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Original research article

The impact of toe-clipping on animal welfare in amphibians: A systematic review

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ABSTRACT

Amphibians, the animal class most threatened by extinction, are frequently studied in order to provide insights informing conservation efforts. This research regularly requires the capture and marking of individual animals or tissue sampling for genetic analyses. A common technique for both marking and collecting DNA from amphibians is toe-clipping, which involves cutting off a portion of one or more toes. Toe-clipping is relatively fast and cheap, but it might have a negative impact on animal welfare. However, results from studies investigating the impact of toe-clipping have been variable; while some studies have reported no effect on movement, survival, or stress levels, other studies showed the opposite. Therefore, the aim of this review was to evaluate all currently available evidence of the potential animal welfare impact of toe-clipping amphibians in a systematic manner. We searched the Web of Science, BioOne, and Agricola databases for relevant studies. Studies were incorporated into the review if they included original empirical data derived from experiments conducted on amphibians, evaluated the impact of toe clipping on welfare-related outcomes, and used either a suitable control group (no intervention or handling only), or compared outcomes in the same group before and after toe-clipping. Conference proceedings, reviews, non-peer-reviewed publications, informal reports and research employing co-interventions that might have affected the results were systematically excluded. The quality of the studies was evaluated with an augmented version of the SYstematic Review Centre for Laboratory animal Experimentation (SYRCLE)'s bias risk assessment tool for animal studies. In total, 14 relevant articles were identified and included in this review. The methodological design of these articles and their outcome measures showed considerable heterogeneity, and we observed an unclear or substantial risk of bias across all examined studies. Consequently, and in line with our protocol, no meta-analysis was performed and the evidence was narratively synthesised. Furthermore, none of the studies included a power or sample size calculation. Among 44 welfare-related outcomes assessed, we identified evidence indicating discomfort associated with toe-clipping, manifested as decreased jump distance immediately after toe-clipping, elevated corticosterone and decreased testosterone levels in urine, as well as long-term repercussions reflected in lower daily weight gain. Nevertheless, the existing evidence is too scarce and the

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methodological design of available studies too heterogeneous to reliably conclude that there is a welfare impact of toe-clipping amphibians. Since the quality of the current evidence remains questionable, there is a critical need for adequately powered, high-quality studies that report reliable and pertinent outcome measures to accurately determine the effects of this still popular marking and sampling method. Until more robust evidence is obtained, the impacts of toe-clipping on the welfare of amphibians used in research and the integrity of study results cannot be conclusively confirmed or dismissed. Following the precautionary principle as well as the legal requirements to implement the 3Rs (Replacement, Reduction, Refinement) in research, it may be preferable to use less invasive methods for marking and DNA collection purposes in amphibians in place of toe-clipping, whenever feasible, although case-by-case decisions for each study might be necessary.

1. Introduction

Amphibians are the most endangered animal class, with an estimated 41 % of species threatened with extinction (IUCN, 2024). Between 1980 and 2004, 91 % of deteriorations in the International Union for the Conservation of Nature's (IUCN) Red List Index status were driven by disease and habitat loss (Luedtke et al., 2023). The alarming decline of amphibian populations has prompted a surge in conservation efforts, requiring accurate data on species distribution, disease outbreaks, population sizes, and genetics. Yet, there is a considerable deficiency in fundamental natural history and geographical distribution data for a substantial number of amphibian species – 11 % are categorized as data deficient by the IUCN (IUCN, 2024), representing one of the highest rates of data deficiency among all vertebrate classes. Over the last few decades, research on amphibians has grown rapidly to fill this gap (Womack et al., 2022). Many of these studies require DNA collection or the unique identification of individual amphibians.

Despite controversy, toe-clipping remains one of the most common methods for marking or genetic sampling in frogs, toads, newts, and salamanders (Zemanova, 2019). Herpetologists have employed toe-clipping since the 1940s in mark-recapture studies, where one or more toes are clipped in a unique pattern allowing identification of individuals and cohorts subsequent recaptures (Woodbury, 1956). This method is favoured because it is relatively easy, inexpensive, and quick compared to other techniques for DNA collection and individual identification (Phillott et al., 2007). Additionally, clipped toes can be used to estimate the age of animals (Peng et al., 2022) or to detect infectious diseases (Retallick et al., 2004). A recent review estimated that over 80 % of genetics studies in amphibians still implement lethal or invasive (sensu lato, i.e., involving puncture of the skin; Cousins et al., 2019) DNA sampling techniques, including toe-clipping (Zemanova, 2019), even though there are less invasive methods for both DNA sampling and marking of amphibians available, for example, buccal, cloacal, or skin swabbing (Broquet et al., 2007; Mucci et al., 2014; Pichlmuller et al., 2013) and photo-identification (Bardier et al., 2020; Gould et al., 2023).

Many animal welfare legislations worldwide require that the impact on animals used in research is minimized (e.g., the EU Directive 2010/63EU, USDA Animal Welfare Regulations, New Zealand's Animal Welfare Act 1999) and make it a prerequisite to implement the 3Rs principles (Russell and Burch, 1959). The Rs stand for Replacing the use of animals in experiments with other approaches whenever possible, Reducing the number of animals used whilst ensuring the statistical power and Refining the experiments to minimise pain, suffering, distress, or lasting harm. In research on free-living amphibians and other wild animals, refinement might take the form of using the least harmful research method available (Zemanova, 2020). Beyond ethical concerns and the legal requirements, the potential impact of research methods on the animal may also compromise the validity of results. Pain and stress might alter an animal's behaviour and physiology, leading to biased data in some study designs (Jewell, 2013). This might be particularly problematic in mark-recapture studies, in which it is preferable that the marking technique does not influence the survival and probability of recapture (Donnelly and Guyer, 2014; Sandercock, 2020).

Adverse animal welfare impacts of toe-clipping, such as inflammation, infection, necrosis, abnormal regeneration of digits, and a reduction in the return rate of marked animals, have been reported in some studies (Golay and Durrer, 1994; McCarthy and Parris, 2004; Olivera-Tlahuel et al., 2017; Stock and Bryant, 1981) but not in others (Bull and Williamson, 1996; Lemckert, 1996; Zamora-Camacho, 2018). The discrepancies in these findings may be attributed to differences in species, study conditions, or methodologies. Despite these conflicting results, the potential harm caused by toe-clipping should not be ignored. Minteer and Collins (2005) designated toe-clipping as an example of an ethically loaded practice in ecology, emphasizing the need for animal welfare considerations and ethical guidance. The potential impact of toe-clipping on the welfare of amphibians has been summarized in the literature review by Perry et al. (2011) but it has still not been systematically assessed. Narrative summaries might be prone to bias and lack of transparency and usually do not assess study quality (Haddaway and Macura, 2018; Haddaway et al., 2015). Systematic reviews can avoid these issues by employing pre-registered protocols, comprehensive searches, robust and reliable methodology, and critical appraisal of all included studies (Higgins et al., 2019).

Therefore, the aim of this study was to conduct the first systematic review to evaluate all currently available evidence of the potential animal welfare impact (e.g., survival rate, hormone levels, growth, locomotor performance, behavioural changes) of toe-clipping amphibians. By identifying research gaps, this review might also guide future studies in addressing unknown animal welfare impacts. The findings can help shape more ethical research practices, balancing the need for reliable scientific data with the imperative of safeguarding the welfare of animals used in research.

2. Methods

2.1. Search strategy

The PICO (population, intervention, comparison, outcome) question was: “What is the effect of toe clipping, in comparison with no clipping, on the amphibian’s welfare?”. The methodology was pre-specified in a review protocol, using the SYRCLE format (de Vries et al., 2015), and registered on the Open Science Framework registration platform (https://osf.io/8er2f) before we started the search for relevant articles. The only modification to the protocol consisted of narrowing the eligibility criteria of a control group to a group undergoing no intervention or handling only. Comprehensive searches were conducted on 24 February 2023, using the subscription of the University of Fribourg. We searched these databases: 1) Web of Science – All Databases (1900-present; databases: Web of Science Core Collection, KCI-Korean Journal Database, MEDLINE®, Preprint Citation Index, SciELO Citation Index), 2) Agricola via EBSCO (1970–2022) and BioOne (1965-present). The search strategy was based on the study by Wever et al. (2017) but modified for amphibians. Likewise, it consisted of separate search blocks for toe clipping, welfare and amphibians, each including multiple synonyms. The full search syntax is outlined in the Supplementary Material (S1 Table). Duplicates were removed manually in Endnote (EndNote Team, 2013). Unique references were imported into Rayyan (Ouzzani et al., 2016) for screening.

2.2. Eligibility criteria

Studies were included in the review if they: 1) reported original research on amphibians with toes (i.e., excluding larval amphibians and caecilians), 2) assessed animal welfare-related impact of toe-clipping in comparison with a control group undergoing no intervention or only handling, or alternatively, in comparison within the same group before and after toe-clipping, 3) were written in English. Excluded were conference proceedings, non-peer-reviewed publications, informal reports, and publications with inaccessible full texts. The eligibility assessment was done in two stages: 1) evaluation of the title and abstract, followed by 2) full-text assessment (Fig. 1). Full texts were retrieved via Google Scholar, the library of the University of Fribourg, and if not available via these routes, we contacted the primary author via email. If none of these routes was successful, the paper was excluded for lacking access (detailed in the Supplementary Material, S3 Table). Both selection stages were conducted by two reviewers (MAZ and RLM) independently and conflicts were resolved through a discussion. We also searched for additional relevant studies by inspecting the reference lists of all included articles and pertinent reviews.

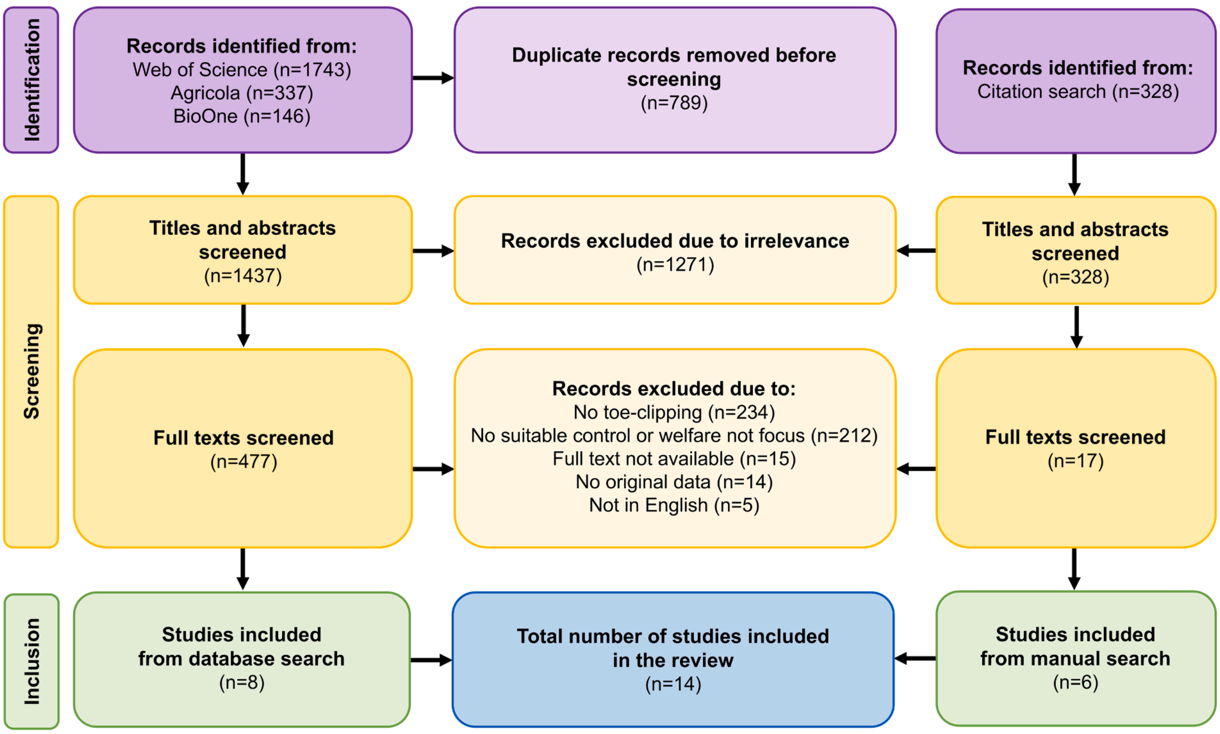

Fig. 1. Flow chart of the study selection process.

2.3. Data extraction

We extracted information related to study design characteristics relevant for inter-studies comparison (e.g., species studied, sex of the animals, sample size, setting, number of toes and legs clipped) and any outcomes related to welfare. Data extraction was performed in Microsoft Excel. The data were extracted from text, tables, or graphs by one reviewer (MAZ) and checked by a second reviewer (RLM). Only one article provided continuous data in a numeric form. We used the WebPlotDigitizer (available at https://automeris.io) to extract values from plots in the remaining studies because actual numbers were not reported.

2.4. Risk of bias and study reporting quality assessment

While uncommon in the fields of herpetology and ecology, risk of bias evaluations are considered crucial in systematic reviews (Higgins et al., 2019). Thus, we performed a formal risk of bias assessment for studies with a separate control group with the SYstematic Review Centre for Laboratory animal Experimentation (SYRCLE)'s risk of bias tool (Hooijmans et al., 2014). With this tool, we evaluated the following types of bias, with the guidance of questions stated in the tool: 1) selection bias (Were individual animals allocated to groups randomly? Were the groups similar at baseline?), 2) performance bias (Were the animals randomly housed during the experiment? Were the experimenters blinded from knowledge which intervention each animal received?), 3) detection bias (Were animals selected at random for outcome assessment? Was the assessor blinded to the treatment?), 4) attrition bias (Were incomplete outcome data addressed adequately?), and 5) other biases (Were there any other potential problems in the study design that could result in risk of bias?). The assessment of selective outcome reporting risk was not conducted, as none of the studies provided a study protocol that delineated primary and secondary outcomes (Wever et al., 2017). In the evaluation of selection bias, groups within a given study were deemed comparable at baseline if there were no significant differences in sex, age, and weight among the animals reported by the authors. Furthermore, we evaluated the reporting of any randomization, blinding, and (*post hoc* or *a priori*) power and sample size calculations as supplementary indicators of study quality. Since the risk of bias assessment tool was designed for studies that employ distinct control and treatment groups, two studies (Hudson et al., 2017; Phillott et al., 2011) were ineligible for scoring due to incompatible study designs (comparison of outcomes within the same group before and after toe-clipping). The assessment was done by one reviewer (MAZ) and checked by a second reviewer (RLM). Discrepancies in the evaluation were solved through a discussion and clarification of the criteria.

2.5. Data re-analysis and synthesis

Whenever comprehensive outcome data could be extracted (i.e., mean, variance, and sample size per group for continuous outcomes, or the count of events and non-events for dichotomous outcomes), we conducted a re-analysis of the data by determining the effect size as a standardized mean difference (SMD) or risk ratio (RR) for continuous and dichotomous outcomes, respectively. In our protocol, we specified that meta-analyses would be performed if a minimum of three articles with comparable interventions and outcomes would be included. No more than two studies per taxonomic group reported the same outcome. Furthermore, the study designs were considered to be too heterogeneous to allow for a meta-analysis of the overall effect (Myung, 2023). Consequently, we report solely the SMD and RR along with the corresponding 95 % confidence intervals for each individual outcome per study. Study effect estimates were computed utilizing a random-effects model and shown in forest plots. All analyses were performed in R 4.4.1 (R Core Team, 2024) via RStudio 2024.09.0 (Posit Team., 2024), using the package metafor (Viechtbauer, 2010).

3. Results

3.1. Study selection

Our search in the three databases retrieved 2226 references. After duplicate removal, 1437 unique references were retained and screened. Following the title and abstract screening, 477 articles were analysed in the full-text screening, and 8 studies included. An additional search of reference lists of the included studies and pertinent reviews identified 328 records, out of which 6 were relevant for our study. In total, 14 articles were included in this review (Fig. 1).

3.2. Study characteristics

Out of the 14 studies, 6 were done in frogs, 5 in toads, and 3 in salamanders (Table 1). Two studies analysed both female and male animals, two studies only males, and the sex of the animals was not reported in ten studies. Three studies described adult animals, three studies sub-adults, and eight studies did not report the animals' age. Nine studies were conducted under laboratory conditions, four under field or semi-field conditions, and one study conducted both laboratory and field experiments. Eleven studies assessed wild or wild-caught, and three studies captive animals. Control treatments consisted of handling only (eight studies) or no intervention (four studies). The two remaining studies compared outcomes before and after toe-clipping. The number of toes clipped varied widely, ranging from one to twelve. The number of legs on which the toes were clipped or whether the toes were clipped on the front or hind legs was often not reported (Table 1).

Table 1
Characteristics of the included studies and outcomes reported by the authors (F: females, M: males, NR: not reported, NRA: not re-analysed).

Study	Species (amphibian type)	Sex	Age	Setting	Source	Toes clipped (n)	Legs clipped (n)	Control	Toe-clipped group size (n)	Control group size (n)	Outcome reported by the authors
Davis and Ovaska, 2001	*Plethodon vehiculum* (salamander)	NR	NR	Lab and field	Wild-caught	3, max. 1 per leg	3 (NR)	No intervention	Laboratory experiment: 20; Field experiment: 32	Laboratory experiment: 20; Field experiment: 32	Weight after 476 days in the laboratory; Survival rate after 476 days in the laboratory (NRA); Daily weight gain in the field experiment within 1 month (NRA); Movement between cover objects in the field experiment within 1 month
Fisher et al., 2013	*Rhinella marina* (toad)	NR	NR	Lab	Captive	3	NR	Handling only	6	6	Corticosterone in blood 350 minutes after toe-clipping
Ginnan et al., 2014	*Rana pipiens* (frog)	NR	NR	Lab	Captive	2–12	NR	Handling only	Group with 2 toes clipped: 10; Group with 3 toes clipped: 10; Group with 4 toes clipped: 10; Group with 8 toes clipped: 10; Group with 12 toes clipped: 10	50	Survival rate 4 months after toe-clipping
Hartel and Nemes, 2006	*Bombina variegata* (toad)	F&M	NR	Field	Wild	1–3	NR	No intervention	27	41	Weight one year after toe-clipping: females, males; Size (snout-vent length) one year after toe-clipping: females, males; Reproductive behaviour (NRA)
Hudson et al., 2017	*Rhinella marina* (toad)	NR	Sub-adults	Lab	Wild-caught	1–2, max. 2 per leg	1 (Hind)	Before and after	10	10	Locomotion: the time required to complete a 5-meter racetrack (NRA); Locomotion: number of hops required to complete the racetrack (NRA); Number of pokes required to complete the racetrack (NRA)
Kindermann et al., 2013	*Litoria wilcoxii* (frog)	M	Adults	Lab	Wild-caught	1–3, not reported per leg	NR	Handling only	12	12	Colour score values (NRA)
Kinkead et al., 2006	*Desmognathus fuscus, Desmognathus monticola* (salamander)	NR	NR	Lab	Wild-caught	1: right hind leg	1 (Hind)	Handling only	Hormone levels: 9; Behavioural observations: 5	Hormone levels: 9; Behavioural observations: 5	Hormones: head adrenaline immediately after toe-clipping; Hormones: torso adrenaline immediately after toe-clipping; Hormones: head noradrenaline immediately after toe-clipping; Hormones: torso noradrenaline immediately

(*continued on next page*)

Table 1 (*continued*)

Study	Species (amphibian type)	Sex	Age	Setting	Source	Toes clipped (n)	Legs clipped (n)	Control	Toe-clipped group size (n)	Control group size (n)	Outcome reported by the authors
											after toe-clipping; Behaviour: position of the affected limb at rest (NRA); Behaviour: movement (NRA); Behaviour: feeding (NRA); Behaviour: gular-pump respiration (NRA)
Liner et al., 2007	*Hyla squirella* (frog)	NR	Adults	Lab	Wild-caught	2: the third toe on the left front leg and the fourth toe on the right hind leg	2 (Front and hind)	No intervention	31	31	Weight six months after toe-clipping: small group, medium group, large group; Size (snout-vent length) six months after toe-clipping: small group, medium group, large group; Survival rate six months after toe-clipping (NRA)
Narayan et al., 2011	*Rhinella marina* (toad)	M	Adults	Field	Wild	3	NR	Handling only	40	40	Corticosterone in urine 6 hours after toe-clipping; Corticosterone in urine 72 hours after toe-clipping; Testosterone in urine 6 hours after toe-clipping; Testosterone in urine 72 hours after toe-clipping; Lymphocytes 6 hours after toe-clipping; Lymphocytes 72 hours after toe-clipping; Monocytes 6 hours after toe-clipping; Monocytes 72 hours after toe-clipping; Neutrophils 6 hours after toe-clipping; Neutrophils 72 hours after toe-clipping; Basophils 6 hours after toe-clipping; Basophils 72 hours after toe-clipping; Eosinophils 6 hours after toe-clipping; Eosinophils 72 hours after toe-clipping
Ott and Scott, 1999	*Ambystoma opacum* (salamander)	NR	Sub-adults	Semi-field	Wild-caught	2 adjacent toes of one leg	1 (NR)	No intervention	80	80	Weight 4 months after toe-clipping; Survival rate 4 months after toe-clipping (NRA)
Phillott et al., 2011	*Litoria genimaculata, L. nannotis, L. rheocola,*	NR	NR	Field	Wild	1–5, max. 2 per foot	1–3 (Front and hind)	Before and after	459 (recaptured after toe-clipping)	777 (before toe-clipping)	Inflammation during 12 months

(*continued on next page*)

Table 1 (*continued*)

Study	Species (amphibian type)	Sex	Age	Setting	Source	Toes clipped (n)	Legs clipped (n)	Control	Toe-clipped group size (n)	Control group size (n)	Outcome reported by the authors
	Nyctimystes dayi (frog)										
Schmidt and Schwarzkopf, 2010	*Litoria nasuta* (frog)	NR	NR	Lab	Wild-caught	1–4, max. 1 per leg	NR (but different for each frog)	Handling only	Immediately after toe-clipping: 21; 14 days after toe-clipping: 15	Immediately after toe-clipping: 20; 14 days after toe-clipping: 17	Jump distance immediately after toe-clipping; Jump distance 14 days after toe-clipping
Swanson et al., 2013	*Psuedacris maculata* (frog)	NR	Sub-adults	Lab	Captive	2, 1–2 per foot: group a) 2 toes on the hind right leg; group b) 1 toe on the front right leg, 1 toe on the hind right leg; group c) 1 toe on the front left leg, 1 toe on the hind left leg	1 or 2 (Hind or both)	Handling only	Group a (2 toes clipped on the same leg): 17; Group b+c (2 toes clipped, each on different legs): 31	51	Survival rate after 12 months: toe-clipped on the same leg, toe-clipped on both front and hind leg
Zamora-Camacho, 2018	*Epidalea calamita* (toad)	F&M	NR	Lab	Wild-caught	2, 1 per leg (third toe)	2 (Hind)	Handling only	61	57	Sprint speed 1 hour after toe-clipping; Run rate 1 hour after toe-clipping

3.3. Outcome data concerning animal welfare

The 14 included studies reported a wide array of outcome data pertinent to animal welfare (Table 1). There were 44 outcomes reported, which can be categorised into parameters associated with survival, weight and size (snout-vent length), locomotion, behaviour, hormonal and immune response, and colour score values (Table 1; Supplementary Material, S2 File). When an outcome was measured repeatedly, we re-analysed data from the last measurement (e.g., survival, size, weight) or the first and last measurement if immediate impact of toe-clipping was assessed as well (e.g., hormone levels, locomotor performance). In several studies, the data for some of the outcomes were either only reported in a descriptive manner or absent. In seven articles (Davis and Ovaska, 2001; Hartel and Nemes, 2006; Hudson et al., 2017; Kindermann et al., 2013; Kinkead et al., 2006; Liner et al., 2007; Ott and Scott, 1999), one or several outcomes could not be re-analysed because of the lack of reporting of mean, variance, the number of animals, or unsuitability for re-analysis (e.g., survival rate with no animals surviving in both toe-clipped and control groups).

The majority of results seem to indicate that toe-clipping has no detrimental effect, with a few exceptions where minor effects were reported. Specifically, toe-clipping was reported not to affect survival either in frogs (Ginnan et al., 2014; Liner et al., 2007), or salamanders (Davis and Ovaska, 2001; Ott and Scott, 1999), but the study by Swanson et al. (2013), found that survival rate 12 months after toe-clipping was lower in frogs with toes removed on the same leg in comparison with frogs with toes removed on different legs. Neither weight nor size were negatively affected by toe-clipping in salamanders (Davis and Ovaska, 2001; Ott and Scott, 1999), toads (Hartel and Nemes, 2006), or frogs (Liner et al., 2007), but Davis and Ovaska (2001) reported lower daily weight gain in toe-clipped salamanders in a field experiment. No effect on locomotion was reported in several movement studies; there was no difference reported in the movement between cover objects for salamanders (Davis and Ovaska, 2001); in time, number of hops, nor number of pokes required to complete a racetrack as well as sprint speed and run rate for toads (Hudson et al., 2017; Zamora-Camacho, 2018); nor in jump distance 14 days after toe-clipping for frogs that exhibited a decreased jumping ability immediately after toe-clipping (Schmidt and Schwarzkopf, 2010). In terms of physiological parameters, Kinkead et al. (2006) reported no difference in head and torso adrenaline and noradrenaline levels in salamanders after toe-clipping. Fisher et al. (2013) found no difference in blood levels of corticosterone in toads after toe-clipping. However, Narayan et al. (2011) observed increased levels of corticosterone and decreased levels of testosterone in toads' urine 6 and 72 hours after toe-clipping. White cell levels remained comparable between toads that were toe-clipped and those that were only handled (Narayan et al., 2011). For reproductive and feeding behaviour, no differences were reported in toads and salamanders (Hartel and Nemes, 2006; Kinkead et al., 2006), nor for inflammation (Phillott et al., 2011) and

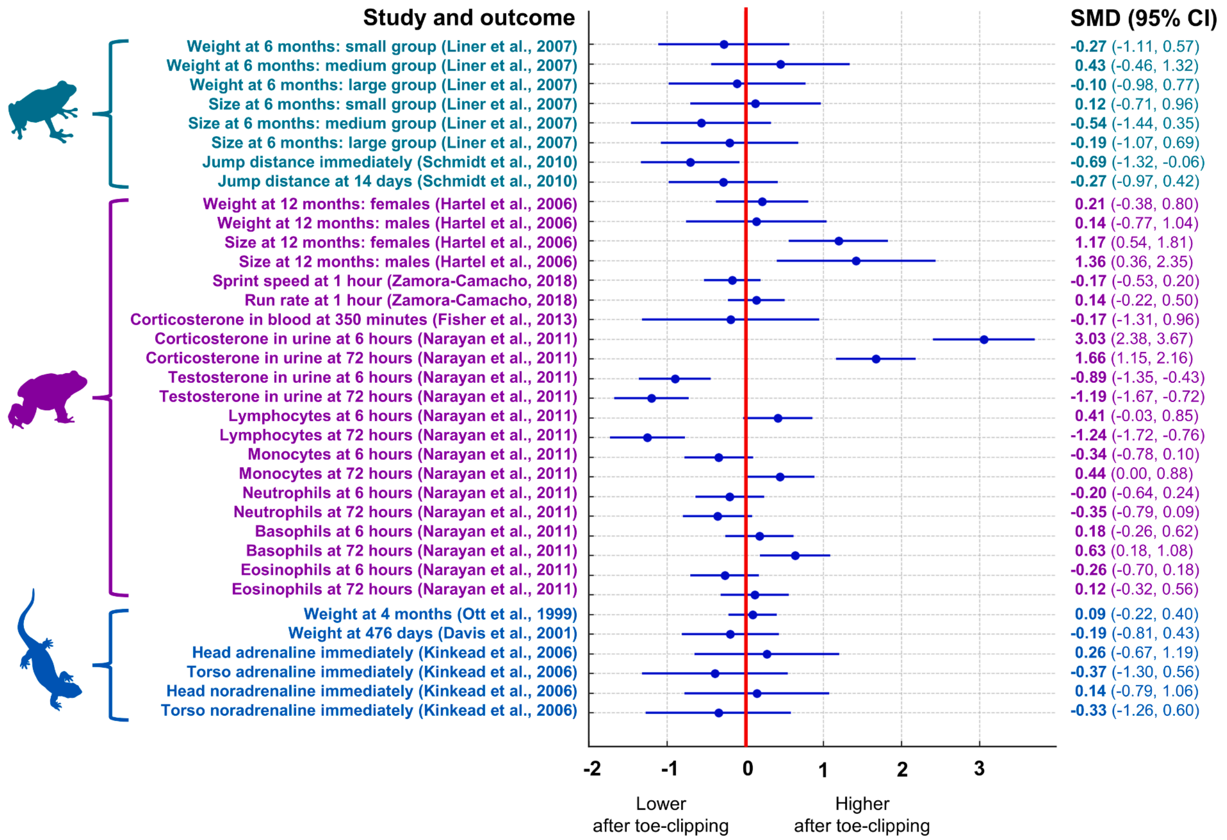

Fig. 2. Forest plot of the continuous outcome data reported in the included studies in frogs (green), toads (purple) and salamanders (blue). Effect sizes were calculated as standardized mean difference (SMD) and corresponding 95 % confidence interval (CI) using a random effects model.

colour score values (Kindermann et al., 2013) in frogs.

Re-analysis of the original data from studies included in this review revealed inconsistencies with the original analyses for four outcomes. We found three additional significant effects not reported by authors (Fig. 2); one on toad size (Hartel and Nemes, 2006), another on lymphocytes (Narayan et al., 2011), and a third on basophil levels 72 hours after toe-clipping (Narayan et al., 2011). The survival rate difference reported in the study by Swanson et al. (2013) was not significant when the data were re-analysed (Fig. 3).

3.4. Risk of bias and study reporting quality assessment

The potential for bias and the quality scores derived from 13 experiments reported in 12 studies with distinct control groups are delineated in Fig. 4 (overall scores) and Fig. 5 (individual scores). While the process of randomization in group allocation was referenced in seven of these experiments (Fig. 4A), only one described the specific methodology utilized for randomization (Fig. 5). None of the experiments mentioned any form of blinding throughout any segment of the research procedure, or conducted a power or sample size calculation (Fig. 4). Due to the inadequate reporting of measures designed to mitigate bias, most items in the risk of bias assessment tool were categorized as 'unclear' (Figs. 4 and 5). The insufficient reporting pertaining to the methodology employed for randomization resulted in an ambiguous risk concerning selection, performance, and detection bias. The baseline characteristics of the subjects were sufficiently documented in five experiments, leading us to evaluate the risk of selection bias as low. In all remaining experiments, one or more baseline characteristics were omitted from reporting, culminating in an unclear risk of bias. Concerning blinding, we determined the risk of performance bias to be high across all experiments, as toe-clipping is practically impossible to conceal during the intervention or subsequent handling of the animal. Consequently, we also assessed the risk of detection bias to be high in 10 experiments, with the exception of three experiments that analysed hormone levels from blood, urine, or tissues. Theoretically, in these cases, some sort of detection blinding could have been implemented on the lab results. In the included experiments of this type, however, it remained ambiguous whether adequate measures had been implemented, resulting in an unclear risk of bias. Concerning the risk of attrition bias, three experiments reported dropouts accurately, thereby achieving a low risk of attrition bias. In one experiment it was mentioned that they experienced attrition but failed to report the exact numbers, therefore being scored as having a high attrition risk of bias. In the remaining nine experiments, the risk of attrition bias was classified as unclear. We did not detect any additional problems with the study designs and therefore the potential for other forms of bias was assessed to be low across all experiments.

4. Discussion

While toe-clipping amphibians has been regarded by some authors as a contentious technique, guidelines on whether this method should or should not be used have not been anchored in a systematic consolidation of currently available data. This article represents the first systematic review of evidence concerning the animal welfare impact of toe-clipping amphibians.

4.1. Currently available evidence

We encountered similar issues as already described in the systematic review on the impact of toe- and ear-clipping in laboratory rodents by Wever et al. (2017). First, research concentrating on the effects of toe-clipping amphibians is limited, notwithstanding the ongoing discourse regarding the possible effects of toe-clipping on the welfare of amphibians. Second, the design of currently available studies is considerably heterogenous. This heterogeneity primarily stems from differences in the population (different sex, different

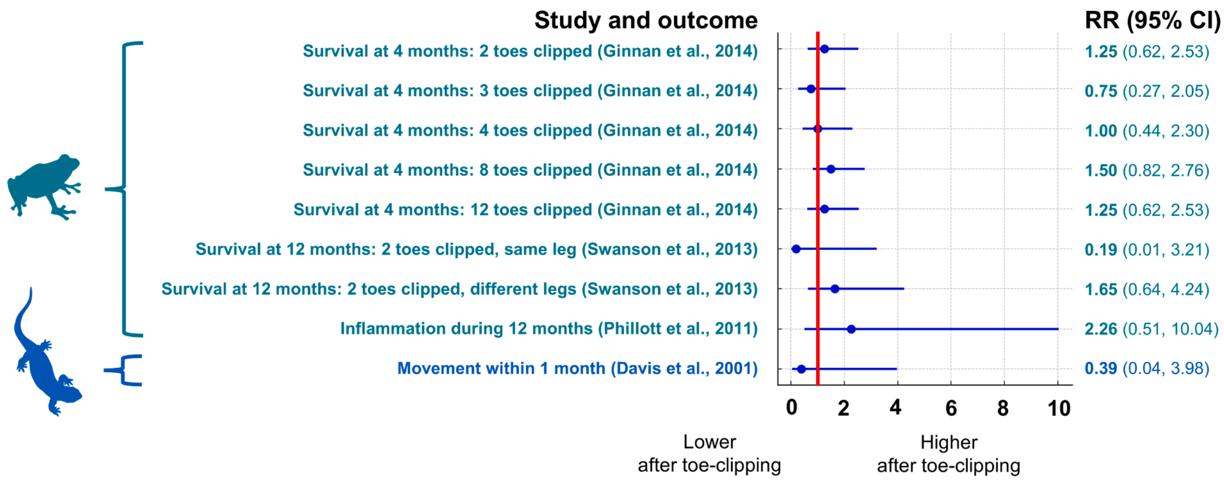

Fig. 3. Forest plot of the dichotomous outcome data reported in the included studies in frogs (green) and salamanders (blue). Effect sizes were calculated as risk ratio (RR) and corresponding 95 % confidence interval (CI) using a random effects model.

Fig. 4. Reporting of quality indicators and risk of bias assessment in 13 experiments conducted in 12 included studies. (A) Reporting of any mention of randomization, blinding, or power or sample size calculation. (B) The risks of selection, performance, detection, attrition, and other types of bias. Three experiments were conducted in the field, therefore random housing risk of bias was not assessed. Two studies were excluded from the assessment due to having study designs incompatible with the risk of bias tool (absence of a control group).

species), the interventions (number of toes clipped, number of legs marked with toe-clipping, control groups with no interaction vs. handling – in which the effect of marking might be conflated), as well as the diverse range of outcome measures evaluated (Table 1). While there is some evidence indicating the impact of toe-clipping on jump distance, daily weight gain, and corticosterone and testosterone levels in urine, the various study design issues inherent in the included studies compromise the reliability of the evidence in favour as well as against animal welfare impact of toe-clipping amphibians.

4.2. Quality of the study designs

Assessing the potential risk of bias within a study and the quality of evidence requires comprehensive documentation of methodology. Our assessment revealed that inadequate reporting of animal research occurs in studies on amphibians to at least the same extent as has been reported in studies on laboratory animals (e.g., Fabian-Jessing et al., 2018; Faggion Jr.. et al., 2011; Landis et al., 2012). The suboptimal documentation of various elements of experimental design led to most indicators of risk of bias being classified as unclear. This situation is alarming, as evidence derived from preclinical animal investigations suggests that the absence of strategies to mitigate bias can substantially affect the outcomes of primary studies (Hawkes, 2015; Hirst et al., 2014). Consequently, the findings of our review might be affected by the potentially high risk of performance and/or detection bias in all included studies. In order to improve the quality of reporting and to minimize any potential bias, we recommend the use of ARRIVE guidelines (Percie du Sert et al., 2020) for any study on animals, including amphibians.

4.3. Lack of power and sample size calculations

Rather surprisingly, not a single one of the included studies described any power or sample size calculations to determine the minimum sample size per group to obtain statistically meaningful results (Figs. 4 and 5). This might have implications for the

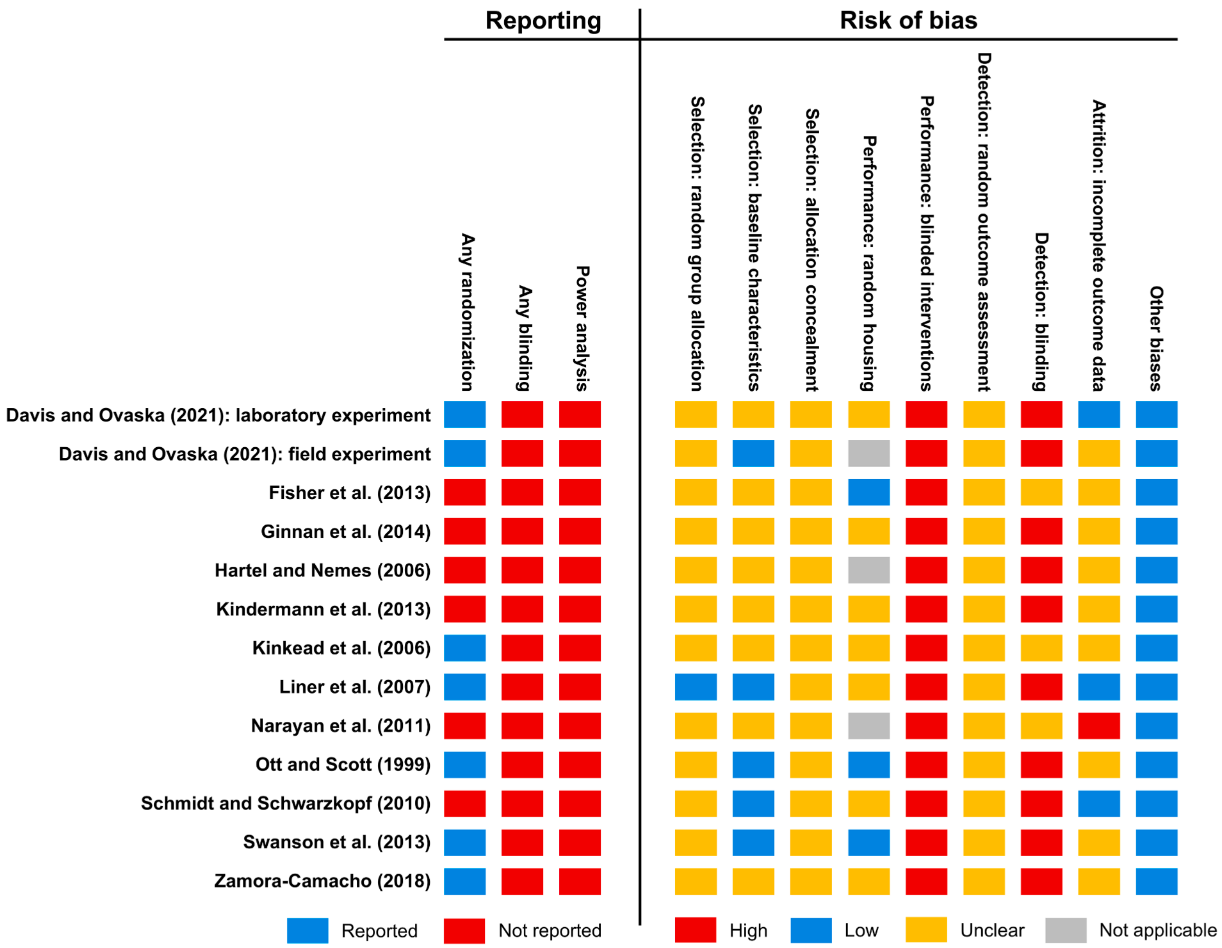

Fig. 5. Individual scores for study quality indicators and risk of bias in 13 experiments conducted in 12 included studies. Three experiments were conducted in the field, therefore random housing risk of bias was not assessed. Two studies were excluded from the assessment due to having study designs incompatible with the risk of bias tool (absence of a control group).

reliability of the findings – if a study is underpowered, the results are likely to be skewed. Furthermore, determining the minimum number of animals necessary to obtain statistically reliable results – and not using more than needed – is not only an ethical obligation but often also a legal requirement (see, e.g., the EU Directive 2010/63/EU).

4.4. Implications for practice

Since the currently available evidence base of the animal welfare impact of toe-clipping amphibians is very limited and heterogeneous, we cannot make any definitive statements. While animal welfare outcomes would be hard to generalize across taxonomic groups, studies conducted on other animal groups could provide some additional insights into potential impacts. Unfortunately, currently available research seems inconclusive also for other taxa, with some studies reporting animal welfare impacts and other studies no effect of toe-clipping. For example, a study of five different species of lizards by Olivera-Tlahuel et al. (2017) showed that toe-clipping significantly reduced survival rate, but no survival effect was observed in a study of the lizard *Cnemidophorus sexlineatus* by Dodd (1993). Similarly, while Pavone and Boonstra (1985) observed lower life-span in toe-clipped male voles (*Microtus pennsylvanicus*), Borremans et al. (2015) found no effects of toe-clipping on survival, movement, or body weight in the Natal multimammate mouse (*Mastomys natalensis*), although some differences between sexes were noted.

Whereas some organisations, such as the American Society of Ichthyologists and Herpetologists or the Society for the Study of Amphibians and Reptiles, state in their guidelines that toe-clipping "may be used for general marking of free-ranging animals when toe removal is not judged (by observation of captives or of a closely-related species) to impair the normal activities of the marked animal" (ASIH, 2004), others (e.g., the Canadian Council on Animal Care, the Association of Reptilian and Amphibian Veterinarians and the British Herpetological Society) have recommended that toe-clipping should be avoided if feasible (ARAV, 2009; BHS, 2017; CCAC, 2004). Possibly resulting from these recommendations, the proportion of studies using toe-clipping has sharply declined in the scientific literature since 2015 (IUCN SSC Amphibian Specialist Group, 2024).

Historically, toe-clipping was often the method of choice to gather essential information about amphibian populations, particularly for genetic analysis. In the meantime, scientific advancements in laboratory protocols and molecular techniques have introduced several non-invasive or minimally invasive approaches for various study objectives, which could be used instead of toe-clipping out of precautionary principle. Such techniques might be also preferable from the legislative perspective; e.g., the EU Directive 2010/63/EU prescribes in the Article 13 that a researcher has to choose between procedures the one that "causes the least pain, suffering, distress or lasting harm". For example, for some amphibian species it is possible to distinguish individual animals based on their natural patterns, scars, or other markings, making the need for marking through toe-clipping superfluous (Zemanova, 2020, 2021a). Such unique chromatophore patterns can be leveraged by photo-identification, which is a highly effective method, particularly when combined with reliable software (e.g., Wild-ID, HotSpotter). For instance, Patel and Das (2020) used photo-identification of dorsal blotch pattern in the Assam sucker frog (*Amolops formosus*), Lima-Araujo et al. (2021) used inguinal colour patterns in the frog *Pithecopus gonzagai*, Reyne et al. (2021) used ventral pattern of Natterjack toad, and Drechsler et al. (2015) used ventral pattern in the great crested newt (*Triturus cristatus*) to reliably distinguish individual animals. This technique is not limited to adult amphibians; even individual tadpoles can be identified based on their tail venation pattern (Gould et al., 2023). Apart from photo-identification, another possibly less invasive, short-term marking approach was suggested by Ottburg and van der Grift (2019), who used small stickers with a unique number attached to the back of common toads (*Bufo bufo*) with biodegradable glue. While no animal welfare impact was reported, this method however needs further validation.

If toe-clipping is used for genetic sampling, methods such as buccal swabs (Angelone and Holderegger, 2009; Broquet et al., 2007; Gallardo et al., 2012), cloacal swabs (Muller et al., 2013) or skin swabs (Pichlmuller et al., 2013; Reyne et al., 2021; Ward et al., 2019) can nowadays be implemented instead. One common argument against the use of non-invasive sampling has been that the quality and quantity of DNA is lower than that obtained through invasive samples (Zemanova, 2021b). Recently, Rainey et al. (2024) compared the DNA yield, purity, amplification success rate, and genotypic data quality among toe clips, buccal swabs, and skin swabs obtained from the Blanchard's Cricket Frog (*Acris blanchardi*). They found that while skin swabbing may produce suboptimal results, buccal swabbing performed equally well as toe clips in terms of DNA yield, purity, and quality of genotypic data. Similarly, Ambu and Dufresnes (2023) compared the performance of buccal swabs and tissue samples obtained from several frog and toad species (*Bombina* spp., *Hyla* spp., *Pelobates* spp., *Pelophylax* spp., *Alytes* spp., and *Discoglossus* spp.). They found that DNA yields from buccal swabs provided even higher DNA yields than tissue samples and RADseq data were not significantly different between toe-clips and buccal swab samples. The DNA yields from non-invasive buccal swabbing might be highly dependent on how skilled the researcher is in the technique (Lavanchy and Dufresnes, 2020), thus proper training may be necessary to achieve optimal results. Training is also important to ensure safe handling of the animals, particularly in species with delicate jaw bones (Ringler, 2018). Apart from buccal, skin, or cloacal swabbing, a recent study by Brustenga et al. (2024) showed that also water baths might be an effective, non-invasive method for DNA collection in amphibians. Although these techniques do not involve injury to the tissue as is the case of toe-clipping, it is important to note that even handling for skin swabbing or photo-taking can be in itself stressful for the animal (Daversa et al., 2024; Fisher et al., 2013), and more research is needed to assess the potential impacts of these methods as well.

Toe-clipping is also often used to determine amphibian age through skeletochronology. This technique involves counting lines of arrested growth in bones, akin to tree rings, to estimate age of species living in temperate climates. While non-invasive approaches to collecting age class data are being developed, e.g., using the DNA methylation patterns to create a species-specific epigenetic clock (Morselli et al., 2023), they require further validation. Consequently, one way to implement the 3Rs principles in these studies would be to include already dead individuals (e.g., roadkill) whenever this is possible, as these might relatively well reflect the age structure of the nearby live population (Kolenda et al., 2018).

In summary, even though other, potentially less invasive methods are available, there is uncertainty about their harmlessness. And admittedly, in some circumstances, toe-clipping would still be the only possible or most efficient method to use in amphibian research due to its relative inexpensiveness and ease of use. The conflict between the potential negative impact on individual animals and the benefit of obtaining data necessary for informing conservation efforts might be unavoidable and requires careful considerations of trade-offs (Minteer and Collins, 2008; Parris et al., 2010). In such cases, minimising the number of toes clipped might be important. Even though the effect of increased number of toes clipped on survival was not clear in the study by Ginnan et al. (2014), research by Waddle et al. (2008) demonstrated an increasing negative effect. Currently, the general recommendation is to clip 1–2 non-adjacent toes in different limbs and to avoid the thumb, which is vital for amplexus, burrowing, and nest excavation for select species, or to clip only the toe pad (so called toe-tipping) without affecting the bones (Lüddecke and Amézquita, 1999; Phillott et al., 2007).

5. Conclusions

In light of the worldwide amphibian crisis, it is crucial to establish, expand, and enhance monitoring programs for amphibian populations to provide essential contemporary data regarding the condition and well-being of amphibians on a global scale. However, the methods used for amphibian monitoring must be carefully considered to account for potential animal welfare impact to ensure that the data collected is both reliable and ethically obtained. Unfortunately, the existing evidence regarding the welfare impact of toe-clipping amphibians is markedly inadequate, overly diverse, and of insufficient rigour to facilitate definitive conclusions. The relevant studies identified in this review, regardless of whether their results do or do not demonstrate an impact of toe-clipping, are impeded by similar constraints: deficiencies in design, lack of detail in methodology, and incomplete presentation of outcome data. It is imperative that forthcoming research addresses these shortcomings. In the absence of such studies, we are unable to ascertain or dismiss any potential effects of toe-clipping on animal welfare in amphibians. This review should also be updated in a few years from now to include currently emerging evidence (e.g., Daversa et al., 2024). In view of this uncertainty, we recommend using less invasive methods instead of toe-clipping whenever this is possible and describing power or sample size calculations – to comply with the legislative requirement to implement the 3Rs principles and out of the ethical concern for the wellbeing of animals used in research.

Ethical statement

Not applicable: This manuscript does not include human or animal research.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.gecco.2025.e03582.

Data availability

Data will be made available on request.

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