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Standard methods for marking caudate amphibians do not impair animal welfare over the short term: An experimental approach*

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Research Article

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Abstract

Major advancements in ecology and biodiversity conservation have been made thanks to methods for marking and individually tracking animals. Marking animals is both widely used and controversial due to the potential consequences for animal welfare, which are often incompletely evaluated prior to implementation. Two outstanding knowledge gaps concerning the welfare consequences of individual marking are their short-term behavioural impacts and the relative impacts from marking versus the handling of animals while carrying out procedures. We addressed these knowledge gaps through an experimental study of alpine newts (*Ichthyosaura alpestris*) in which we varied handling and marking procedures. Examining individual responses to handling, toe clipping and visible implant elastomer (VIE) injection over 21 days showed that handling and marking elicited increased newt activity and hesitancy to feed compared to animals that did not get handled or marked. These effects were apparent even when animals were handled only (not marked), and marking did not further increase the magnitude of responses. Increases in newt activity and feeding hesitancy were transient; they were not observed in the weeks following handling and marking. While previous studies emphasise the welfare impacts of marking procedures themselves, these findings highlight that handling alone can elicit behavioural changes with possible costs to welfare. Yet, the transient nature of behavioural responses suggests that immediate costs of handling may be subsequently compensated for in the short term.

Introduction

Confronting the global biodiversity crisis requires a critical understanding of how threatening processes impact wildlife populations. Demographic studies are the most common approach for investigating impacts, studies which frequently require the capacity to discriminate between individuals (Major 2020). For many species, this requires handling and the application of an artificial mark. The minimising of pain and distress is a fundamental principle of wildlife marking and is also a legal requirement under numerous animal protection legislations; the mark ideally should not significantly impair the welfare of the marked individual (e.g. Locatelli *et al.* 2019). Despite the widespread acceptance of prioritising welfare (Dawkins 2006; Hecht 2021; Soryl *et al.* 2021), the impacts of capture and marking are not always tested, or at least not revisited regularly to reassess the consequence of marking methods under different circumstances (Soulsbury *et al.* 2020). As a result, some marking techniques have gained widespread application across taxonomic groups without explicit tests of impacts on each species, or reapplication in new populations, with examples including colour-coded bird banding, fish adipose fin clipping and amphibian and reptile toe clipping (Perry *et al.* 2011; Tinbergen *et al.* 2014; Uglem *et al.* 2020).

Amphibians are the most threatened vertebrate taxon (Luedtke *et al.* 2023), a conclusion largely justified through the outputs of numerous studies of population dynamics utilising individual marking strategies (e.g. Storfer 2003; Bucciarelli *et al.* 2020). Although less invasive methods are available, their use is frequently hampered by a range of limitations. For instance, individual skin patterns, while an option, sometimes lack distinctiveness or prove suitable solely for short-term studies due to their tendency to change over time (Arntzen *et al.* 2004; Ferner 2007; Aevarsson *et al.* 2022; Kenyon *et al.* 2009); conversely, radio tracking, while a powerful tool, presents size restrictions, feasible only for relatively larger specimens, while bearing substantial economic costs (Ferner 2007; Andreone *et al.* 2013; Daversa *et al.* 2017). As a result, researchers

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have traditionally resorted to established methods like toe clipping (the complete or partial removal of digits; e.g. Perry *et al.* 2011) and, more recently, visible implant elastomer (VIE) injection (subcutaneous injection of silicone-based polymer that hardens after injection), which are attractive due to the inexpensive costs and relatively fast execution that permits large sample sizes.

Toe clipping and VIE injection, while instrumental in assessing how threatening processes impact demography, have sparked controversies concerning welfare implications (Narayan *et al.* 2011; Perry *et al.* 2011; Palmer *et al.* 2023). Critical studies of their impacts have revealed real and potential impacts on individual performance and survival (Bloch & Irschick 2005; Narayan *et al.* 2011). Marking may directly reduce survival through physical impairment (Bloch & Irschick 2005), for example, how improperly cured elastomer can migrate to organs where it can presumably impair organ function (McCarthy & Parris 2004; Cabot *et al.* 2021). Even when no physical impairment occurs, marking may elicit behavioural responses immediately post-marking that have the potential to mediate downstream welfare and survival. Short-term behavioural responses to marking have been largely unexplored (but see Sapsford *et al.* 2014), a knowledge gap which may overlook opportunities to improve welfare without abandoning methods, particularly for cases where the effects are transitory.

While most extant amphibians are anurans, caudates are disproportionately more threatened (57.3% caudates vs 33.2% anurans; IUCN 2023). Despite this, the majority of assessments of marking techniques have focused on anurans (but see Ott & Scott 1999; Davis & Ovaska 2001; Kinkead *et al.* 2006). Here, we examined the behavioural impacts of two invasive marking techniques on the European alpine newt (*Ichthyosaura alpestris*), a caudate species that is often the subject of numerous demographic and behavioural studies (for recent examples, see Diego-Rasilla & Phillips 2021; Gvoždík 2022; Bernabò *et al.* 2023). The overriding aim of the study was to systematically evaluate the short-term effects of handling versus toe clipping and VIE marking on newt behaviour. We achieved this aim through an experiment designed to discriminate between the effects of handling from the impacts of marking on newt activity, shelter use, and feeding.

Materials and methods

The following procedures were approved by the Zoological Society of London's Ethics Committee prior to commencement and licensed by the Home Office (PPL 80/2466 to TG). Alpine newts were collected from invasive populations in the UK and treated prophylactically to eliminate infections with *Batrachochytrium dendrobatidis*: individuals were placed in tanks and treated with an itraconazole solution bath (1 mg L^{-1}; Sporanox, Janssen-Cilag, Titusville, NJ, USA) for 5 min, followed by 10 min in ringer solution once a day for seven days (Garner *et al.* 2009). Our previous work showed that post-treatment newt behaviour, including their activity levels and habitat use, was still informative for comparative studies examining health and welfare (Daversa *et al.* 2018).

Newts (n = 40; 32 female, 8 male) were weighed to the nearest 0.1 g, measured snout-to-vent (SVL) to the nearest mm and then individually housed in 5-L plastic tubs (Really Useful Boxes, Castleford, UK; 340 × 200 × 125 mm; length × width × height), where they were given five days to acclimate. Each tub was divided in half, one half containing 1.5 L aged tap water while the other was filled with autoclaved gravel (5–20-mm diameter). Cover objects (small PVC shelters) were embedded on gravel substrate and submerged in water. During acclimation and throughout the experiment, 1/3 of the tub water was replaced twice per week and debris removed from the aquatic side using sterile turkey basters to maintain sanitary aquatic conditions. We fed newts earthworms (*Lumbricus terrestris*) after water changes (twice per week; total mass at each feed 0.4–0.5 g). The housing and husbandry protocols followed our previous experimental studies (Daversa *et al.* 2018) and pilot work (unpublished) with alpine newts in the same facilities, which indicated no adverse welfare impacts to newts. To further ensure the minimising of adverse welfare impacts, all work was overseen by licenced veterinarians and trained herpetologists.

Experimental treatments were designed to discriminate between the behavioural effects of handling versus the effects of either marking protocol. Treatment #1 (placebo) involved capturing and holding newts in the hand before returning to the housing unit. Treatments #2 (toe clipping) and #3 (VIE) comprised hand-capture followed by distinct procedures: complete removal of the middle digit of the rear right foot and return to the housing unit, and hand-capture followed by injection of approximately 0.015 mL of elastomer (Northwest Marine Technology, Anacortes, WA, USA) in the ventral side of the thigh of the right rear leg and return to the housing unit. These first three treatments were standardised to each last approximately 4 min, while newts in the #4 treatment (control) were left undisturbed during the marking period. We used a sex ratio of eight females for every two males across the four treatments, but within sex, newts were assigned randomly to treatments.

Newt behaviour was sampled for 10 min immediately following the return to the aquatic part of the housing unit (Winandy & Denoël 2011; Bateson & Martin 2021). One observer recorded the position (aquatic versus terrestrial, hidden with head and greater than half the body under cover versus in view with head and greater than half the body exposed) and activity (active versus stationary) for 30 s at 60-s intervals (Winandy & Denoël 2011; Bateson & Martin 2021). The same observer repeated focal sampling 48 h and seven days after the initial sampling. To eliminate treatment effects of time of day on sampling, we assigned one individual per treatment to one of ten sampling groups and collected behavioural data simultaneously across all four treatments in a sampling group. We recorded feeding behaviour immediately after handling/marking and once weekly after that for three weeks, as a yes/no event and as latency (time taken to show interest in food up to 10 min after the food was added to the enclosure). We also recorded total consumption. The experiment was concluded 21 days after marking, with euthanasia performed by immersing the animals in buffered MS-222 (American Veterinary Medical Association [AVMA] Panel on Euthanasia 2020).

For data analyses, we characterised three behavioural responses: activity level (the proportion of time active), visibility level (proportion of time visible), and habitat use (proportion of time aquatic). We chose these responses for their functional role in newt avoidance of threats; activity and visibility level are commonly measured responses to predation risk (Winandy & Denoël 2013; Daversa *et al.* 2021), and habitat use relates to reproductive opportunities, predation and parasitism risk (Winandy *et al.* 2015; Daversa *et al.* 2018). We ran Generalised Linear Mixed Models (GLMMs) in R (R Core Team 2023; lme4 package), one each for each response, to examine: (1) how newt behaviour changed over the course of the experiment; and (2) how handling and marking influenced newt behaviour. We used a binomial error structure for GLMMs and included a unique newt identification as a random effect to account for repeated sampling. We also included experimental treatment, observation number, and their interaction as fixed effects. We assessed the influence of the fixed effects on newt responses by performing likelihood ratio tests with a Chi-squared distribution, using the dropterm() function in R (MASS package). In cases where newt behaviour depended upon the timing of observation, we also assessed the effect of experimental treatment on newt behaviour separately for each observation. To do so, we ran GLMMs with a binomial error structure and an observation-level random effect to account for overdispersion of the data (Harrison 2014). These models included experimental treatment as a single, fixed effect. We compared model coefficients for the three treatment groups (placebo, toe clipping, VIE) against coefficients for the control groups to determine whether newts that were handled and/or marked behaved differently than controls.

To test for feeding latency, we first performed a 'time-to-event' analysis (i.e. survival analysis). The response variables used were: (1) the time within the 10-min observation period when newts were first observed feeding on worms (0–10 min); and (2) an event status indicating whether newts fed within the 10-min observation period (0 = no observed feeding, 1 = observed feeding). For example, newts that were not observed feeding were assigned values of ten for the observation period and 0 for the event status. Second, we ran Generalised Linear Models (GLMs) to detect treatment effects on total consumption as a proportion of the total food provided, including experimental treatment as the fixed effect and again using a binomial error structure. We again used likelihood ratio tests with a Chi-squared distribution and the dropterm() function in R to examine the overall influence of experimental treatment on total worm consumption. We also compared model coefficients for the three treatment groups against coefficients for the control group to determine whether newts that were handled and/or marked consumed more or less than controls. We explored feeding trends over time by running GLMMs with a binomial error structure and including individual ID as a random effect to account for repeated sampling and interactive effects of treatment and sampling week. We tested for interactive and additive effects by performing likelihood ratio tests with a Chi-squared distribution and the dropterm() function.

Results

The proportion of time that newts were active varied over observations (fixed effect of sampling event, $\chi^2 = 37.28$; $P < 0.001$; Figure 1[a]), though handling/marking did not generally influence the proportion of time that newts were active (treatment: sample interaction, $\chi^2 = 3.89$; $P = 0.273$; main effect of treatment, $\chi^2 = 4.98$; $P = 0.174$). However, examining activity patterns on a sample-by-sample basis revealed the effects of all experimental treatments at a specific time-point. Newts in all three handling/marking treatments were more active than newts in the control group immediately following handling/marking ($t \geq 2.06$; $P \leq 0.039$), an effect not detected in subsequent samples (Figure 1[a]). Newt visibility varied across different observations (fixed effect of sampling event, $\chi^2 = 43.36$; $P < 0.001$); yet again, handling/marking newts did not generally influence the proportion of time that newts were visible versus hidden in shelters (treatment: sample interaction, $\chi^2 = 4.96$; $P = 0.175$; main effect of treatment, $\chi^2 = 4.26$; $P = 0.236$). Examining visibility on a sample-by-sample basis did not reveal any event-specific effects (Figure 1[b]). Newt

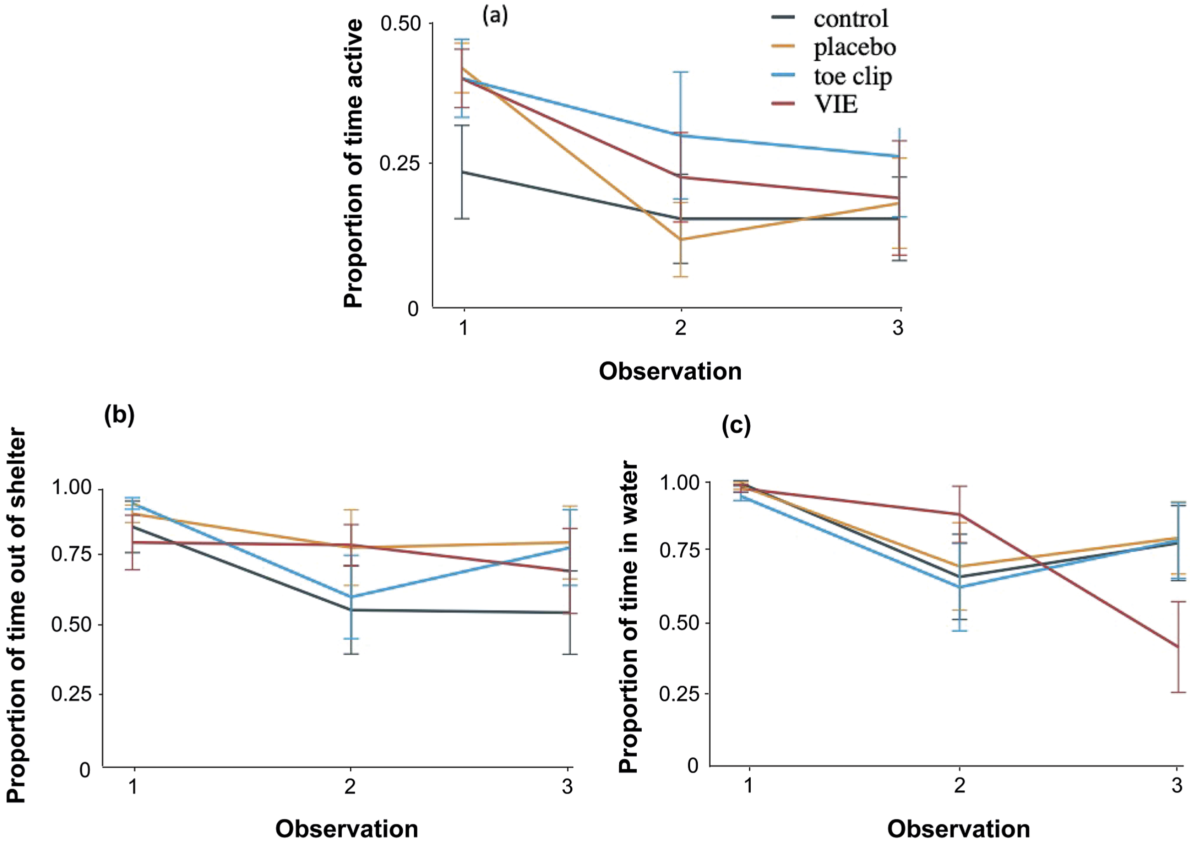

Figure 1. The proportion of observations that newts were (a) active (i.e. moving), (b) outside of their shelter and (c) in water as opposed to on land. Newts were sampled on three occasions: (1) immediately after treatment; (2) 48 h after treatment; and (3) one week after treatment. VIE: visible implant elastomer.

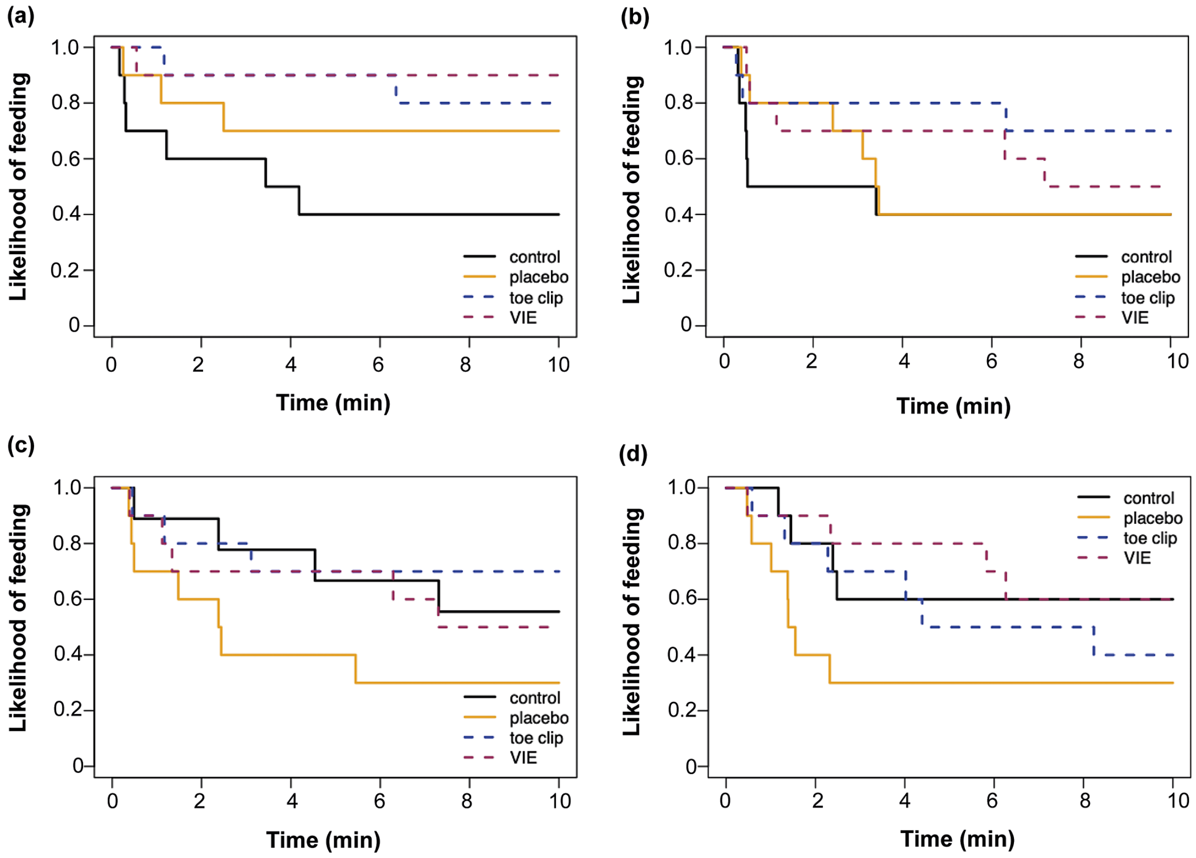

Figure 2. Probability that newts were feeding at a given time during each ([a]–[d]) of the 10-min observation periods (Feeding 1–4, respectively) performed weekly for four weeks (Kaplan-Meir plots for the time-to-event) for each of the four handling/tagging treatments: control, placebo, toe clip, and visible implant elastomer (VIE). Observations began immediately after food was administered into new tanks.

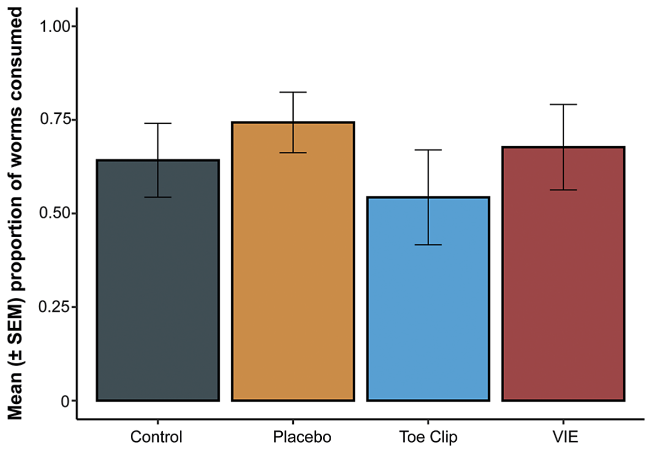

Figure 3. The mean proportion of worms consumed by newts for each of the four handling/tagging treatments. Newts were fed a total of 12 worms over the course of the experiment (four feedings of three worms). Error bars show the standard error of the mean. VIE = visible implant elastomer.

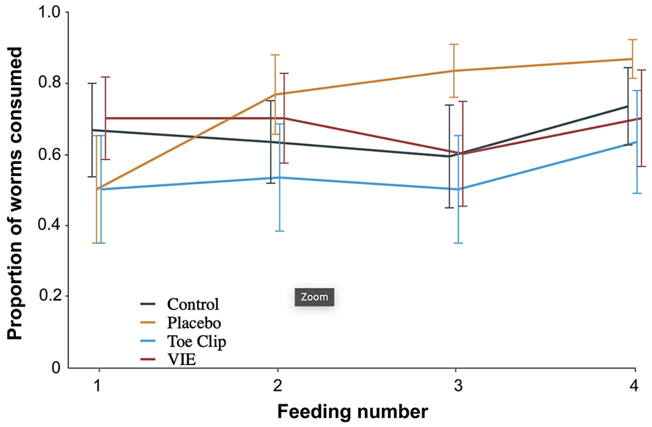

Figure 4. The mean proportion of worms consumed by newts on a weekly basis, with different lines distinguishing the specific marking/handling treatment received. Newts were fed three worms per feeding across four feedings (n = 12). The lines denote mean values within the treatment groups and error bars denote the standard error of the mean. VIE = visible implant elastomer.

habitat use was influenced by handling/marking, depending on the sampling period and marking protocol (treatment:sample interaction, $\chi^2 = 72.05$; $P < 0.001$). Newts that were VIE-tagged spent proportionally less time in the water than control and other treatment newts at the last sampling ($t = -4.28$; $P < 0.001$; Figure 1[c]).

Although there was no statistically significant treatment effect on feeding latency (including experimental treatment in Cox Proportional Hazards model only marginally improved fit, $\chi^2 = 6.60$; $P = 0.086$), in qualitative terms, newts in the control group were, on average, quicker to start feeding after handling/marking than newts in treatment groups (Figure 2[a]). The above trends in feeding latency were not apparent after the first week (Figure 2[b]–[d]). There was a general treatment effect on the proportion of worms that newts consumed across the experiment (dropping treatment from the model reduced goodness of fit, $\chi^2 = 10.99$; $P = 0.012$), but this effect was due to differences between newts receiving a placebo and newts that were toe clipped ($z = 3.20$; $P = 0.001$; Figure 3). The proportion of worms that newts consumed in the three treatment groups generally did not differ from observed consumption in the control group ($P > 0.05$ in all cases; Figure 3). Examining worm consumption on a weekly basis revealed interactive effects between marking/handling and the time of sampling ($\chi^2 = 10.61$; $P = 0.014$), with effects largely driven by temporal changes in feeding by newts in the placebo group (Figure 4). Newts in the placebo group (handled only) initially consumed fewer worms on average compared to subsequent observations (Figure 4).

Discussion

Assessments of the welfare impacts involved in the process of marking amphibians do not always discriminate between handling and marking (but see Oropeza Sánchez *et al.* 2020). This overlooks the directionality of the welfare assessment process, embedded in Soulsbury et al.'s (2020) decision tree for marking wildlife. Here, the determination of both the necessity and welfare implications of capture precedes that of the impact of marking (Soulsbury *et al.* 2020; Figure 1). For a more meaningful assessment of the marking technique itself, then, the impacts of animal capture and restraint first need to be ascertained. Our study illustrates this, as we showed that the initial changes in activity by alpine newts elicited by handling and marking are largely attributable to the handling itself, and transient. Nevertheless, the initial increase in activity immediately after marking may not be in the best interests of the marked animal. Typically, reduced activity and immobility are amphibian responses to predator risk, and so increased activity possibly exposes animals to predators (Winandy *et al.* 2015; Chapman *et al.* 2017; Passos *et al.* 2017; Daversa *et al.* 2021). Yet, animals employ diverse anti-predator behaviours depending on the context (Polčák & Gvoždík 2014; Daversa *et al.* 2021). In the context of handling by humans, the increased activity we observed is likely an anti-predator escape behaviour initiated in response to what newts perceive as a predation attempt (Polčák & Gvoždík 2014). This hypothesis is supported by studies of other wildlife that mount anti-predator responses to human stimuli (Clinchy *et al.* 2016; Palmer *et al.* 2022). Still, increased activity and feeding reductions were not associated with an increased propensity to seek refuge under cover objects or to move onto land. The reasons for these behavioural patterns are unclear, but together they may be indicative of human interventions causing aquatic habitats to be preferential for newts, perhaps because adult newts can flee faster in water than on land (Gvoždík & Van Damme 2008).

The escape-like behaviours that we observed immediately after handling are characteristic of responses made in 'fear' and permit the hypothesis that human handling acts as a fear stimulus for newts (Zanette & Clinchy 2019; Daversa *et al.* 2021). Fear-like responses in wild animals are well-studied and have widespread ecological consequences (Zanette & Clinchy 2019), and frameworks for understanding fear in wildlife hold value to welfare science. For example, fear-like escape behaviours involve physiological stress responses that cause temporary distress, heightened energetic demands, and internal damage (i.e. 'wear and tear'; McEwen & Wingfield 2003; Wingfield 2005). Stress responses involve a recovery period and may decrease investment in reproduction and feeding in caudates (Moore 1984; Bliley & Woodley 2012). In addition to relatively low initial feeding rates by newts in the placebo group, we observed increasing hesitancy to forage in two of the three handling treatments immediately after handling/marking, a trend that was strongest in the two treatments where marking was involved. Stress-associated inappetence may explain this hesitancy, an argument supported by evidence in badgers (*Meles meles*) that human noise causes delayed feeding (Clinchy *et al.* 2016). Alternatively, given that newts increased activity immediately after the marking/handling, they may have simply been too distracted to eat. In either case, the responses that we observed provide evidence that human handling induces fear-like responses in caudate amphibians, as they do in other animal groups (Clinchy *et al.* 2016; Palmer *et al.* 2022).

There have long been calls for longitudinal studies in research of the ecology of fear (Daversa *et al.* 2021). Longitudinal data are essential for measuring the duration of pain and/or distress and the how either might be behaviourally manifested. This study marks a step toward addressing these calls by tracking individual responses over time. Doing so, we found that behavioural responses to handling and marking were transient, providing hope that distress caused to animals is only temporary. The transient nature of the responses also suggests that newts may be able to develop tolerance to human handling that mediates fear and associated impacts to welfare. Nevertheless, repeated human handling can have covert physiological costs that accumulate over individual lifespans (Wingfield 2005) and should be explored more deeply to understand the extent of welfare impacts of handling and marking. Extending our experimental design to longer time-periods, integrating measures of physiological rates (metabolic rate, hormone levels, etc) and assessing physical damage from fear responses would mark a further step toward understanding how human handling and marking impact welfare in caudates and other wild animals.

Longitudinal studies of welfare could help to resolve open questions concerning the ethics of amphibian research. The ethical landscape of animal use in research has a long history, with procedures like amphibian toe clipping undergoing particular scrutiny (for a review, see Perry *et al.* 2011). Our study establishes amphibian handling as an additional facet in need of ethical consideration independently of any procedures. The ethics of handling animals is a function of its impact on an animal's ability to carry out adapted behaviours, its impact on the affective states of animals, and the duration of those impacts (Fraser *et al.* 1997). Our findings underscore the relevance of considering the duration of these impacts. Procedures yielding transient impacts, such as the heightened activity and reduced feeding observed in newts post-handling and marking, present unresolved ethical questions. Therefore, prior to drawing conclusions about the ethical treatment of transient welfare impacts, progressive long-term costs to animal function and affective states should be evaluated (Fraser *et al.* 1997). Longitudinal studies play a crucial role in this objective of future research into animal welfare.

Animal welfare implications

Animal welfare is defined in large part by how individuals cope with their environment (Broom 1991). While marking methods have received considerable attention in terms of the pain they inflict on animals (Palmer *et al.* 2023), marking may also affect functional behaviours in ways that compromise the capacity of animals to cope with the environment and maintain sound health. The behavioural effects of marking methods used in field research are particularly understudied in amphibians despite the common usage of the methods and the high degree of concern this group of animals receives from biodiversity conservationists. We show in alpine newts that behavioural effects arise largely from the handling process, irrespective of the specific marking method used. From a behavioural perspective, handling seems to be as consequential to animal welfare as does the actual marking of animals.

The transient nature of the behavioural changes should be factored into cost-benefit analyses of marking animals and provide promise that methods need not be abandoned to uphold strong welfare standards concerning animal behaviour (not factoring in pain and suffering of the procedures). This is especially relevant with studies posing a high conservation benefit, for which certain levels of impact on individuals may be warranted. However, we only considered a single handling and marking event, which overlooks possible costs that arise distinctly from repeated interventions. In terms of welfare science, the transience of altered behaviour permits the hypothesis that immediate behavioural impacts of handling do not compromise long-term welfare of newts, at least when handling is not repeated over time. Testing this hypothesis was beyond the scope of this study, but the hypothesis could be tested in future studies via monitoring of welfare across age classes of handled and marked newts. We see such studies as imperative to progress in our understanding of the long-term effects of human interventions on the ability of wild animals to cope with their environment and, in turn, maintain good health and welfare.

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Competing interest. None.

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