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The use of visual and automatized behavioral markers to assess methodologies: a study case on PIT-tagging in the Alpine newt

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Abstract

In various research fields, biomarkers are now widely used as tools for assessing individual integrity. The recent advances in quantification methods for behavioral patterns, such as computerized video-tracking procedures, make them valuable biomarkers. However, the corollary of these novelties is that they remain relatively unknown and unused. In this study, we show that such tools can assess the validity of research methods, such as individual recognition. To demonstrate this, we employed, as a model, a marking method (passive integrated transponder [PIT] tagging) widely used in amphibians. Detailed visual observations and video-tracking methods were complementary in highlighting components at different behavioral scales: locomotion, feeding, and breeding. We illustrate the scientific and ethical adequacy of the targeted marking method but also suggest that more studies should integrate behavioral analyses. Such biomarkers are a powerful tool for assessing conservation concerns when other techniques cannot detect detrimental effects.

Keywords: Amphibian - Behavior - Biomarker - PIT tag - Video tracking

Researchers have studied behavioral patterns in order to understand the characteristics of species and individuals, categorized into tactics and adaptations, and they also have sometimes been used in other fields, such as conservation biology (Caro, 2007; Shuster & Wade, 2003). Similar to what is done on a molecular or physiological basis (Cooke & O'Connor, 2010), an emerging practice has been to use them as biomarkers—that is, as a tool in evaluation and assessment processes (Fasulo et al., 2010). For instance, behavioral patterns thus have become model systems used to understand how pesticides and other chemicals can affect the integrity of organisms (Denoël et al., 2010; Giusi et al., 2010; Scott & Sloman, 2004). Yet despite the great potential of behavioral markers, they are still little used in many research fields where other life marks, such as life history traits, have historically been preferred (Sutherland, 1998). Video-tracking, a technique benefitting from the recent developments in computer sciences, is now available for quantifying various behavioral patterns that are derived from space use across time (Delcourt, Becco, Vandewalle, & Poncin, 2009; Kato et al., 2004). Its recent use as a biomarker shows its powerful efficacy in laboratory assessments (Denoël et al., 2010; Eddins, Cerutti, Williams, Linney, & Levin, 2010). In a large number of physiological, ecological, and behavioral studies, individual recognition of each member of a studied population is a necessity (Barron, Butler, McDonnell & Ward, 2009; Coltherd, Morgan, Judge, Smith & Hutchings, 2010; Gubili et al., 2009). This allows repeated measures on the same organisms, identification of tactics, and application of effective conservation measures (Caro, 2007; Martin & Bateson, 2007; Shuster & Wade, 2003). When natural marks cannot be used for individual identification, many techniques are now available to mark a large variety of animals, such as fish (Winter, Jansen, Adam, & Schwevers, 2005), amphibians (Donnelly, Guyer, Juterbock & Alford, 1994; Pope & Matthews, 2001; Schulte, Küsters, & Steinfartz, 2007), reptiles (Jemison, Bishop, May, & Farrell, 1995;Keck, 1994), birds (Nicolaus, Bouwman, & Dingemanse, 2008), and mammals (Morley, 2002). The most commonly used methods are tag attachment, tattooing, toe-clipping, burning, and PIT-tagging (Faber, 2001). A PIT-tag is an electronic microchip with a unique code (Donnelly et al., 1994). It is implanted directly in the animal's body and has the advantage of being permanent, reliable, and easily readable. Because of these benefits, PIT-tags are becoming widely used and preferred over techniques such as tattooing and toe-clipping, which are temporary because of color attenuation and toe regeneration (Faber, 2001; Gibbons & Andrews, 2004). The PIT-tag also allows distance detection and thus prevents potential stress of recapture and manipulation (Cucherousset, Marty, Pelozuelo, & Roussel, 2008; Hill, Zydlewski, Zydlewski, & Gasvoda, 2006; Mellor, Beausoleil, & Stafford, 2004). However, PITtags are expensive (Arntzen, Goudie, Halley, & Jehle, 2003) and require a minimal animal size for insertion (Gibbons & Andrews, 2004).

A major component of marking technique choice is that it should not affect the integrity of organisms (Dennis, Newberry, Cheng, & Estevez, 2008; Ferner, 2010; Gibbons & Andrews, 2004). This is important in order to avoid biases in analyses (Winter et al., 2005), but also for ethical considerations (May, 2004). In this respect, most research programs now undergo an ethical evaluation and are obligated to minimize disturbance on animals (May, 2004; Wolfensohn & Lloyd, 2003; see also Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010).

Most research on the possible effects of the PIT-tag has been conducted on fish and has indicated an adequacy of PIT-tagging. No detrimental effect has been found on survival (Bolland, Cowx, & Lucas, 2009; Navarro et al., 2006; Ombredane, Baglinière, & Marchand, 1998), growth (Acolas, Roussel, Lebel, & Baglinière, 2007; Knaepkens, Maerten, Tudorache, De Boeck, & Eens, 2007; Lee, Park, & Cho, 2009), reproduction (Baras, Malbrouck, Houbart, Kestemont, & Mélard, 2000; Mahapatra et al., 2001), feeding behavior (Newby, Binder, & Stevens, 2007; Park & Park, 2009), and swimming ability (Moore, Russell, & Potter, 1990; Mueller, Moursund, & Bleich, 2006;). The few studies on snakes have shown that PIT-tagging does not affect growth, movement, and speed (Keck, 1994; Jemison et al., 1995). In newts (Faber, 1997; Jehle & Hödl, 1998; Perret & Joly, 2002), studies have focused primarily on mortality, growth, fertility, and wound recovery inflicted by the implantation. Neither survival nor growth was affected by the electronic chip (Cummins & Swan, 2000; Fasola, Barbieri, & Canova, 1993; Ott & Scott, 1999). It appears that PIT-tagged individuals survived under both natural and captive conditions and that PIT-tagged salamanders became pregnant under natural conditions (Steinfartz, Stemshorn, Kuesters, & Tautz, 2006). Behavioral patterns have thus been too rarely used to evaluate the effect of PIT-tagging on organisms (Winter et al., 2005). Particularly in newts—although no abnormal behavior has been observed in the field (Faber, 1997)—no controlled experiments have been conducted to assess the impact of PITtagging on behavior. Although there has been no evidence of negative impact on the mortality and fitness of newts (Jehle &Hödl, 1998), behaviors might be altered and, thus, affect fitness in a natural situation. Therefore, there is a need for complementary studies to assess potential effects at the behavioral level.

In this study, we show the value of using various behavioral markers to assess the potential effect of a marking technique, PIT-tagging. We took into account simple and complex behaviors at different scales, such as locomotion, feeding, and breeding. We integrated standardized visual observations, as well as new computerized techniques,—that is, the latest software in video-tracking analysis (Noldus Ethovision XT7). We used newts as models to test our methods because (1) they are used in several research fields (Kopecki, Vojar, & Denoël, 2010; Šamajová & Gvoždik, 2010), (2) marking them has not yet been evaluated at the behavioral level, and (3) video tracking has never been used on these organisms.

Methods

Study organism

Amphibians exhibit a large diversity of patterns and habitat use and reveal potential medical or veterinary applications (Clarke, 1997; D'Agostino et al., 2007). The Alpine newt (*Mesotriton alpestris*) is a widely spread species in Europe (Denoël, 2007), in which PIT-tagging has been repeatedly used (Denoël, Lena, & Joly, 2007; Faber, 1997; Perret & Joly, 2002). Alpine newts are aquatic during the breeding season and terrestrial throughout the rest of the year. In the aquatic phase, they use various microhabitats and prey (Denoël & Andreone, 2003). Sexual interactions consist of several male courtship displays toward the female (Denoël, Mathieu, & Poncin, 2005) and occur mainly during the morning in association with high locomotor activity (Martin, Joly, & Bovet, 1989).

Sampling and laboratory maintenance

We caught 32 adult Alpine newts (16 individuals of each sex) in a pond in Pays de Herve (Province of Liege, Belgium; 50°34'20"N, 5°42'40"E; elevation a.s.l. 201 m) at the start of the breeding season (April 1, 2010). Newts were brought directly to the laboratory after a 20-min drive in boxes (3 L) containing aquarium cotton filter and water kept at a low temperature. The newts were randomly distributed, 4 by 4 (2 males and 2 females) in eight tanks (60 × 60 cm, 40-cm water level). The bottoms of the aquariums were covered with flat stones. We also provided three types of shelter: behind an oblique stone, under a roller device (for spawning), and on the ground between the stones. The water temperature was maintained at an average of 15.1°C (SE = 0.3°C), and the photoperiod followed the natural cycle of the capture place, starting at 13-h light: 11h dark and ending at 14-h 30-min light: 9-h 30-min dark at the end of the experiment (30-min increments of the day phase every 15 days). Subjects were fed every 2 days, in the afternoon, with 500 mg of Chironomus larvae per tank. All newts were released into their habitat of capture after the end of the experiment (May 21, 2010).

Pit-tagging

The day after capture, half of the newts (four of the eight tanks) were marked with a PIT-tag (RFID Mark, 134.2 kHz, Reseaumatique, 9×1.4 mm, 33 mg). The PIT-tag was injected under the skin at the level of the hindlimbs and pushed in direction of the forelimbs. The presence of the PIT-tag was verified throughout the experiment with a RFID reader (Reseaumatique, RT 100). The marked newts constituted the experimental group (n= 16), while the other newts were used as a control group (n = 16).

Experimental procedure

Visual observation The observation of newts for a few days prior to the experiment allowed us to establish a list of behavioral units displayed by newts during daytime. By using sketches of unique spots and special features of the newts, we were able to visually recognize the 4 individuals from each tank. We selected the following behavioral units for our study: courtship (sexual acts of the male toward the female), shelter use (presence in one of the shelters), and feeding (eating chironomid larvae).

Courtship and shelter use data collection consisted of an observation session in the morning (9:30 to 11:30 a. m.) and an observation session in the afternoon (14:30 to 16:30 p.m.). At each session, we used a focal sampling method (Martin & Bateson, 2007) 10 times for a minute for each aquarium. The observation sessions took place during the first 10 days after implantation of the PIT-tag (period 1) and during the last 10 days before release of the newts (period 2). The observations were replicated 5 times at each morning and afternoon session of the two periods (a total of 10 days).

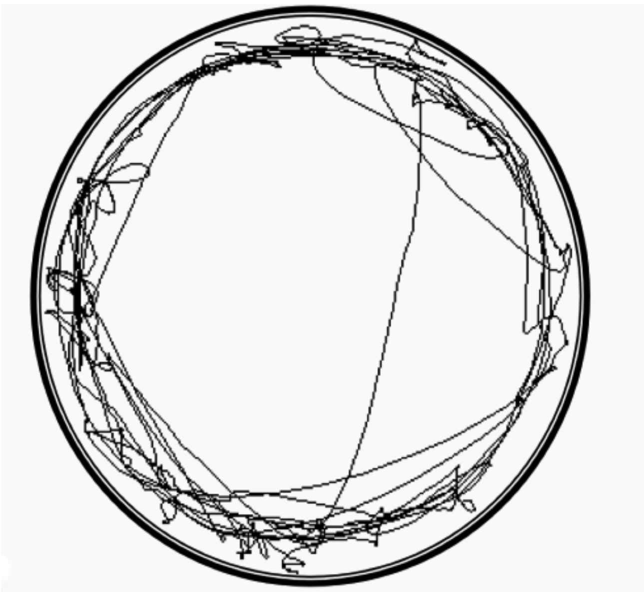


Fig. 1. Example of a video track using Ethovision XT7.

We assessed feeding behavior by measuring the food detection latency (time from the introduction of *Chironomus* larvae at the center of the aquarium to the first capture by each newt). We observed four aquariums simultaneously for 20 min and replicated this during 5 days for the two periods (a total of 10 days). The feeding sessions did not take place on the same days as the other behavioral observations

Video tracking We used Ethovision® XT 7 (Noldus Information Technology), which is an automated video-recording system that allows analysis of movement and activity. We recorded 4 newts at the same time. Each newt was placed in a separate arena with a 19-cm diameter filled with water (15-cm depth). We conducted this experiment between the two periods of visual observations. Newts were given 30 min of habituation before we recorded their behavior for 10 min.

A Sony video camera (DCR-HC90E) recorded the subjects' movement and converted it to a digital signal. Then the system detected the subject by grayscale contrast with the background and determined its size and position on each image (5/sec). From these images we established individual tracks (Fig. 1) and transformed them into a series of dependent variables that quantified the behavior. The selected variables were distance (in centimeters), space use (distance to center point, in centimeters), moving duration (in seconds), and velocity (in centimeters per second).

Statistical procedure

The replicated visual observations of behaviors were tested using a general linear model that accounted for repeated measures. We tested the video-tracking analysis using general linear models, introducing size as a covariate. To achieve normality, continuous data from visual observations were normalized by the square-root transformation before computing the parametric significance test (Sokal & Rohlf, 1995). For all tests, we set an a priori maximum error risk of .05. We conducted all statistical analyses in Statistica 9.1 (Statsoft-France, 2010).

Results

Visual observation

PIT-tag and its interaction with sex and time period had no significant effect on food detection latency (Mean ± SE = 464 ± 70 sec and 507 ± 75 sec, respectively, for marked and control newts; see Table 1 and Fig. 2a). There was also no significant effect of marking and its interaction with time on courtship behavior, regardless of time period (0.99 ± 0.27 per session of observation and 0.78 ± 0.19 per session of observation, respectively, for marked and control newts; see Table 1 and Fig. 2b). There was a significant effect of PITtag on shelter use, but not of the interaction with sex and time (see Table 1 and Fig. 2c): Marked newts used shelters less than did controls (6.31% ± 1.04% and 9.5% ± 1.94%, respectively, for marked and control newts). Fifteen percent of the difference between groups was explained by PIT-tags

Video-tracking analysis

We had to pull out two tracks of the analysis because 2 newts (1 female and 1 male) escaped from the arena during the recording. There was no significant difference between the marked and control groups on the total distance (722 ± 77.7 cm and 544 ± 83.3 cm, respectively; see Table 2 and Fig. 3a), distance to center point (6.39 ± 0.13 cm and 6.32 ± 0.14, respectively; Fig. 3b), moving duration (125 ± 16.2 sec and 89 ± 17 sec, respectively; Fig. 3c), and velocity (1.21 ± 0.13 cm/sec and 0.92 ± 0.14 cm/sec, respectively; Fig. 3d).

Discussion

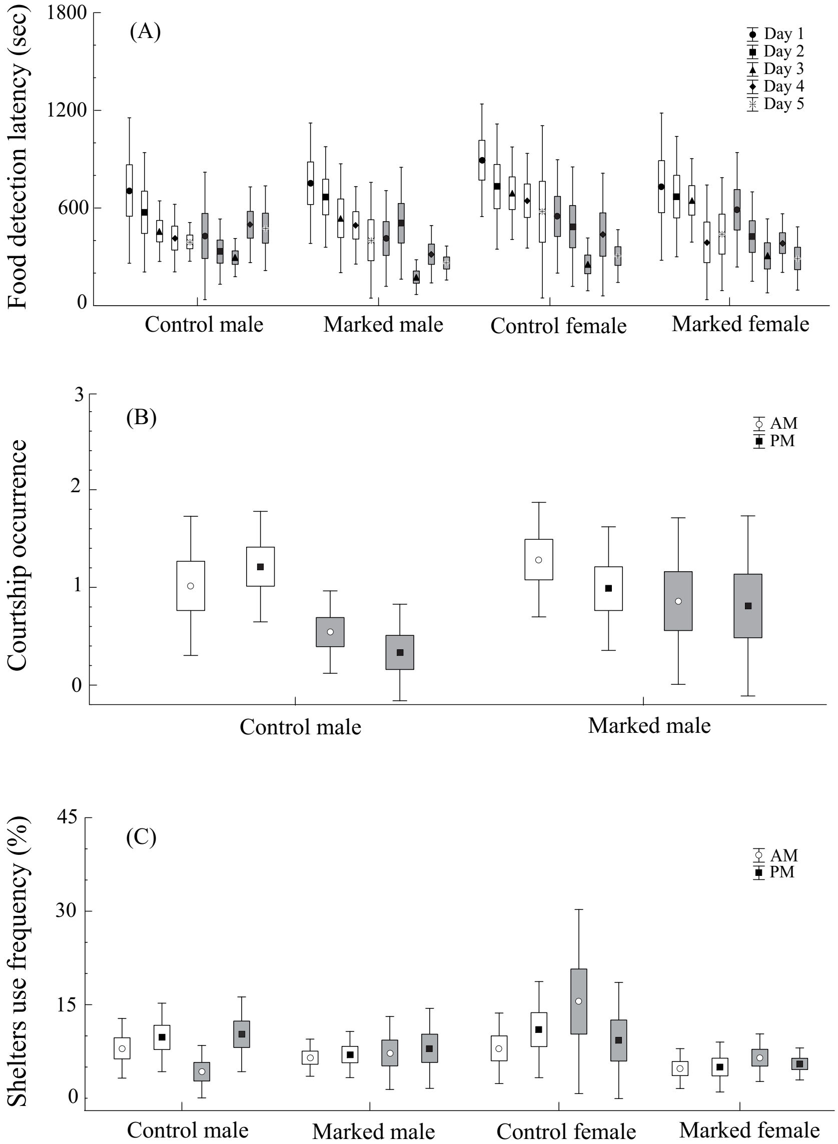
In this study, we used two types of methods to measure behavior. The replicated visual observations allowed analysis of very important and consistent behavior, such as courtship, feeding, and shelter use. The use of a newly developed video-tracking software (Ethovision® XT 7) allowed us to quantify variables such as distance, movement, and velocity. This computerized method has an advantage over standard visual methods in that it determines in a very accurate way the position of an individual and allows processing of a large

Table 1.Visual observations: GLM with repeated measures evaluating the effect of PIT-tag and its interaction with sex and time on behavioral patterns.

Behavior	Factors	*F*	*P*
Feeding	PIT-tag	$F_{(1,28)}$= 1.19	.28
	PIT-tag x sex	$F_{(1,28)}$= 0.93	.34
	PIT-tag x time	$F_{(9,252)}$= 0.34	.96
	PIT-tag x sex x time	$F_{(9,252)}$= 0.80	.62
Courtship	PIT-tag	$F_{(1,14)}$= 0.55	.47
	PIT-tag x time	$F_{(3,42)}$= 1.91	.14
Shelter use	PIT-tag	$F_{(1,28)}$= 5.00	**.03**
	PIT-tag x sex	$F_{(1,28)}$= 2.63	.12
	PIT-tag x time	$F_{(3,84)}$= 0.18	.91
	PIT-tag x sex x time	$F_{(3,84)}$= 1.53	.21

Significatant values are highlighted in bold.

Fig. 2. Visual observations: food detection latency (A), courtship occurrence (B) and shelter use frequency (C) of newts as a function of PIT-tags presence (mean ± SE and SD values). See table 1 for statistical results. Open boxes: first period of observation, shaded boxes: second period of observation.



amount of data in a relatively short time period (Delcourt et al., 2006; Delcourt et al., 2009; Denoël et al., 2010; Eddins et al., 2010). Both methods proved to be complementary with respect to each other in analyzing different behavioral aspects: Visual observations assess complex behaviors such as feeding and breeding, while video tracking assesses more quantitative patterns of locomotor activity. No individuals died during the experiment, and marked newts recovered quickly after surgery. No loss or expulsion of PIT-tags occurred during the whole study. Although surgical glue could be used to avoid loss (Jehle & Hödl, 1998), PIT-tag persistence without glue in our study shows that, if well inserted, they work without using glue (see also Gibbons & Andrews, 2004). Furthermore, surgical glue could have a dermatological, allergic, and respiratory toxicity (Leggat, Smith, & Kedjarune, 2007). Since a PIT-tag can remain on a newt for the duration of its life, it is thus regarded as an appropriate method for marking newts. PIT-tagging reduces stress imposed by handling the animals, because the portable PIT antenna negates the need for recapture (Charney, Letcher, Haro, & Warren, 2009; Faber, 1997). The portable detector (which can be water resistant) allows identification of marked newts directly in their aquatic habitat (Cucherousset et al., 2008).

The feeding behavior of marked newts was not disturbed, resulting in similar food detection latency in both groups. These results are consistent with those obtained in the framework of fish research (Moore et al., 1990; Newby et al., 2007) and sea urchins (Lauzon-Guay & Scheibling, 2008). Another essential behavioral pattern, courtship, occurred frequently during our experiment. It also was not affected by the marking procedure, an aspect that has so far not been covered by previous studies. In terms of shelter use, we observed a difference between groups. While unmarked newts preferred to hide, marked newts were more often in open areas. In the wild, this difference could have predation consequences, but the effect is unknown. It is possible that more active newts could find better prey and ates but could also be more vulnerable to predators such as fish. Fraker (2008) found that the activity level of green frog tadpoles reflects a trade-off between predation risk and feeding: The tadpoles reduced their activity level when predators were present. In a study on salmon, Adams, Rondorf, Evans, Kelly, and Perry (1998) evaluated predator avoidance of juvenile marked fish and found that predators (small-mouth bass) caught significantly more marked fish than unmarked fish. In

Table 2. Video tracking analyses: GLM evaluating the effect of PIT-tag and its interaction with sex on locomotors activity patterns: distance moved (cm), distance to center point (cm), moving duration (sec) and velocity (cm/sec). Size was introduced as covariate.

Variables	Factors	*F*	*P*
Distance moved	PIT-tag	$F_{(1,25)}$= 2.38	.14
	PIT-tag x sex	$F_{(1,25)}$= 2.95	.10
	Size (covariate)	$F_{(1,25)}$= 0.39	.54
Distance to center point	PIT-tag	$F_{(1,25)}$= 0.14	.71
	PIT-tag x sex	$F_{(1,25)}$= 2.39	.13
	Size (covariate)	$F_{(1,25)}$= 0.01	.92
Moving duration	PIT-tag	$F_{(1,25)}$= 2.25	.15
	PIT-tag x sex	$F_{(1,25)}$= 2.51	.13
	Size (covariate)	$F_{(1,25)}$= 0.35	.56
Velocity	PIT-tag	$F_{(1,25)}$= 2.11	.16
	PIT-tag x sex	$F_{(1,25)}$= 2.90	.10
	Size (covariate)	$F_{(1,25)}$= 0.48	.50

contrast with our visual observation highlighting a different use of habitat, our video-tracking analyses did not show any difference between groups: Both marked and control groups moved similarly along the edge of their arena. Although our analysis of video tracks did not show any significant effect of marking on movement, distance, and velocity, we can observe in the Fig. 3 that marked females showed a tendency to be more active. Perret and Joly (2002) also studied the effect of PIT-tagging on the Alpine newt and found that marked females laid significantly more eggs than did unmarked females. They hypothesized that the increased production of eggs could be a response to stress caused by implantation of the PIT-tag. It is possible that stress induced by marking affects the fertility and activity of females, but no definite conclusions can be made. Winter, Jansen, Adam and Schwevers (2005) also detected a difference in the activity of marked eels: They were less active than the control group. They assumed that the fish had not recovered from injury after surgery. However, with time, this difference in activity between groups did not decrease. It is difficult to assess whether stress is a result of surgery or whether of the presence of the PIT-tag itself. Only surgery with all the newts, without inserting a chip in the control group, could test this hypothesis. Close, Fitzpatrick, Lorion, Li, and Schreck (2003) addressed this in a study on the lamprey. They did not compare behavior between marked and control groups but compared blood glucose level, which is a physiological way to assess stress response (Mesa, Bayer, & Seelye, 2003). They found no short-term and long-term difference in glucose concentration between the two groups. This suggests that the marking in question was not more stressful than handling the animal.

However, it should be noted that the small size of Alpine newts makes them more vulnerable to potential pressure of the chip on internal organs. This could cause stress and explain the observed variations. Most studies that have taken into consideration body size have assessed the impact of PIT-tagging not on behavior, but on growth. The absence or delay of growth can be explained by stress induced by tagging. In fish studies, while some authors did not report any effect of PIT-tagging on growth (Acolas et al., 2007; Lee et al., 2009; Navarro et al., 2006), others researchers found a reduction in short-term growth (Baras, Westerloppe, Mélard, & Philippart, 1999; Lacroix, Knox, & McCurdy, 2004; Sigourney, Horton, Dubreuil, Varaday & Letcher, 2005). Cucherousset, Paillisson, and Roussel (2007) found that the effect on growth was independent of the size of their studied fish, whereas Greenstreet and Morgan (1989) determined that a minimum size of 160 mm for Atlantic salmon prevented a tag effect on growth. A tag can represent from 4.6% to 10.4% of the weight of a fish smaller than 120 mm (Adams et al., 1998). However in our study, we used PIT-tags that represented only 0.1% of the weight of the newts—that is, much less than in the previously mentioned studies.

Conclusions

These results add to those of previous studies in suggesting that PIT-tagging is not destructive and has no major invasive effects on behavior. Nevertheless, since some differences were outlined, more in-depth studies on marked animals, both in the laboratory and in the field, are recommended to assess potential invasive aspects of marking on behavior. Since PIT tagging is a preferred marking method in

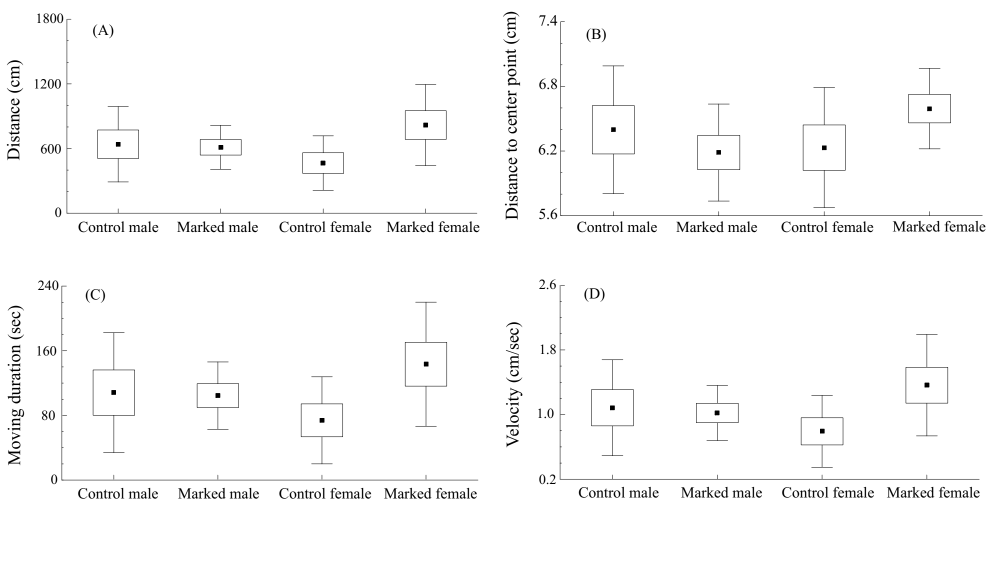


Fig. 3. Video tracking analyses: total distance moved (cm) (A), total distance to center point (cm) (B), moving duration (sec) (C) and velocity (cm/sec) (D) of newts as a function of PIT-tags presence and sex (mean ± SE and SD values). See table 2 for statistical results.

the field, it is essential to ensure that it does not influence the behavior of the studied organisms. The size of animals is an essential factor when a microchip implantation method is used. Fortunately, technological progress now has reduced the size of PIT-tags: only 1.4 × 9 mm in this study, as against 2 × 12 mm up until only a couple of years ago. The smallest PIT-tags are much more expensive and can minimize effects, yet no prior studies have compared the effects of various mark sizes. Since they are now available, we recommend using small marks, except for large organisms or when detection distances are important (Cucherousset, Roussel, Keeler, Cunjak, & Stump, 2005).

Finally, this study demonstrates that more studies should integrate quantitative behavioral analyses, such as video tracking, to estimate the adequacy of methodologies. These behavioral methods proved to give complementary, reliable, and straightforward results. In our study, we applied them to a marking method, but they could also be similarly applied in other fields. Like the biomarkers used in physiology (Cooke & O'Connor, 2010), quantitative ethology is an important tool for validating experimental research and assessing conservation concerns when other techniques lack the sensitivity necessary to detect detrimental effects (Denoël et al., 2010).

Author note

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