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PIT-Tags as a technique for marking fossorial reptiles: insights from a long-term field study of the amphisbaenian *Trogonophis wiegmanni*

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Abstract. Many field studies of ecology or conservation require individual identification of the animals, and for this, several marking techniques have been developed. However, no specific labeling technique has been tested for fossorial reptiles, such as amphisbaenians. We describe the use of Passive Integrated Transponder (PIT) tags as a long-term labeling method of the amphisbaenian *Trogonophis wiegmanni.* We present the details of the marking procedure and examine the benefits and drawbacks of the technique considering the fossorial environment. After marking many individuals in a long-term field study, we can ensure that the marks were easily applicable and were not lost over a period of at least four years. Moreover, PIT tags did not negatively affect the body condition of amphisbaenians. We conclude that PIT tags are useful for doing field studies of this and similar fossorial species.

Keywords. Amphisbaenians, *Trogonophis wiegmanni*, PIT-tagging, body condition, fossorial reptiles.

INTRODUCTION

Many field studies of ecology, behavior or conservation require individual recognition of the subjects that make up a population. Being able to distinguish individuals allows the assessment of diverse ecological traits such as the size and dynamics of the population, survivorship, movements, home ranges, activity patterns, social interactions, etc. (reviewed in Plummer and Ferner, 2012; Ferner and Plummer, 2016). For that reason, labeling individuals is often necessary, and diverse tagging techniques have been developed depending on the species and/or the traits that are the object of study. Ideally, these marks should allow a correct identification and be easily applicable, but without causing suffering to the animals, and they should last for at least the duration of the entire field study, but without affecting survival or behavior of the marked animals (reviewed in Ferner and Plummer, 2016).

Diverse methods of marking individuals have been described for reptiles. Some are intended for short-term studies, such as external painting marks, beads, adhesive tapes, elastic bands, metal or plastic discs, buttons, etc. (Gibbons and Andrews, 2004; Ribeiro and Sousa, 2006; Ferner and Plummer, 2016). While others are focused on long-term studies, such as toe clipping, scale clipping, shell notching on turtles, heat/freeze branding, photo identification based on natural markings, Visible Implant Elastomer (VIE) tags and/or Passive Integrated Transponder (PIT) tags (Daniel et al., 2006; Hutchens et al., 2008; Ekner et al., 2011; Ferner and Plummer, 2016).

Several groups of reptiles and amphibians, comprising as much as 20% of the global herpetofauna, or nearly 3,000 species, are fossorial (Measey, 2006). However, as is the case with other fossorial animals, their ecology and conservation status are much less well understood than those of their epigeal relatives (Copley, 2000; Wolters, 2001; Böhm et al., 2013). This may be explained because

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of the difficulty of doing field studies of fossorial animals (Measey, 2006; Henderson et al., 2016), which includes difficulties in individually marking these animals, given their burrowing habits. Although several marking techniques have been tested in fossorial caecilians (Measey et al., 2001) and *Ambystoma* salamaders (Connette and Semlitsch, 2012), to our knowledge, no specific labeling technique has been tested for limbless fossorial reptiles such as amphisbaenians (Henderson et al., 2016).

Due to the morphology of most amphisbaenian species (i.e., elongated body without limbs in most species), it is obviously not possible to use many types of marking methods. Further, given the fossorial habits of amphisbaenians, most external markings (painting, beads, adhesive tapes, etc.) may be incompatible with the burrowing behavior of these animals and will be quickly lost by repeated contact of the body with the soil. Therefore, potential methods that could be used for long-term marking of amphisbaenians might be restricted to scale clipping, heat/freeze branding, VIE tags and/or PIT tags (Camper and Dixon, 1988; Jemison et al., 1995; Hutchens et al., 2008; Ferner and Plummer, 2016). Here, we describe the use of PIT tags as a labeling method for long-term field studies of the checkboard amphisbaenian *Trogonophis wiegmanni*, Kaup 1830. A PIT tag is a microchip with an electromagnetic coil encased in a biocompatible glass cylinder, encoded alphanumerically in an unique way, that is implanted in the animal (Gibbons and Andrews, 2004). We present here the detailed marking procedure that we applied to amphisbaenians, examine the potential benefits and drawbacks of the technique, considering the peculiar characteristics of the fossorial environment, and discuss its utility for doing ecological studies of this and similar fossorial species.

MATERIALS AND METHODS

Study species

The checkboard amphisbaenian *T. wiegmanni*, Kaup 1830 is a representative of the family Trogonophidae (Gans, 2005) (Fig. 1a) that inhabits arid areas from southwest Morocco to northeast Tunisia (Bons and Geniez, 1996). These amphisbaenians live all their life buried in the soil, but they are frequently found under rocks (Civantos et al., 2003; Martín et al., 2013a). Little research has been carried out on this species, as on other amphisbaenians, but there is now a growing body of information on aspects such as its thermal biology (López et al., 2002), microhabitat and soil selection (Civantos et al., 2003; Martín et al., 2013a), reproduction (Bons and Saint Girons, 1963), social behavior and population structure (Martín et al., 2011b, c) or diet (Martín et al., 2013b; Baeckens et al., 2017). However, all these studies have been made by randomly sampling unmarked amphisbaenians. More detailed studies would require to individually identify the amphisbaenians that are being examined. This is important, not only because of the scientific interest in understanding the ecology and behavior of amphisbaenians, but because several conservation problems that may potentially affect their populations have been noted (Martín et al., 2011a, 2015, 2017), and a detailed long-term monitoring of these populations require the ability to individually identify and follow the study subjects.

Field study and marking procedure

We have carried out field and laboratory studies of *T. wiegmanni* amphisbaenians on the Chafarinas Islands (Spain) for almost twenty years. This is an archipelago, formed by three small islands, located in the southwestern area of the Mediterranean Sea (35°10'N, 02°25'W), 2.5 nautical miles to the north of the Moroccan coast (Ras el Ma, Morocco) and 27 miles to the east of the Spanish city of Melilla. The islands have a dry, warm, Mediterranean climate, and vegetation is dominated by bushy plants (*Suaeda, Salsola*, *Lycium* and *Atriplex*) adapted to salinity and drought. *Trogonophis wiegmanni* is very common and is represented by very large populations on these islands (Martín et al., 2011a).

During the years 2015-2018, we made field campaigns twice a year, during two weeks in spring (March-April) and two weeks in Autumn (September-October), to capture, mark and recapture *T. wiegmanni*. We delimited three study plots (surface area = 0.14 Ha, 0.40 Ha and 0.58 Ha) on different islands, which we walked systematically and intensively during the morning and afternoon of different days. Amphisbaenians were found by carefully lifting almost all rocks located inside the study plots. Individuals were captured by hand, measured and immediately after marked in the field with PIT tags. We used one of the smallest available PIT tags (Biomark MiniHPT8; Biomark, Inc., Boise, Idaho, USA), with a length of 8.4 mm, 1.4 mm in diameter and a weigh of 0.03 g. This weigh represents 0.6 % of the mean body mass (i.e., around 5 g) of a typical adult amphisbaenian in our population (Martín et al., 2011c). We gently implanted PIT tags subcutaneously in the upper right side of the body of amphisbaenians (Fig. 1). For this, we made a small puncture at around 3 cm from the snout (mean SVL of adult amphisbaenians is around 14 cm) using a stainless steel needle (Biomark N165 needle; length = 5.1 cm, needle diameter = 1.49 mm), disinfected with alcohol before and after puncturing each individual, which was fitted to a specially designed syringe style implanter (Biomark MK165 syringe). We gently lifted the skin from the underlying muscle and then inserted the transponder subcutaneously using the implanter. During the insertion of the PIT tag, the needle was maintained parallel to the body to ensure that the tag remained under the skin and did not enter the coelomic cavity (Fig. 1). The injection site was immediately disinfected with alcohol after the implant. According to Brown (1997), losses of PIT tags may occur immediately after the implant is done, while the wound is still open. To avoid this, incisions may be sealed with medical grade suture glue. However, in our case, this was not needed as the incision was

Fig. 1. An adult amphisbaenian (*Trogonophis wiegmanni*) as it was found under a stone (left); PIT tag implantation procedure (right).

very small and the tags showed no evidence of becoming displaced. Further, at least in lizards, the glue may slow the healing process (Le Galliard et al., 2011). Moreover, the long needle pushed up the tag under the skin towards the posterior part of the animal. Thus, the tag was implanted at least 2 cm posterior of the small puncture point, which precluded tits loss when the amphisbaenian burrowed forward. All the marking procedure could be easily made by a single experienced researcher, holding the amphisbaenian with one hand and the implanter with the other. However, the presence of an additional researcher, who prepared the equipment and took notes, made the process easier and quicker, decreasing the manipulation time and disturbance to the animals.

This marking technique is particularly appropriate for amphisbaenians, as their skin attachment is quite loose and leaves a subcutaneous space where the pit-tag is inserted. The skin is connected to the axial mass by costocutaneous and vertebrocutaneous muscles, that allow the skin to move independently from the body, mainly in rectilinear locomotion (Gans, 1978; Gasc, 1981; see illustrations in Smalian, 1884), As those muscles are numerous and redundant, the insertion of a strange body (or even the damage of some muscle fibers) should not interfere with the normal locomotion or excavation.

Although amphisbaenians obviously "felt" and showed a small aversive response to the puncture with the needle, we did not observe any subsequent additional negative behavioral responses (e.g., stress, immobility, forced unnatural movements, or attempts to remove the tag) (Warwick et al., 2013). Amphisbaenians behaved normally when they were released at their capture points a few minutes after being captured and marked. The implant procedure very rarely resulted in a small drop of blood, but in that case the wound was cleaned with alcohol and bleeding stopped rapidly. We avoided the use of local anesthesia, because the duration of the recovery time from anesthesia could be much longer than the natural recovery from the implanting procedure. Moreover, the administration of anesthesia *per se* is an additional procedure that requires increasing manipulation time and careful control of conditions, and it could have negative physiological side effects for small reptiles (Heard, 2001; Chatigny et al., 2017).

A hand-held portable reader (Biomark 601 Reader) was used to read the individual unique code of the tag (the tags have a 134.2 kHz, ISO FDX-B, frequency), The code can be provided either as a hexadecimal or as a decimal number (15 digits). In the practice, the four last digits were enough for a reliable identification of individuals in each study population. The reader works in the field with AA rechargeable batteries but it may be also used in the lab with an AC power supply.

To test the long-term effect of PIT tags in amphisbaenians, we compared the body condition of individuals at first capture, when they were untagged, and when they were recaptured one year after being implanted with a PIT tag. Body condition was assessed as the residuals of an ordinary least squares linear regression of log-transformed mass (measured with an electronic balance to the nearest 0.1 g) against log-transformed total length (measured with a metallic ruler to the nearest 1 mm). To ensure that amphisbaenians had empty stomach and intestines before being weighed, we gently compressed their vents to force the expulsion of feces (used for a study of diet). The small weight of the tag was considered negligible.

RESULTS AND DISCUSSION

In the four years of marking amphisbaenians, we have implanted PIT tags in a mean of 45 $\pm$ 4 amphisbaenians per study plot and campaign (3 plots and 7 campaigns of 15 days each), which so far leads to a grand total of 930 marked individuals in the four years. The number of individuals found and marked was significantly higher in spring than in autumn for a similar search effort ($F_{1,19} = 11.82$, $P = 0.003$).

Recapture rate was, however, relatively low; only around 15% of individuals found had already been marked. This is likely attributable to the difficulty of finding the same individual on several occasions in a relatively short field campaign (i.e., each study plot is surveyed only during 3-4 days per campaign) and the high density of amphisbaenians, rather than to the fact that the marking procedure might affect survivorship or that the tags were lost and we were not be able to identify previously marked individuals. In fact, when we captured an unmarked individual, we always ensured that it had no scars at the usual injection point, which may indicate that it had been marked previously but had no tag inside. Such scars are typical of marked individuals, but they have never been observed in unmarked individuals. Also, we have not noted a decrease of population size, as assessed from the number of individuals usually found in a working day, which might reflect low survivorship of marked animals. Moreover, nearby populations on the islands, where we sampled amphisbaenians without marking them, show similar trends to the marked populations (unpublished data). Therefore, we are confident that the marking procedure is effective and it is not adversely affecting the populations.

Several authors have considered that PIT tags are not always permanent (Brown, 1997; Ott and Scott, 1999), while others claim permanence for more than 20 (Germano and Williams, 1993) or 70 years (Ferner and Plummer, 2016). In our study, we have recaptured individuals marked in the first year of the fieldwork after four years and we are confident that the mark will persist during the entire life of the amphisbaenian. Gibbons and Andrews (2004) postulated that tag migration may complicate code checking when it is not possible to find the tag, and can also lead to health problems when migrating through the digestive or urinary systems (Jemison et al., 1995). This problem may be greater in fossorial burrowing animals due to the constant friction with the substrate (Measey et al., 2001). In our study, although tag migration occurred in several individual amphisbaenians, in all cases, the tag had stayed just under the skin and was relocated posteriorly of the injection point, reaching a point close to the cloaca in the longest observed migrations. This movement of the tag seems to be along the subcutaneous space typical of amphisbaenians (see above) (Gans, 1978; Gasc, 1981). We did not detect injuries or health problems (e.g., infection, sores, bleeding, low body condition, etc) in any case. Besides, we did not encounbter any problems in reading the tag, even in cases where its exact location was not easily detected at first sight, probably because the small size of *T. wiegmanni* allowed us to scan the entire body surface under the reader at the same time.

On the other hand, long-term effects of PIT tags have been described for several species. However, Lobos et al. (2013) did not find significant impacts on growth rates, mating, or risk of predation when PIT tagging different species of *Liolaemus* lizards. Brown (1997) also concluded that PIT tags did not make any difference in survivorship nor body condition of diverse amphibians, and Keck (1994) obtained similar results for growth rates and mobility in several snake species. Nevertheless, females of the newt *Ichthyosaura alpestris* laid significantly more eggs when marked, which seems to be related to a stress response (Perret and Joly, 2002). Also, measures of corticosterone in blood have shown that the PIT tag implanting procedure can be stressful for small skinks at least 14 days after the implant (Langkilde and Shine, 2006), but have no effects on stress five days after in common lizards (Le Galliard et al., 2011). Our data show that PIT tags do not have a negative long-term impact on the body condition of *T. wiegmanni* (body condition of the same individuals, initial vs. recapture: 0.05 ± 0.03 vs. 0.06 ± 0.04; one-way repeated measures ANOVA: $F_{1,96} = 0.34$, $P = 0.56$). This lack of change of the body condition is a good indication of the absence of long-term negative effects of the PIT tag on health of individuals, as it is known that natural and anthropomorphic alterations of the soil are reflected in a low body condition of these amphisbaenians (Martín et al., 2015, 2017).

Another disadvantage associated with PIT tagging may be related to the price of the reader and each transponder (Gibbons and Andrews, 2004). In our case, the current price of each PIT tag is $2.58 (they are provided in packs of 100 units), the implanters cost $5 each (each one is useful for many markings), the needles costs $2 each (for an optimal functioning, we used a different needle for every 20 punctures), and the reader costs $595. These costs may be normally easily assumed by research or conservation projects financed by the government or other institutions.

With respect to the invasiveness of the procedure, it has been recommended that it should not be used for individuals smaller than 8 cm (Camper and Dixon, 1988; Gibbons and Andrews, 2004; Ferner and Plummer, 2016). In our study, the small size of the PIT tags allowed us to mark amphisbaenians as small as 90 mm SVL without problems, the suitability for being marked depending more on the diameter of the body than on the length. These "small" amphisbaenians are second year young subadult individuals (Martín et al., 2001c). Only newborn individuals (SVL<70 mm when they born in autumn) seem unsuitable for marking with these PIT tags, considering the size of the currently available tags. This can be a problem that precludes the study of aspects of population dynamics, such as growth rates and survivorship of juveniles in their first year. However, given the low movement rate of amphisbaenians, is still possible to assess the number of individuals born in a population if we control for the geographic location of the newborn individuals found, to ensure that we do not repeat the same individual on different days. We expect that the future development of smaller PIT tags will allow them to be used in all individuals.

In contrast to PIT tags, scale clipping and heat branding may be cheaper (Winne et al., 2006; Ekner et al., 2011), whereas VIE tags can be seen without capturing the subject (Daniel et al., 2006; Hutchens et al., 2008), and none of these techniques are as invasive as PIT tagging (Gibbons and Andrews, 2004; Ferner and Plummer, 2016). However, PIT tags offer numerous advantages compared with the other long-term techniques potentially useful for marking amphisbaenians. PIT tags are permanent and marks are unmistakable, while brands made by scale clipping or heating/freezing procedures may be confounded with natural marks or become unreadable as time passes due to external agents (Winne et al., 2006; Ekner et al., 2011). Similarly, VIE tags might be hard to detect in darkly pigmented tissues (Hutchens et al., 2008; Petit et al., 2012). Also, PIT tags are able to provide data even after the death of the marked individual, and/or can be even reused (Gibbons and Andrews, 2004). Finally, PIT-tag telemetry (i.e., detecting the radiofrequency signal of the tag at distance; e.g., Connette and Semlitsch, 2012; Ousterhout and Semlitsch, 2014) may allow the detection and relocation of fossorial animals burrowed underground without physically contacting them. However, for this technique a special, and more expensive, detector with an attached antenna is needed to carefully scan the soil surface, and the detection range for the smallest 8 mm PIT tags is only 16 cm in depth increasing to 30 cm for a 12 mm PIT tag (Ousterhout and Semlitsch, 2014).

In conclusion, although further and more specific studies are needed to test the usefulness and effectiveness of PIT tagging in fossorial reptiles, it seems to be a valid procedure for individual recognition in long-term field studies of amphisbaenians such as *Trogonophis wiegmanni*, and, therefore, we suggest that it may be also applied to other similar limbless fossorial species.

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