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Long-term reliability of Visual Implant Elastomers in the Italian cave salamander (*Hydromantes italicus*)

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Capture-Mark-Recapture (CMR) methods are key tools for ecological studies on various animal species (Mazerolle et al. 2007, Bradbury et al. 2008, Link & Hesed 2015). These techniques help researchers to obtain information on population dynamics and even on single individual behaviour (Fitzgerald et al. 2004, Sanchez-Camara & Booth 2004, Phillips & Fries 2009, Pierce et al. 2014). Several methods have been developed to mark individuals (e.g., toe-clipping, implantation of visual signals or transponders, photographic identification), with each one coming with different pros and cons and only being useful for certain species and/or individuals with appropriate features (Heard et al. 2008, Rocha et al. 2013, Lunghi & Veith 2017). Therefore, prior to performing any CMR study, it is fundamental to test both the efficiency and reliability of the selected marking method on the target species, to reduce time and financial expenditure and to limit potential negative effects on the study population (Brannelly et al. 2014, Carpentier et al. 2016, Lunghi & Veith 2017).

Visual Implant Elastomers (VIE; Northwest Marine Technology, Inc., Shaw Island, Washington) are among the most commonly used approaches for marking different vertebrate groups even under extreme conditions (Olsen & Vøllestad 2001, Davis et al. 2004, Campbell Grant 2008, Butt et al. 2009, Silva de Freitas et al. 2013, Balázs et al. 2015). VIE consist of a two-component silicone-based material that, after having been mixed, is injected subcutaneously like a coloured liquid; the liquid will then solidify and form a pliable biocompatible material within a short period. Visibility of tags is greatly enhanced using ultra-violet lights (Fitzgerald et al. 2004). VIE has been identified as one of the best marking approaches for amphibians, because of their reliability due to low invasiveness and high retention rate even after metamorphosis (Davis & Ovaska 2001, Bailey 2004, Campbell Grant 2008, Balázs et al. 2015, Iannella et al. 2017). This marking methodology has only been applied a few times to European cave salamanders (genus *Hydromantes*, subgenera *Speleomantes* and *Atylodes*; Wake 2013), generally providing good results over a short period (Salvidio 2013, Lunghi et al. 2014a, Lunghi et al. 2018). Still, the retention of implanted tags and their potential use in long-term studies has not been examined yet (Price et al. 2012, Muñoz et al. 2016). Here we evaluate the durability of VIE tags in the Italian cave salamander, *Hydromantes italicus*, over a period of five years.

During the period February–June 2013, we used VIE to individually mark 34 adults of *Hydromantes italicus*; we considered adults all salamanders with total length ≥ 70 mm (Lanza et al. 2006, Lunghi et al. 2015). The period of tagging included the season in which the species is known to be most active (Lunghi et al. 2015). We marked salamanders observed inside four caves (8–9 individuals per cave) located in the north of the Tuscan Apennines in the provinces of Prato (N = 1) and Pistoia (N = 3) (between Lat. 43.99° Long. 11.09° and Lat. 44.16° Long. 10.99°). The three caves located in Pistoia are situated very close to each other, representing the vertexes of a polygon with a surface area of just 0.02 km². Salamanders were captured and handled using disposable gloves; a single operator individually applied the tags. After tag application, individuals were released in their capture locations to let them experience regular natural events that might affect tag retention (e.g., stress caused by inter/intraspecific competition, environmental conditions). The marking procedure is the follow-

ing. We used seven marking points located on the ventral side of the salamanders (Fig. 1A) as this portion of the body is usually less pigmented and the risk of altering inter- and intraspecific interactions is lower (Lunghi et al. 2014a). We injected only one tag per marking point to avoid any possible mixing (Brannelly et al. 2014). The code attributed to each salamander was composed of: i) a letter identifying the sex (Male/Female), ii) a letter identifying the used colour (Red/Green/Yellow/Blue), and iii) the serial number corresponding to the marking point used (Fig. 1B). We applied a total of 77 VIE tags (2.2 ± 1.0 per individual) resulting in a unique combination of marking codes to avoid misidentification due to a) tag loss (Brannelly et al. 2014), and b) potential immigrations from caves nearby. After tagging, the wound was massaged for few seconds to promote closure and correct tag placement; the needle of the syringe was disinfected with pure ethanol wipes prior to marking the next individual (Webb et al. 2007). Then, the salamanders were released and observed for a few minutes in order to check for any possible harm from stress (Samimi et al. 2016). Sites were randomly surveyed until May 2018; each recaptured salamander was massaged above the tag insertion point to check for tag stability.

VIE tags were injected quickly into each marking point: the estimated time to inject a single tag was ca. 5 seconds. In total we recaptured 26 salamanders over a period in excess of five years: fifteen were recaptured in the caves in Pistoia and eleven in Prato. Individuals were recaptured on average 1.3 ± 1.0 times (a single individual was recaptured seven times); eight salamanders were never recaptured. Out of the 42 recaptures, 29 were made within the first year after tagging, seven within the second, and six within the fifth/sixth (after 59, 60 and 65 months). None of the tags was lost, and we did not detect any tag movement after we massaged the salamanders' skin. All tags remained in their positions and were perfectly readable with bare eyes (Fig. 2). All salamanders were recaptured inside the caves where their tagging had been performed.

Visual Implant Elastomers were easily injected into cave salamanders, and the procedure did not require any help

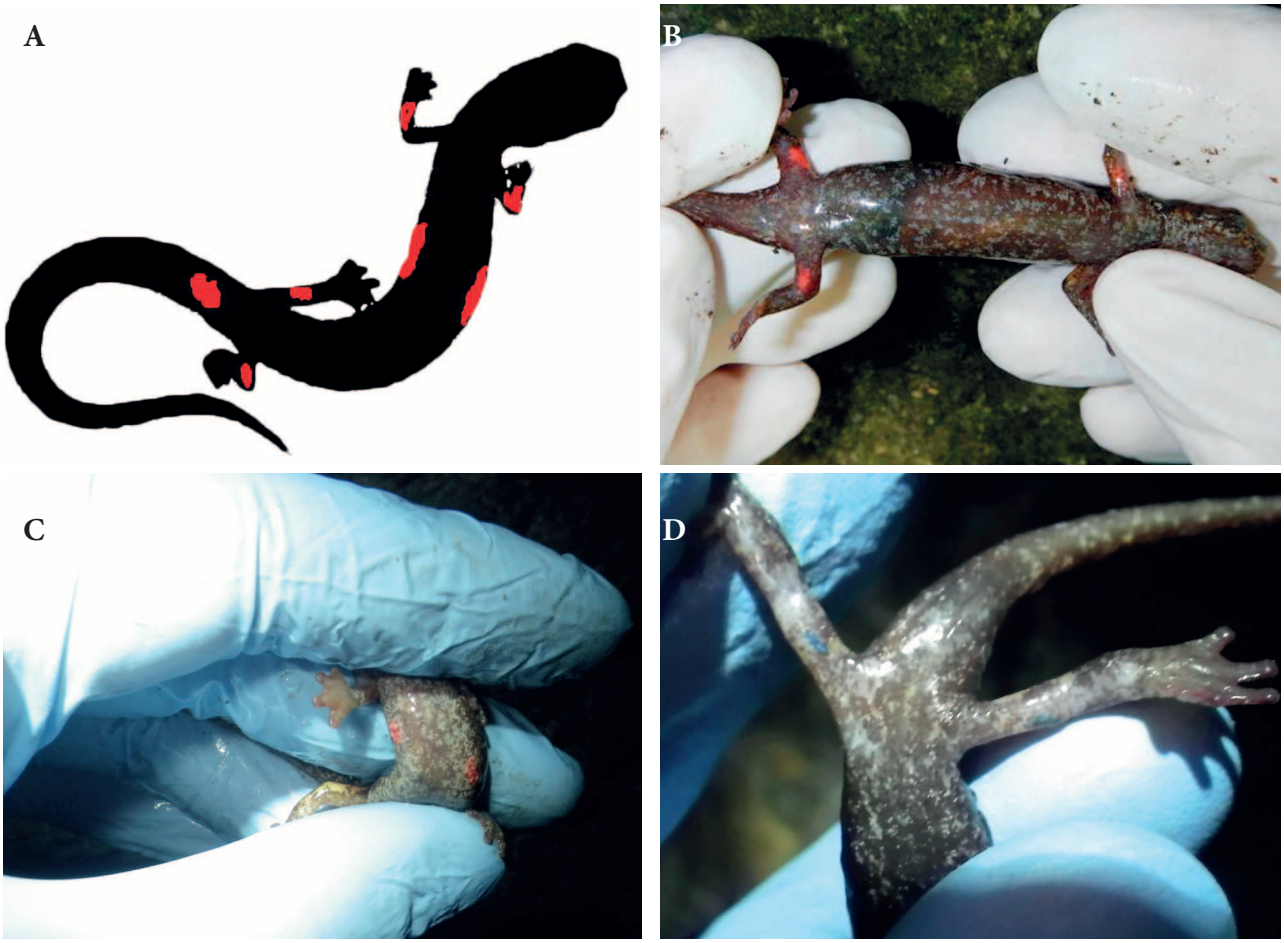

Figure 1. Example of the marking method used on *Hydromantes italicus*. A) Outline of a salamander pointing out the tagging zones (modified from http://pinicola.ca/jourbook.htm); B) an individual showing the positioning of the tags (code: FR156); C) and D) FR34 and MB56, respectively, are two individuals recaptured after five years.

from a second operator, so that handling times were short and therefore minimally stressful to the animals (Kinkead et al. 2006, Caipang et al. 2014, Weber et al. 2015, Lunghi et al. 2016, Lunghi & Veith 2017). Recaptured salamanders (76.5 % of all marked) were in a healthy condition and none of the tags were lost. VIE tags never slid or changed position even if we stimulated their movement. Our observations support the reliability of VIE tags to individually mark European plethodontid salamanders (Salvidio 2013, Lunghi et al. 2014a, Lunghi et al. 2018). Furthermore the recapture of four individuals after five years (Fig. 2) corroborates the reliability of this marking technique also for long-term studies (Price et al. 2012, Muñoz et al. 2016). However, we should point out that besides compelling information on recaptured tagged salamanders, we cannot provide any data on those we did not recapture, as the study was performed under natural conditions and thus, several factors (e.g., death, emigration) may have affected the observation of these specific individuals.

Hydromantes seek refuge underground during periods characterized by adverse climatic conditions (too hot and/or harsh) (Lunghi et al. 2015); however, when outdoor conditions are suitable, they usually exploit different typologies of epigean environments (Manenti 2014, Costa et al. 2016, Salvidio et al. 2017b). During their outdoor activity, *Hydromantes* seem to not move far from their selected refuges (Salvidio 2013) as to be able to return to them quickly when needed (Lunghi et al. 2014b, Salvidio et al. 2017a). Indeed, during our study, marked salamanders were never recaptured in a different cave, even though three of the surveyed sites were located at very short distances from each other. Our observations support the hypothesis that these salamanders exhibit a high site fidelity towards their underground refuges (Salvidio 2013). However, the factors that may induce site fidelity in *Hydromantes* are still unknown.

Herein we provide first information on the long-term reliability of Visual Implant Elastomers in a species of European plethodontids. VIE tags proved to be highly reliable for years after their first application; however, further studies are needed to confirm whether tag loss does or does not occur. Future VIE tagging will help to understand the spatial ecology of *Hydromantes*, and to assess their home range behaviour and site fidelity in their natural environments.

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