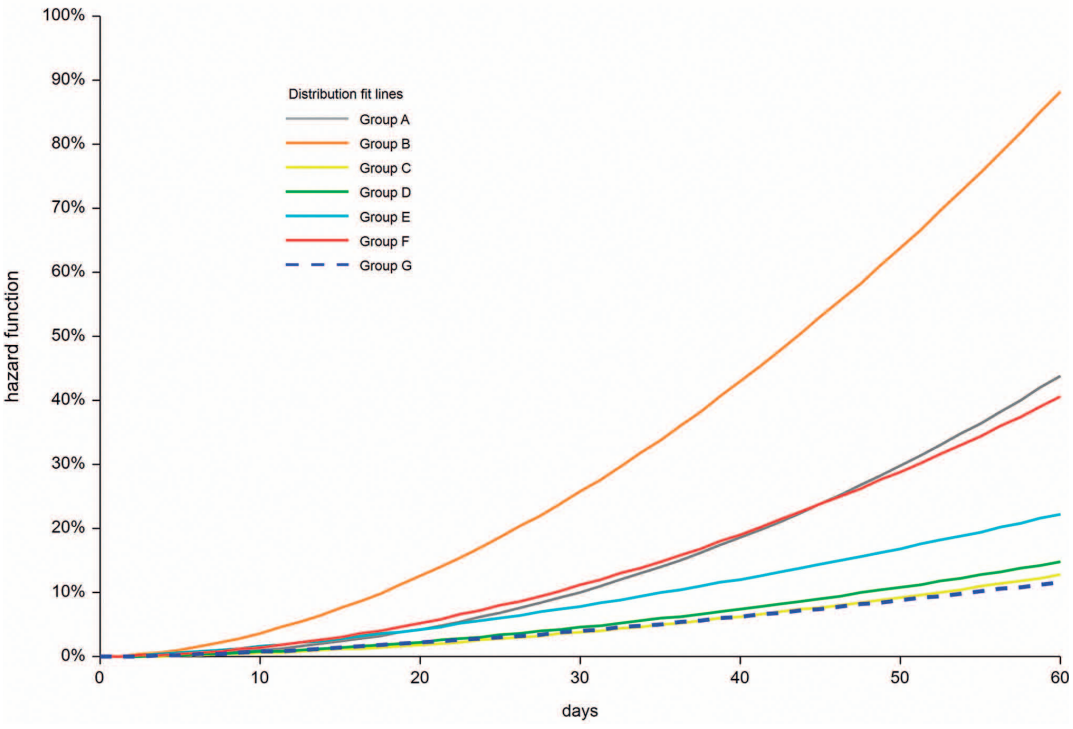

Figure 4. Hazard functions for each tag group considered (A–F) vs. control group (G).

Tag migration and tag loss are other important factors useful to evaluate the behavior and the effectiveness of the VIE tags in the different marked groups considered. Apart from the groups A and B, where tag migration and loss are both particularly high in the toadlets because of the reabsorption of the tail during metamorphosis, group C (dorsal area of body) also shows a very high rate of tag migration in tadpoles, preferably towards left (70% of the cases) or right (20%) side of body, probably due to the swimming movements involving the dorsal musculature, as suggested by Bainbridge et al. (2015) as well. Grant (2008) reported events of tag migration mainly from the tail to the dorsal area of body, instead.

About the tag retention, the highest stability was observed in the D and E groups (respectively ventral and right side areas of body), where the interference of the VIE tags in the normal life activities and movements, both in the tadpole and toadlet stages, is very probably lower (Supplementary Fig. 3). Every tag position required different abilities. Both markings on the tail were the easiest and fastest to make, while marking dorsal and ventral areas of the body was definitely the most difficult modality because of greater precision needed in inserting the needle. Slightly easier was marking the tadpoles on the sides of their body, even if the left side (group F) required more precision and a longer time than the right one, because of the operculum.

In conclusion we mainly suggest two different procedures using the VIE tags, depending on the focus of the experimental design, to keep rate of mortality and tag migration lower:

a) to mark larvae in the upper tail position (group A), using different colors or combinations of them, when the identification of groups of tadpoles is needed. Tagging on dorsal area and left side of body (respectively groups C and F) is suggested when the identification of marked/unmarked individuals is needed. Tag migration is high, but rate of mortality is low;

b) to mark ventral area and right body positions (groups D and E, respectively) for studies on toadlets; these two tag positions showed the lowest rate of mortality and the highest percentage of tag retention during the initial phases of the metamorphosis.

However, on the basis of our study, tagging on the ventral area (group D) seems to be less deadly than the right side position (group E), even if tagging on D position is more difficult than on E position. Future studies should consider the use of more than one VIE tag per single tadpole, in order to increase the number of combinations through different colors and positions, so as to evaluate possible synergies, hazard variation and errors in reading tags.

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Supplementary material

Supplementary Table S1. Temporal variations.

Supplementary Table S2. VIE tags variations.

Supplementary Fig. 1. Variations in tadpoles' SVL.

Supplementary Fig. 2. Histograms.

Supplementary Fig. 3. Tag retention rates.