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In situ underwater tagging of aquatic organisms: a test using the cave-dwelling olm, *Proteus anguinus*

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In situ ecological studies on obligate cave-dwelling aquatic animals are scarce at best. This is particularly true for capture-mark-recapture (CMR) studies that form the basis of understanding population structure and dynamics. Here, we report on the *in situ* underwater application of the Visible Implant Elastomer (VIE) tagging system on the olm, *Proteus anguinus*, an obligate cave-dwelling aquatic amphibian. We tagged seven adult individuals and monitored the population during 31 dive transects during four years. We found that VIE tagging is applicable underwater. Based on our recaptures, the tags were recognisable after four years and the recaptured three individuals exhibited extreme site-fidelity. Our results indicate that CMR studies are feasible in underwater cave ecosystems without even temporarily removing individuals. *In situ* underwater tagging also holds great potential for studies of other aquatic ecosystems, where removing animals from water, their habitat or territory is problematic for ethical, logistic or scientific reasons.

Introduction

Cave ecosystems have generated long-lasting biological interest. While the 21st century can be seen as the "golden age of the study of subterranean biology" (Culver & Pipan 2009), several authors agree that modern ecological and evolutionary studies are relatively few in this field (Culver & Pipan 2009, Romero 2009). For a long period of time, the Lamarckian approach to troglomorphic adaptations was overwhelmingly accepted — ironically, this was also propagated by Darwin himself (Darwin 1859). Aquatic cave ecosystems, particularly those that cannot be accessed without diving techniques, are usually understudied due to the obvious logistic constraints. This is indeed an unfortunate fact, given that their biodiversities usually exceed that of the non-aquatic caves or cave passages (Culver & Pipan 2009). However, studying aquatic cave ecosystems are important for several reasons, including (i) general evolutionary biological interest in understanding the mechanisms of adaptations to this extreme habitat, (ii) conservation of these unique ecosystems and (iii) securing an important source of potable water needed for human use (Herman *et al*. 2001).

An important approach in gathering ecologically relevant information from any species or population is to conduct capture-mark-recapture (CMR) studies. A well-designed CMR study can provide answers about population parameters such as the number of individuals, their sex ratio, age distribution, survival, recruitment, dispersal and both the short-term (seasonal effects) and long-term changes (population trends) in these, which are all inevitable for basic and applied science regarding the species or population in question. Only a few CMR studies have been carried out on natural aquatic cave-dwelling populations, typically targeting easy access cave streams and operating via the temporary removal of the individuals (e.g. Knapp & Fong 1999).

The olm (*Proteus anguinus*), also known as the "human fish" and "blind cave salamander", is a unique member of the European herpetofauna. It is the only member of the family Proteidae and the only obligate cave-dwelling vertebrate in Europe, showing remarkable adaptations to its unique environment. The species is blind, totally depigmented (apart from *P. a. parkelj* Sket & Arntzen 1994), neotenic, shows high tolerance to anoxia and has long been known for its extreme life-history traits such as its unmatched longevity among anurans (probably over 100 years; only the Japanese giant salamander, *Andrias japonica*, being comparable) and its resistance to starvation (Hervant *et al*. 2001, Issartel *et al*. 2009, Speakman & Selman 2011, Voituron *et al*. 2011). Despite the long lasting scientific interest, biological knowledge of the species has been gathered mainly from observations and experiments made with captive populations in laboratories. We are not aware of any CMR attempts in wild populations.

The aim of the present study was to test the *in situ* underwater applicability of the Visible Implant Elastomer (VIE, Northwest Marine Technology, Inc., Shaw Island, WA, USA) tagging system in aquatic cave environments. VIE has already been successfully employed with several fishes (Buckley *et al*. 1994, Bonneau *et al*. 1995, Frederick 1997, Leinonen *et al*. 2011), frogs (Hoffmann *et al*. 2008, Schmidt & Schwarzkopf 2010, Branelly *et al*. 2013, Swanson *et al*. 2013) as well as with small shrimps (Godin *et al*. 1995). Here, we used *P. anguinus* as a model. By tagging seven adult individuals underwater and following them for four successive years in the "Vruljak 1" Cave, Eastern Herzegovina, Bosnia and Hercegovina, we aimed to see whether the marks persisted and could be identified visually over longer periods of time without having to catch the animals and to collect preliminary data on the movements of *P. anguinus* in the wild. In a wider sense, our study also provides a test as to whether it is possible to apply VIE underwater without having to remove the animals from their natural habitat.

Material and methods

One year before the actual fieldwork, we tested the method on two captive albino Mexican cave salamanders (axolotl, *Ambystoma mexicanum*). There is a growing body of literature on how to perform VIE tagging (e.g. shrimp (Brown *et al*. 2003); echinoderms (Martinez *et al*. 2013); fishes (e.g. Leinonen *et al*. 2011), and we chose to apply the standard procedure using the Trial Pack (*see* http://www.nmt.us/products/vie/vie.shtml) with black colour both here and again later in the actual study. This mark does not need UV (ultraviolet) lamps for recognition. We chose it to simplify the underwater equipment needed for identification. Albino axolotls show several similar features to *P. anguinus* and therefore it was an excellent test species. The marking was successful (Fig. 1a), as the axolotls showed no signs of infection or immune response resulting in the rejection of the mark. The VIE implants remained clearly visible a year later, before the onset of the field study targeting *P. anguinus*. In actual fact, the tags continue to be visible four years later (Fig. 1b).

As part of the large scale "Proteus Project" (a joint strategy for the protection of the endangered underground endem *Proteus anguinus* and its natural karst habitat in the Trebišnica River Basin; http://www.devonkarst.org.uk/proteus%20project/PTPH1.html), numerous caves in the Trebišnjica River Basin have been mapped and monitored for the presence of *P. anguinus* from the year 2000 onwards. Based on these visits, the "Vruljak 1" cave was chosen as the study site, due to its relative ease of access

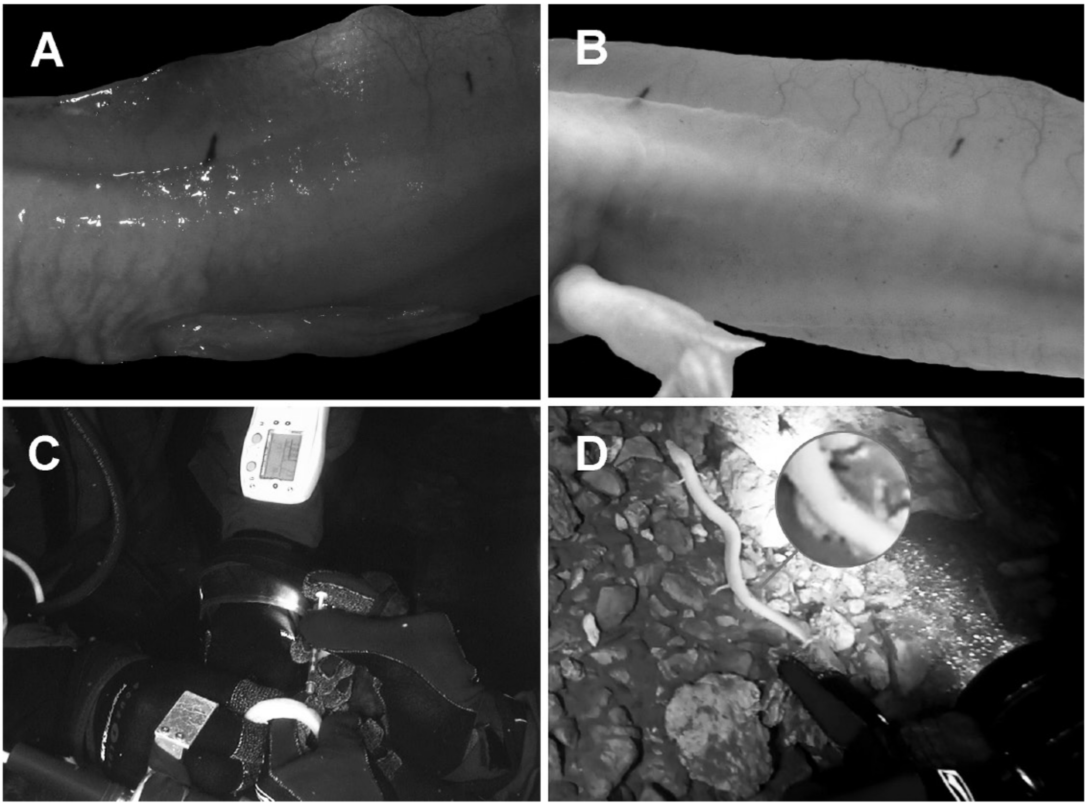

Fig. 1. Underwater tags. (**A**) Axolotl (*Ambystoma mexicanum*) Visible Implant Elastomer tags in July 2009. (**B**) Same individual in January 2014. (**C**) *In situ* underwater tagging of a *P. anguinus* on 26 July 2010. (**D**) Recapture of the same individual 309 days after the tagging.

and the high number of *P. anguinus* (more than 50) found within a ca. 350 m transect routinely monitored (Lewarne *et al.* 2010). The VIE reagents were mixed immediately before the dives. The VIE was administered with a standard 29 gauge needle. The mixed VIE was useable for approximately one hour at 11 °C water temperature.

Between 26 July and 12 September 2010, seven adult olms were caught in the middle part of the transect (Fig. 2) and marked underwater exactly at the site of capture during three marking sessions (first: 26 July [n = 2]; second: 5 September [n = 4]; third: 12 September [n = 1]). The individuals were released immediately after the marking procedure. As the current study aimed to test the method without risking a significant negative impact on the population, we decided to use a relatively low sample size that is still enough to answer the proposed question, i.e. are the VIE marks applicable underwater, persistent and recognisable after long periods? Since the first exploration of the cave in 2007, we had accumulated 558 minutes of video footage recorded during the many survey dives. These videos contained 127 unintentional observations of *P. anguinus*, in which the caudal region was clearly visible in 125, the midbody in 110 and the anterior region in 105 cases. Hence, we decided to mark the caudal region for easy recognition. This region seemed also a good choice, as marking the tail-fin membrane is supposed to be the potentially least harmful for the individuals (note that applying subcutaneous marking underwater is less precise than it would otherwise be under laboratory conditions). We applied a simple marking design to facilitate easy recognition under low visibility. We used black marks to maximise the contrast and individual recognition was possible based

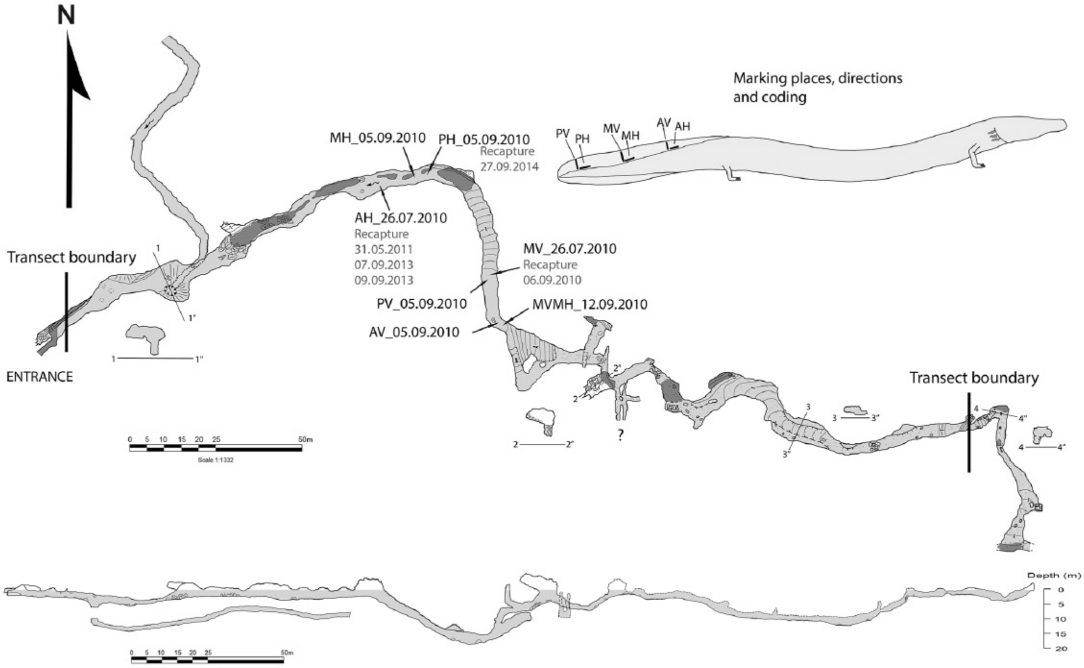

Fig. 2. Map of "Vruljak 1" cave with the locations and dates of markings and recaptures Explanations of the codes: A = anterior, M = middle, P = posterior, H = horizontal, V = vertical.

on the position (anterior, middle and posterior parts of the upper membrane of the tail fin) and direction (horizontal *vs*. vertical) of the marks (Fig. 2). Each *in situ* marking procedure lasted for less than two minutes. None of the marked individuals showed any visible sign of stress and all remained close to the site of capture/release. We made five dives between 5 and 12 September 2010 to look for any negative effects of the procedure and found no sign of injured individuals or carcasses. Three individuals were caught at "easy" and four at "challenging" locations. We characterized a location as being "challenging" when the passage was wide, with lots of rocks at the bottom and side passages and crevices in the close vicinity acting as shelters, whilst the "easy locations" corresponded to narrow passages with an even bottom surface and with a lack of known side passages and crevices in the close vicinity.

Results

The transect was monitored 31 times after 12 September 2010 (4 times in 2011, 12 times in 2012, 11 times in 2013 and 4 times in 2014) during which we were aiming for recaptures. It should be noted that we never physically recaptured the individuals later, only passively observing the marked individuals. We only use the term "recapture" to comply with CMR terminology. Two individuals were recaptured once; the first 42 days after marking (on 5 September 2010 during the second marking dive) and the second 1848 days after marking. A third individual was recaptured three times (309, 1139 and 1141 days after marking; Fig. 2). All recaptured individuals were caught at easy locations. All recaptures were done within 5 m of the original capture site. Visual recognition of the marks was possible within a distance of 1.5–2 m from the animals (Fig. 1d). These patterns show that repeated observation of the same individual is possible through different years.

Discussion

Our most salient findings were that (i) *in situ*, underwater marking is possible and can be done quickly, despite the difficult circumstances originating from cave diving, (ii) VIE marks are easily recognisable after more than four years, and (iii) *P. anguinus* individuals can show extreme site-fidelity. We did not observe four out of seven individuals after the marking and one of the recaptures happened in the year of the marking. We can only speculate about the lack of recaptures of the remaining individuals. Based on our preliminary study with axolotls and the fact that we never observed a carcass or visibly damaged individuals during several diving sessions following the dives used for marking, marking-induced mortality is unlikely. The un-recaptured individuals might have either moved away or remained undetected. The fact that most of the unobserved individuals were marked at "challenging locations" can support both scenarios. Further studies are needed for a better resolution of the patterns. We are fully aware that the sample size of the present study is insufficient to draw solid scientific conclusions, but despite the observational nature of the data, our preliminary findings hold great scientific potential for the future.

Studying cave ecosystems can be important from various aspects. In several geographically and taxonomically independent cases, adaptations to the cave environment (i.e. troglomorph adaptations) happened both within species and among sister species. Studying cave-dwelling *vs*. surface-dwelling population or species pairs hold potential not only for fundamental science, but also has strong applied dimensions. Cave ecosystems are under heavy conservational threat, mainly due to anthropogenic disturbance (Romero 2009). Such negative changes will not only affect nature but man alike, since water supply often relies on karst-waters (Herman *et al*. 2001, Bonacci *et al*. 2009). To properly assess the state of these ecosystems, short- and long-term biomonitoring is crucial. Since VIE marking can be adopted to highly divergent taxa of different sizes (Bonneau *et al*. 1995, Godin *et al*. 1995, Leinonen *et al*. 2011) and monitoring of a population via diving is possible in both open water and cave environments (e.g. Buckley *et al*. 1994, present study) a better utilization of cave-dwelling *vs*. surface dwelling evolutionary models and cave-dwelling bioindicator species is an emerging option.

In the particular case of *P. anguinus*, many aspects of the biology of this unique species have been revealed from the results of laboratory experiments and studies based on captive populations (e.g. Bouquerel & Valet 2003). Hence, even though we know a lot about its functional anatomy and histology (Schlegel *et al*. 2006) and now even from its life history (Voituron *et al*. 2011), our knowledge about even the most basic ecology of the species (e.g., population number, dispersion ability, prey, activity) in the wild is lacking. Furthermore, the species is listed in Appendix II of the Bern Convention and in Annexes II* and IV of the EU Habitats Directive and indentified by IUCN as vulnerable. In its typical habitats (cave systems of the Dinaric Karst), *P. anguinus* is the top predator, therefore it is also an important element and possible bioindicator of the fragile cave ecosystem (Bulog *et al*. 2002).

Several marking methods are known from amphibian studies, ranging from toe-clipping through applying tattoos or freeze and hotwire banding to inserting Passive Integrated Transponder (PIT) tags under the skin (Mellor *et al*. 2004). However, many of these methods raise ethical issues due to the severe stress imposed on the specimens during their application, resulting in the acquisition of questionable data due to such methods affecting individual behaviour and fitness. Other methods are simply inapplicable for underwater use. It may be considered by some that tagging without anaesthesia can be of ethical concern, but we think that the mild pain experienced during tagging (the whole process including capture, tagging and release, is less than 2 min.) is apparently less stressful than the alternative of removing the animals from their habitat for at least several hours, if tagging under anaesthesia is done right next to the water body, or days if the animals are transported to a scientific facility. By using only one colour, three positions and two directions for the tagging marks, 61 combinations are possible based on a maximum of three marks per individual. In our experience, 61 individual marks should be sufficient for most known natural populations, however, the number of combinations can be drastically increased if marks are also applied to other parts of the body. Due to identification issues underwater in bad visibility and also when considering that the number of easily observable and safely-markable body parts is limited, we do not advise using more than one colour for tagging. However, adding one or two marking locations on the dorsal part of the body is realistic. Being mindful that completing just a single transect in an underwater cave requires highly trained personnel and expensive equipment, data of the population structure and population dynamics of *P. anguinus* can be retrieved even without temporarily removing the individuals from their habitat. We plan such a follow-up study in the future and we hope that the present note will also encourage other researchers to utilize the possibility, resulting in comparable data from different populations. Finally, apart from cave ecosystems, *in situ* underwater VIE tagging can be important in numerous situations when the temporary removal of aquatic organisms is better avoided for logistic (e.g., extreme depth), ethical (e.g., stress/mortality) or scientific (e.g., to avoid the disturbance of territories) purposes.

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