Asian Journal of Conservation Biology, December 2020. Vol. 9 No. 2, pp. 188-195 AJCB: FP0135
ISSN 2278-7666 ©TCRP Foundation 2020

Methodological guidelines for minimally invasive tail-clipping: a case study on *Rana huanrenensis* tadpoles

Siti N. Othman[1], Ming-Feng Chuang[2], Hakyung Kang[1], Yoonhyuk Bae[1], Ajoung Kim[3], Yikweon Jang[1], Amaël Borzée[4*]

[1]*Department of Life Sciences and Division of EcoScience, Ewha Womans University 03760, Seoul, Republic of Korea*
[2]*Department of Life Sciences and Research Center for Global Change Biology, National Chung Hsing University, Taichung 402, Taiwan*
[3]*Division of Interdisciplinary Program of EcoCreative, College of Natural Sciences, Ewha Womans University 03760, Seoul, Republic of Korea*
[4]*Laboratory of Animal Behaviour and Conservation, College of Biology and the Environment, Nanjing Forestry University, China*

(Received: June 30, 2020; Revised: August 25 & September 15, 2020; Accepted: October 16, 2020)

ABSTRACT

Tail clipping of amphibian tadpoles is one of the minimally invasive, non-lethal procedures, (apart from skin swabbing) used to collect tissues without euthanising the target individual. It is commonly used for species identification, especially when the continuity between the tadpoles and adult stages is not known. However, there is a lack of published standard and safe protocol for tail clipping of anuran tadpoles. To determine the efficiency of the protocol defined herein, we tail clipped 3.0 mm of four *Rana huanrenensis* tadpoles (Fei, Ye & Huang, 1990), two at each of the Gosner stages 34 and 41. We observed the tails resorbing from tail length = 20.625 ± 0.64 mm on day 0 post-clipping to 5.75 ± 3.49 mm on day 6 post-clipping. During this period, metamorphosis progressed for individuals tail-clipped at Gosner stage 34 (total length: 33.75 ± 2.35 mm; day 0 post-clipping) to Gosner stage 43 (total length: 28.5 ± 3.47 mm; day 6 post-clipping); and individuals tail-clipped at Gosner stage 41 (total length: 35.75 ± 0.35 mm; day 0 post-clipping) to Gosner stage 46 (total length: 15.00 ± 0.00 mm; day 6 post-clipping). We did not record any fatality during the experiment. DNA extracted from 3.0 mm of tail tip tissue yielded gDNA concentrations between 10 and 32 ng/µl, a sufficient amount for barcoding and fingerprinting. We conclude that this protocol is adequate for *R. huanrenensis* and Ranidae in general, and it is safe for tadpoles at Gosner stage 34 and above.

Key words: tail clip, Huanren Frog, larvae, DNA collection, minimally invasive

INTRODUCTION

Acquiring high quality tissue is fundamental for species identification through DNA barcoding. Low amounts and quality of DNA during Polymerase Chain Reactions (PCR) have a negative impact on subsequent genetic analyses (Wong *et al.,* 2012). As a result, to ensure the obtention of sufficient DNA, oversampling is the general rule. This practice burdens both the focal animal and the population (Picazo & García-Olmo, 2015). Specifically, the handling procedure is stressing to the captured individual (Zemanova, 2019), and often lethal (Knesl *et al.,* 2017).

Despite the possibility to rely on non-destructive methods, most DNA sampling procedures are still invasive, such as non-lethal methods of toe clipping (Gonser & Collura, 1996) and blood sampling (García-Feria *et al.,* 2015). However, non-invasive sampling for genetic analyses should be a requirement for the welfare of the organism studied (Müller *et al.,* 2013) and would also increase the chances of legal clearance with permission in many countries. Thus, specific guidelines aiming at minimizing the impact on animals have been developed in agreement with ethical research standards (Ferdowsian & Beck, 2011). For instance, skin swabbing and buccal sampling of adult amphibians are considered non-destructive and ethical, providing DNA usable for microsatellites genotyping (Broquet *et al.,* 2007; Prunier *et al.,* 2012; Pichlmüller *et al.,* 2013), and even next generation sequencing (Dufresnes *et al.,* 2019). However, skin swabbing can result in DNA contamination (Breacker *et al.,* 2017) and buccal swabbing is currently used preferentially for adults rather than anurans larvae due to the small size of buccal cavities and the current technical restrictions to collect epithelial cells (Goldberg *et al.,* 2003). In tadpoles, tails provide the most appropriate tissue type for DNA collection (Gray *et al.,* 2012), providing good quality DNA, due to the absence of bony and cartilaginous elements (Koch & Wilcoxen, 2018).

Amphibian tadpoles regularly get their tail damaged by failed predation attempts (Koch & Wilcoxen 2018; Zamora-Camacho *et al.,* 2018), and some species even increase tail visibility to decrease attacks to vital organs (Mccollum & Leimberger, 1997). The tails usually grow back after being damaged, and regeneration-organizing cells present in the epidermis, as shown for *Xenopus* tadpoles, may act as key factor for tail regeneration (Aztekin *et al.,* 2019). Despite the invasive nature of tail clipping, its impact is comparable to that of failed predations events (Polo-Cavia & Gomez-Mestre 2014), where it has a minimal consequences to the tadpoles fitness (Clarke *et al.,* 2019), no adverse effects on the tadpoles growth (Segev *et al.,* 2015), and highly facilitates molecular analyses, being one of the most effective sample collection methods (Degani *et al.,* 2013). However, while the minimal amount of tissue usable for DNA extraction will not vary, the length and body-percentage of tissue that can be clipped from an ethical point of view will be different for each

*Corresponding Author's E-mail: amaelborzee@gmail.com

individual, based on length and development stage. For instance, the recommended length of tail to be clipped on salamander larvae is from 4.0 to 5.0 mm (~30% of total body; Segev *et al.,* 2015), while it is 3.0 to 10.0 mm for tadpoles (Leyse & Lind, 2003). The objectives of this study are: 1) to measure the percentage of tissue and tail length necessary for DNA collection and fingerprinting in *R. huanrenensis,* and 2) to exemplify the most appropriate larval stage that is safe (with respect to existential performance) for tail clipping in tadpoles.

MATERIAL AND METHODS

Species introduction

Here, we selected *Rana huanrenensis* (Fei, Ye & Huang, 1990) as a model species for tail clipping due to the wide representation of the *Rana* genus in the anuran order (Bossuyt *et al.,* 2006). In addition, we considered the species to be important based on: (i) the complexity in morphological identification of tadpoles in the family (Grosjean *et al.,* 2004); (ii) the absence of data on clear ecological preference for the species (Na *et al.,* 2017); (iii) the parapatric distribution of *R. huanrenensis* with the morphologically similar *Rana uenoi*, *Rana coreana* and *Rana chensinensis* (Do *et al.,* 2018; Dong *et al.,* 2016). *Rana huanrenensis* is listed as Least Concern by the IUCN Red List of threatened species (IUCN SSC Amphibian Specialist Group, 2019). The species ranges from North Eastern China to Southern Korea (Ki *et al.,* 2016; Kim *et al.,* 2002), and in the Republic of Korea, The species generally breeds in stream pools in valleys of high montane region (Na *et al.,* 2017).

Ethics statement and sample collection

The sampling site was based on data retrieved from the georeferenced database Global Biodiversity Information Facility (GBIF). We sampled four *R. huanrenensis* tadpoles in the valley of Mount Buram, in Nowon-gu, Seoul (37.654 N, 127.091 E). We provided each individual a unique identification: (a), (b), (c) and (d). After that, the tadpoles were raised in the lab with the IACUC approval (Permit number: 20-025).We determined the developmental stage following Gosner (1960). Based mainly on toes indentation, we determined that two individuals sampled were at Gosner stage 34 (average total body length: 31.75 mm; identifying trait: indentation 2-3; n = 2) and the other two at stage 41 (average total body length: 35.75 mm; identifying trait: visible forelimbs; n = 2). To minimise the risk of injuries and prevent the death of tadpoles, we limited our investigation to tadpoles between early metamorphosis (Gosner stage > 31; identifying trait: emergence of limb buds; Araújo *et al.,* 2016) and muscle apoptosis (Gosner stage < 44; identifying trait: characterised fingers and toes with induced tails; (Simmons & Horowitz, 2007).

Tail clipping and metamorphosis observation

We clipped 3.0 mm of the tail tip of each individual with sterile forceps on a sterilized petri dish. In order to measure the area of clipped tissues, we photographed the tadpoles before and after tail clipping using an optical camera embedded in the digital microscope Dimis-M with 1x zoom (Dimis v.7.0 software; Siwon Optical Technology, Republic of Korea). The tail tips were stored in 100% alcohol at -20 ºC until DNA extraction. Next, we processed the captured images of the tadpoles to calculate the percentage area of the clipped tail by converting the image to vectors using the scientific image measurement software Digimizer v.5.3.5 (Schoonjans, 2012).We then computed the percentage of clipped tail tip area from the total body area based on the formula: area of clipped tail tip/area of entire body x 100.

In order to observe the morphological changes and to track the tail resorption post clipping, we measured the daily variations in total length, body length and tail length for seven days using the same digital microscope camera (Dimis v.7.0 software; Siwon Optical Technology, Republic of Korea). We observed morphological changes over seven days based on the duration of tail resorption for closely related species (Nakai *et al.,* 2017). The first day of observation was indicated as day 0 post-clipping and the last day of observation was indicated as day 6 post-clipping. Each tadpole was housed separately in identical cylinder PVC tanks (230 mm in diameter and 130 mm in depth, with ventilated lid), under constant water temperature (20.0 ºC) and air supply. All tadpoles were fed once per day with finely ground *Malva verticillata*. All individuals were released back to the original site seven days post tail clipping.

Genomic DNA and PCR yield assessment

We extracted DNA within 24 hours after tissues collection to ensure high quality gDNA (Straube & Juen 2013; Singh *et al.* 2018). DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Germany) according to the instructions of the manufacturer. Following the extractions, we ran an electrophoresis with 3 µl of extracted DNA on a 1.2% agarose gel. Using the same volume, we measured the DNA concentration for each sample (Thermo Scientific™ NanoDrop™ 2000/2000c Spectrophotometers; Thermo Scientific; USA) with direct A280 absorbance measurements, a standard for DNA nucleic acid. This measurement were repeated twice and averaged for consistency.

To confirm that the collected gDNA was adequate we used the DNA from the four tail clips as template in PCR amplification. We amplified 550 bp of the mitochondrial gene *16S* rRNA using the universal primer pair F51 (5'-CCC GCC TGT TTA CCA AAA ACA T-3') and R51 (5'-GGT CTG AAC TCA GAT CAC GTA-3'; Sumida *et al.,* 2002). We performed all PCRs in 20 µL volumes of total reaction, each containing approximately 50 ng/µl of DNA, 1µl Ex taq (5 units/µl; HR001A, Takara; Shiga, Japan), 1.6 µl of 10mM DNTP Mix (Takara; Shiga, Japan), 1 µl of 10 µM forward primer and reverse primer and 1.5 µl of 2.5 mM $MgCl_2$. PCR amplifications were conducted with an initial denaturation of 95 °C for 5 min followed by 35 cycles at 94°C for 30s, 55°C for 30s, 72°C for 60s and a final extension at 72 °C for 10 min. All amplifications were carried with a PCR machine SimpliAmp™ Thermal Cycler (Model PTC1, Applied Biosystems; USA). All PCR amplicons were purified and sequenced for both forward and reverse strands on an ABI platform (CosmoGenetech Company Co., Ltd.; Republic of Korea). PCR amplicons were visualised by electrophoresis on a 1.5% (w/v) agarose gel with 3 µl for each sample, and the gel was documented using a Blue Illuminator (Thermo Fisher Scientific, USA). We determined the PCR efficiency and the Sanger sequencing data quality based on the percentage of nucleotide base obtained from sequencing reads-outs (HQ/%). We tested the consistency of sequencing result obtained by aligning the combined sequences of forward and reverse reads with de-novo assembly to obtain the percentage of pairwise similarity.

Table 1. Percentage of area of clipped tail tip tissue from all *Rana huanrenensis* tadpoles. The percentage of clipped tail tip over total body area and over tail only area were recorded on the day of the tail clipping performed for all four *R. huanrenensis* tadpoles ($n = 4$; day 0 post-clipping).

Individual	Gosner stage (day 0 post-clipping)	Percentage of tail-clipped area over total body area (%)	Percentage of tail-clipped area over tail only area (%)
a	34	4.85	7.67
b	34	4.21	7.92
c	41	4.99	11.93
d	41	4.79	9.88

Tadpoles metamorphosis post tail-clipping

RESULTS

The 3.0 mm of tail tip clipped from all individuals was equivalent to a percentage area of 7.67% and 7.92% for individuals (a) and (b), respectively (Gosner stage 34; day 0 post- clipping; Table 1; Figure 1). The tail clipped area was equivalent to 11.93% for individual (c) and 9.88% measured for individual (d) (Gosner stage 41; day 0 post-clipping; Table 1; Figure 1).

Within seven days post tail-clipping, all individuals metamorphosed (Figure 1). The individuals (a) and (b), tail clipped at Gosner stage 34 (day 0 post-clipping; Table 2; Figure 1), reached Gosner stage 42 by day 6 post-clipping (Table 2; Figure 1). The individuals (c) and (d), tail-clipped at Gosner stage 41 (day 0 post-clipping; table 2; Figure 1), reached the Gosner stage 46 and 45, respectively by day 6 post-clipping (Table 2; Figure 1).

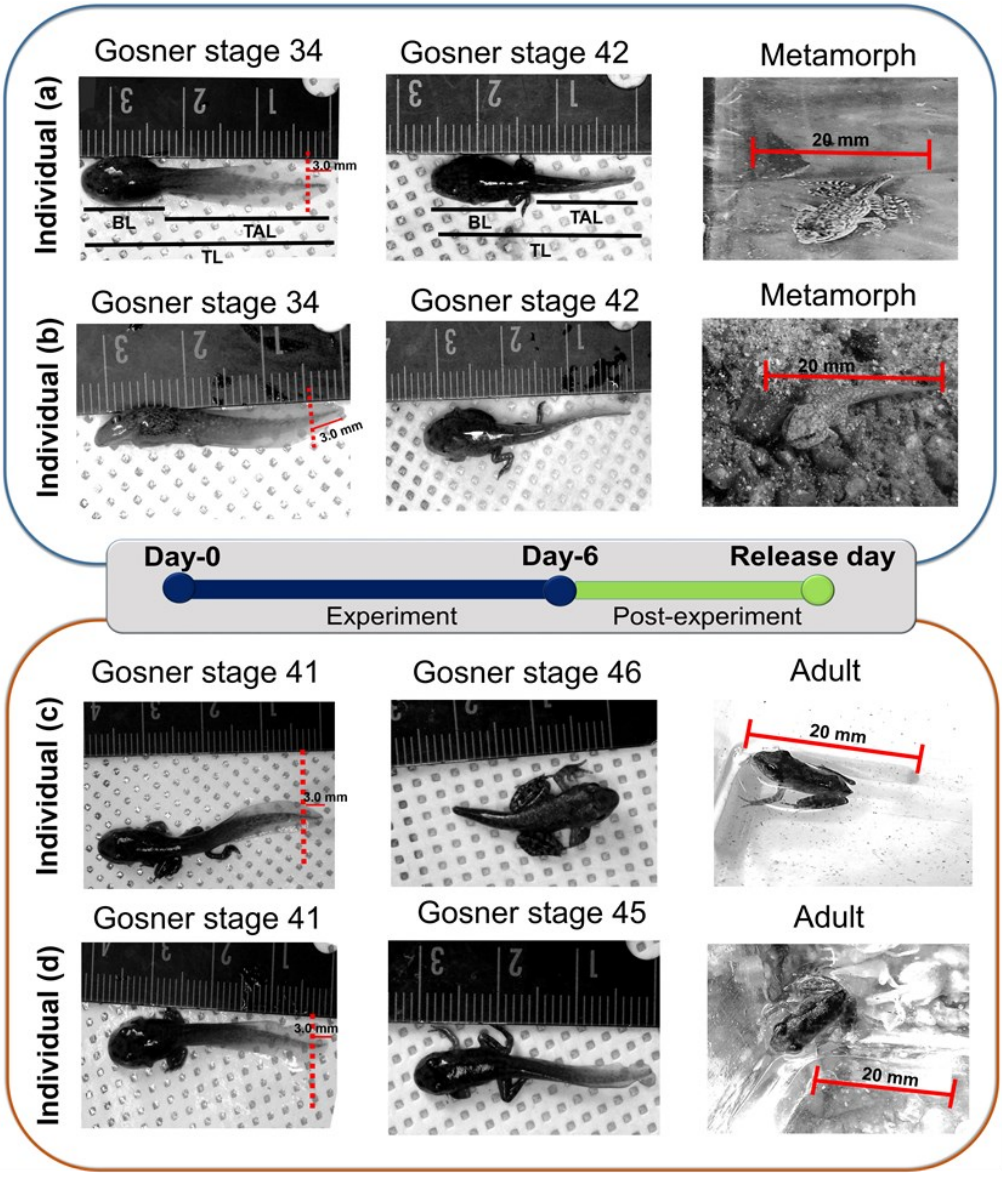

Figure 1. Impact of metamorphosis on morphology of *Rana huanrenensis* tadpoles post tail clipping. All tadpoles metamorphosed and survived the experiment and were released to the capture point. The abbreviations used in the figure stand for; BL: body length, TL: tail length and TL: total length.

Table 2. Daily measurement of morphological data and recorded progress in developmental stages post tail clipping. We recorded the measurement of tadpoles' total length, body length and tail length along the seven days of experiment. We observed the morphology of toes, forelimbs and tail development to infer the metamorphic stage of all *Rana huanrenensis* tadpoles (n = 4).

Gosner stage	Ind-ividual	Observation (Day)	Total length (mm)	Body length (mm)	Tail length (mm)	Identifying trait
34	a	Day 0	31	10	21	Indentation 2-3
34	b	Day 0	32.5	11	21.5	Indentation 2-3
41	c	Day 0	35.5	15.5	20	Visible forelimb
41	d	Day 0	36	15	21	Visible forelimb
35	a	Day 1	28	10	18	Indentation 3
35	b	Day 1	29.5	11	18.5	Indentation 3
42	c	Day 1	32.5	15.5	17	Mouth anterior
42	d	Day 1	33	15	18	Mouth anterior
36	a	Day 2	29	12	17	Five separated toes
36	b	Day 2	30	12	18	Five separated toes
44	c	Day 2	22.5	15	7.5	Tail almost absent
43	d	Day 2	30	13.5	16.5	Visible forelimb
39	a	Day 3	27	15	12	Subarticular patches
39	b	Day 3	30	13	17	Subarticular patches
45	c	Day 3	20	18	2	Tail stub
44	d	Day 3	28	15	13	Visible Tail
40	a	Day 4	28	15	13	Visible forelimb
40	b	Day 4	28	14	14	Visible forelimb
45	c	Day 4	15	15	2	Tail fully resorbed
44	d	Day 4	17	15	11	Tail resorbing
42	a	Day 5	28	16	12	Mouth anterior
42	b	Day 5	30	17	13	Mouth anterior
45	c	Day 5	15	15	2	Tail stub
44	d	Day 5	15	13	10	Tail fully resorbed with visible mouth
43	a	Day 6	26	20	6	Tail fully resorbed
42	b	Day 6	31	20	11	Tail fully resorbed
46	c	Day 6	15	13	1	Metamorphosis almost complete
45	d	Day 6	15	13	5	Pre tail stub, with differentiated mouth

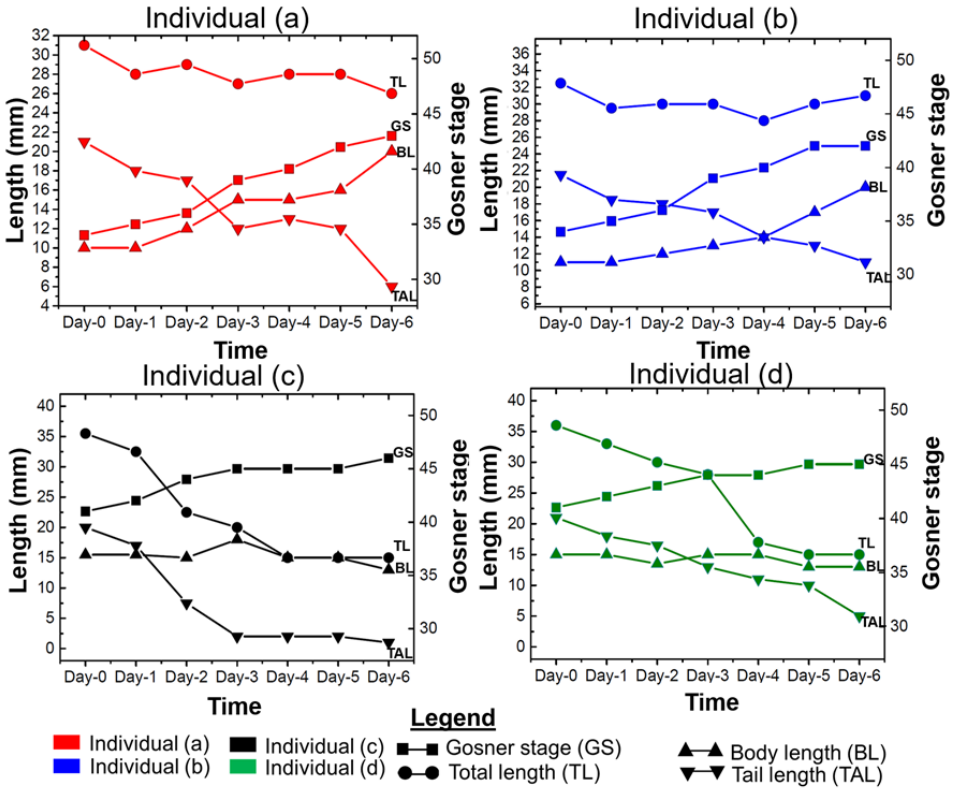

Figure 2. Summary of the seven days of observation on the metamorphosis progress and morphometric measurements of *Rana huanrenensis* tadpoles post tail clipping (n = 4).

In the present study, we found a general reduction in total length and tail length for all tadpoles as they developed and reached higher Gosner stages. We recorded a noticeable reduction of tail length for individuals (a), (b) and (d) during the seven days of the experiment, as the tails resorbed through muscle apoptosis (Table 2, Figure 2). Specifically, a drastic tail resorption reflected muscle apoptosis two days after tail clipping of individual (c) with tail length decreasing from 17.0 (Gosner stage 43; day 2 post-clipping) to 2.0 mm (Gosner stage 45; day 4 post-clipping; Table 2, Figure 2). We found the opposite pattern in body length with the body length of individuals (a) increasing from 10.0 (Gosner stage 34; day 0 post-clipping, Table 2, Figure 2) to 20.0 mm (Gosner stage 43; day 6 post-clipping; Table 2, Figure 2) and the body length of individual (b) increasing from 11.0 (day 0 post-clipping; Table 2, Figure 2) to 20.0 mm (Gosner stage 42; day 6 post-clipping; Table 2, Figure 2). Whereas, the body length of individual (c) decreased after clipping, reducing from 15.5 mm (Gosner stage 41; day 0 post-clipping; Table 2, Figure 2) to 13.0 mm (Gosner stage 46; day 6 post-clipping; Table 2, Figure 2). Similarly, the body length of individual (d) decreased from the original length of 15.0 (Gosner stage 41; day 0 post-clipping; Table 2, Figure 2) to 13.0 mm (Gosner stage 46; day 6 post-clipping; Table 2, Figure 2).

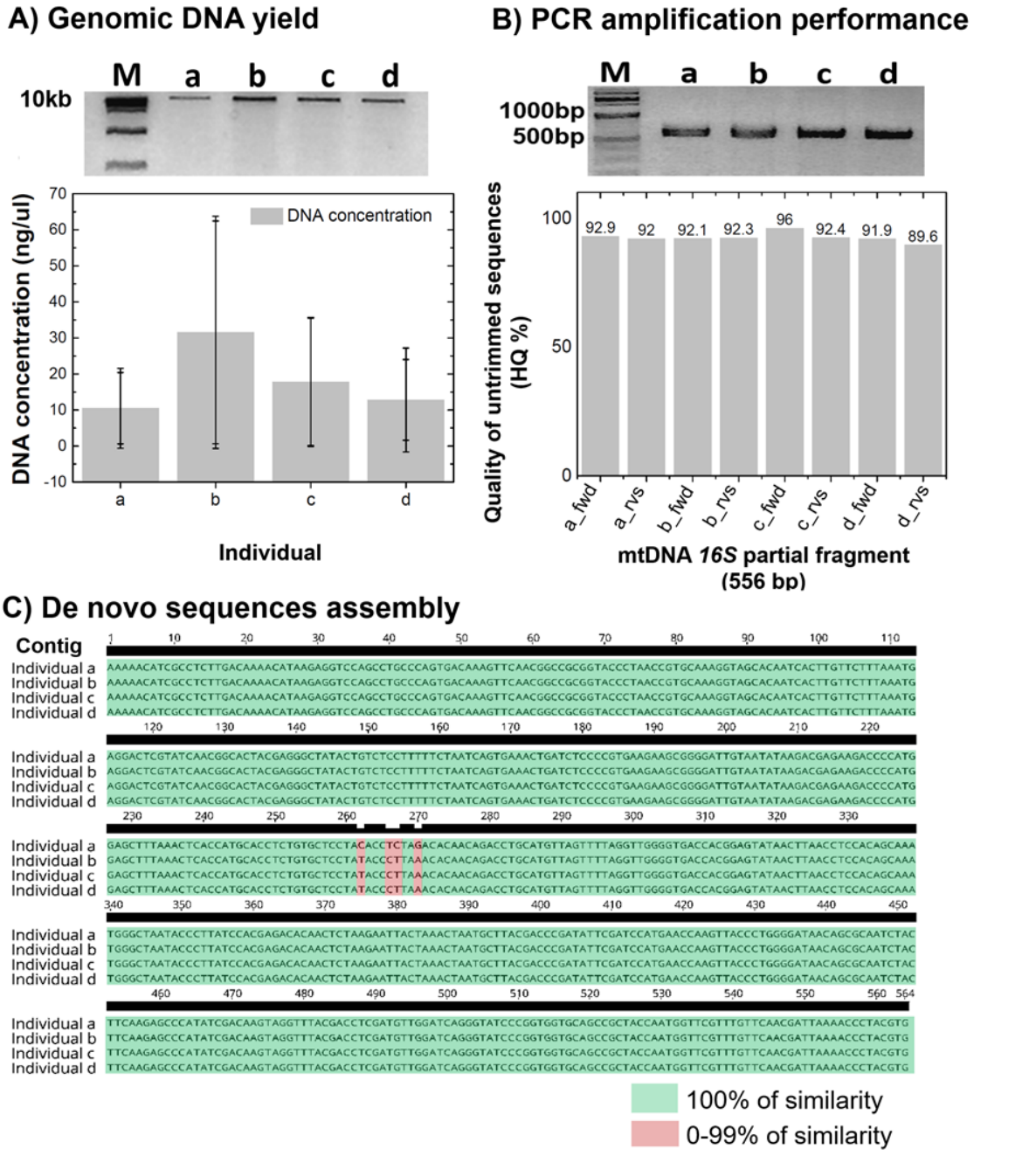

Figure 3. Genomic DNA (gDNA) concentration, PCR amplification results and sequencing data quality. A) Electrophoresis result of gDNA samples extracted from individuals (a), (b), (c) and (d) with DNA concentration measured with NanoDrop Spectrophotometer. B) PCR amplification of partial mtDNA *16S* fragment for the four individuals with their percentage of untrimmed base in a sequence chromatogram (HQ/%). C) De novo assembly of the *16S* partial fragment for contiguous sequences of forward and reverse reads obtained from sequencing of PCR amplicons of the four individuals. The colours highlight the quality of pairwise identity (%) obtained from the sequences alignment.

Table 3. Mean gDNA concentration (ng/µl) extracted using commercial QIAGEN blood and tissue DNA extraction kit. The DNA was extracted from the clipped tail tips of four *Rana huanrenensis* tadpoles.

Individual	DNA concentration reading 1 (ng/µl)	DNA concentration reading 2 (ng/µl)	DNA concentration (ng/µl) (Mean ± SD)
a	11.10	9.90	10.5 ± 0.60 (± 5.71%)
b	30.90	32.20	31.55 ± 0.65 (± 2.06%)
c	17.90	17.70	17.8 ± 0.10 (± 0.56%)
d	11.20	14.40	12.8 ± 1.60 (± 12.50%)

DNA quality and PCR performance

Extracted genomic DNA (gDNA) from the tail tissues yielded an average concentration of 10.50 ± 2.94 ng/µl (mean ± SD) for individual (a), 31.55 ± 3.18 ng/µl for individual (b), 17.80 ± 0.49 ng/µl for individual (c) and 12.8 ± 7.83 ng/µl individual (d) (Table 3, Figure 3A). For each sample, a clear single band of amplified PCR products was visible on the agarose gel, free of smears or primer dimer due to sufficient DNA template. The sequencing results showed a relatively high percentage of identification on the nucleotides sequencing read (HQ/%) for each forward and reverse reads (92.4 ± 1.75%; Figure 3B). The de-novo assembly alignment for all four contig sequences for the four individuals showed excellent pairwise percentage (99.30 %) with a percentage identity of 99.60% (Figure 3C).

DISCUSSION

Despite the debated effect of tail clipping on fitness (Earl & Whiteman, 2015), we demonstrated that tail clipping at Gosner stage 34 and 41 does not have an adverse effect on the visible morphology, metamorphosis progress and survivorship of *Rana huanrenensis* tadpoles (Figure 1). Each anuran species follows a specific ontogeny (Anganoy-Criollo 2013) with variations in development patterns (Johansson *et al.,* 2010), and the pattern described here may be specific to *R. huanrenensis*. However, similar patterns of hind limbs visibility, number of margins in toes and tail resorption were observed in other ranid species, such as *Rana japonicus*, *Rana* [*Lithobates*] *catesbeiana* (Gosner stage 36), and *Hylarana leptoglossa* (Gosner stage 31-39; Saha & Gupta 2011). This consistency is likely a result of the shared life histories in Ranidae (Urszán *et al.,* 2015). Furthermore, tail regeneration over the course of several days post-clipping was also observed in late Gosner stage *Xenopus* tadpoles (Busack & Zug, 1976) and *Osteopilus septentrionalis* tadpoles (Koch & Wilcoxen, 2018). This commonality further indicates only minor variations in tail recovery in amphibian tadpoles, suggesting that the tail clipping procedure used here is applicable to *Rana* spp. in general and is likely to apply to other amphibian species with similar developmental stages (Behr & Rödder, 2018).

The differences in the tail length of clipped individuals at the end of experiments shows that the stress responses of tadpoles to tail-clipping is similar to that of failed predation events. Tail clipping, like failed predation, is a potential trigger to an increase in development speed (Kearney *et al.,* 2016), and to induce tail growth (Maher *et al.,* 2013).

We suggest to perform tail-clipping when the forelimbs are completely visible (Gosner stage 40; Table 2) as it matches with an increase in development speed and switches between Gosner stages (Gosner 40-46; Oielska 2009), but before the apoptosis of the tail muscles (Gosner stage 46; Nakajima *et al.,* 2005). Generally, tissues apoptosis of amphibian larvae happens at the latest metamorphosis stages, when the regenerative capability is almost absent (Ishizuya-Oka *et al.,* 2010), making tadpoles more susceptible to tail injuries in comparison to earlier stages (Tapia *et al.,* 2017). In addition, tail-clipping at a very late stage of metamorphosis (Gosner stage 46 and above) also potentially prolongs the inflammation of the injured muscle (Mescher *et al.,* 2016).

Our study shows that 3.0 mm length of clipped tail tip (Figure 1) is enough to yield sufficient DNA, with a negligible concentration of impurities (Figure 3) and to identify the species using DNA fingerprinting (Figure 3B and Figure 3C). Overall, this study demonstrates the efficiency of minimal tail clipping as an ethical practice for non-lethal tissue samplings.

ACKNOWLEDGEMENTS

We thank the anonymous reviewers for the helpful comments on this manuscript. We are grateful to the local authorities of Nowon-gu for the permission to conduct this study on the tadpoles. This study was supported by a research grant from the Korean Environmental Industry and Technology Institute through 'Development of behavioural ecological methods for population regulation of invasive species' funded by Korea Ministry of Environment (KEITI 2017002270003), and a research grant from Rural Development Administration (PJ01507102) to YJ. The funders had no role in the design and conduct of the study.

REFERENCES

Anganoy-Criollo, M. 2013. Tadpoles of the High-Andean *Hyloxalus subpunctatus* (Anura: Dendrobatidae) with description of larval variation and species distinction by larval morphology. *Papéis Avulsos de Zoologia* 53: 211–224. https://doi.org/10.1590/s0031-10492013001500001

Araújo, O.G.S., Haddad, C.F.B., Da Silva, H.R. & Pugener, L.A. 2016. A simplified table for staging embryos of the pipid frog Pipa arrabali. *Annals of the Brazilian Academy of Sciences* 88: 1875–1887. https://doi.org/10.1590/0001-3765201620150593

Aztekin, C., Hiscock, T.W., Marioni, J.C., Gurdon, J.B., Simons, B.D. & Jullien, J. 2019. Identification of a regeneration-organizing cell in the *Xenopus* tail. *Science* 364: 653–658. https://doi.org/10.1126/science.aav9996

Behr, N. & Rödder, D. 2018. Larval development stages and husbandry of the Rice Frog *Microhyla*

mukhlesuri Hasan et al., 2014 (Anura: Microhylidae). *Bonn Zoological Bulletin* 67: 109–116.

Bossuyt, F., Brown, R.M., Hillis, D.M., Cannatella, D.C. & Milinkovitch, M.C. 2006. Phylogeny and biogeography of a cosmopolitan frog radiation: Late cretaceous diversification resulted in continent-scale endemism in the family ranidae. *Systematic Biology* 55: 579–594. https://doi.org/10.1080/ 10635150600812551

Breacker, C., Barber, I., Norton, W.H.J., McDearmid, J.R. & Tilley, C.A. 2017. A low-cost method of skin swabbing for the collection of DNA samples from small laboratory fish. *Zebrafish* 14: 35–41. https://doi.org/10.1089/zeb.2016.1348

Broquet, T., Berset-Braendli, L., Emaresi, G. & Fumagalli, L. 2007. Buccal swabs allow efficient and reliable microsatellite genotyping in amphibians. *Conservation Genetics* 8: 509–511. https://doi.org/10.1007/s10592-006-9180-3

Busack, S.D. & Zug, G.R. 1976. Observations on the Tadpoles of Pelobates cultripes from Southern Spain. *Herpetologica* 32: 130–137.

Clarke, G.S., Phillips, B.L. & Shine, R. 2019. Clipping the tail fin enables cohort identification of small anuran tadpoles. *Copeia* 107: 71–77. https://doi.org/10.1643/CE-18-128

Degani, G., Goldberg, T., Gasith, A., Elron, E. & Nevo, E. 2013. DNA variations of the green toad *Pseudepidalea viridis* (syn. *Bufo viridis*) from various habitats. *Zoological Studies* 52: 1–15. https://doi.org/10.1186/1810-522X-52-18

Do, M.S., Jang, H., Kim, D., Koo, K., Lee, S. & Nam, H. 2018. The study on habitat analysis and ecological niche of Korean Brown Frogs (*Rana dybowskii*, *R. coreana* and *R. huanrensis*) using the species distribution model. *Korean Journal of Herpetology* 9: 1–11.

Dong, B., Zhou, Y. & Yang, B. 2016. The complete mitochondrial genome of the *Rana huanrensis* (Anura: Ranidae). *Mitochondrial DNA Part A: DNA Mapping, Sequencing, and Analysis* 27: 4551–4552. https://doi.org/10.3109/19401736.2015.1101558

Dufresnes, C., Strachinis, I., Suriadna, N., Mykytynets, G., Cogălniceanu, D., Székely, P., Vukov, T., Arntzen, J.W., Wielstra, B., Lymberakis, P., Geffen, E., Gafny, S., Kumlutaş, Y., Ilgaz, Ç., Candan, K., Mizsei, E., Szabolcs, M., Kolenda, K., Smirnov, N., Géniez, P., Lukanov, S., Crochet, P.A., Dubey, S., Perrin, N., Litvinchuk, S.N. & Denoël, M. 2019. Phylogeography of a cryptic speciation continuum in Eurasian spadefoot toads (*Pelobates*). *Molecular Ecology* 28: 3257–3270. https://doi.org/10.1111/mec.15133

Earl, J.E. & Whiteman, H.H. 2015. Are Commonly Used Fitness Predictors Accurate? A Meta-analysis of Amphibian Size and Age at Metamorphosis. *Copeia* 103: 297–309. https://doi.org/10.1643/ch-14-128

Fei, L., Ye, C.Y. & Huang, Y.Z. 1990. *Keys to Chinese amphibians*. Chongqing Branch, Publishing House of Science and Technology Materials, Chongqing

Ferdowsian, H.R. & Beck, N. 2011. Ethical and Scientific Considerations Regarding Animal Testing and Research. *PLoS ONE* 6: e24059. https://doi.org/10.1371/journal.pone.0024059

García-Feria, L.M., Ureña-Aranda, C.A. & Espinosa de los Monteros, A. 2015. Minimally invasive blood sampling method for genetic studies on *Gopherus tortoises*. *Animal Biodiversity and Conservation* 38: 31–35.

Goldberg, C.S., Kaplan, M.E. & Schwalbe, C.R. 2003. From the frog's mouth: Buccal swabs for collection of DNA from amphibians. *Herpetological Review* 34: 220–221.

Gomez-Mestre, I., Kulkarni, S. & Buchholz, D.R. 2013. Mechanisms and consequences of developmental acceleration in tadpoles responding to pond drying. *PLoS ONE* 8: 1–12. https://doi.org/10.1371/journal.pone.0084266

Gonser, R.A. & Collura, R. V. 1996. Waste Not, Want Not: Toe-Clips as a Source of DNA. *Journal of Herpetology* 30: 445. https://doi.org/10.2307/1565192

Gosner, K.L. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* 16: 183–190.

Gray, M.J., Miller, D.L. & Hoverman, J.T. 2012. Reliability of non-lethal surveillance methods for detecting ranavirus infection. *Diseases of Aquatic Organisms* 99: 1–6. https://doi.org/10.3354/dao02436

Grosjean, S., Vences, M. & Dubois, A. 2004. Evolutionary significance of oral morphology in the carnivorous tadpoles of tiger frogs, genus *Hoplobatrachus* (Ranidae). *Biological Journal of the Linnean Society* 81: 171–181. https://doi.org/10.1111/j.1095-8312.2003.00272.x

Ishizuya-Oka, A., Hasebe, T. & Shi, Y.B. 2010. Apoptosis in amphibian organs during metamorphosis. *Apoptosis* 15: 350–364. https://doi.org/10.1007/s10495-009-0422-y

IUCN SSC Amphibian Specialist Group. 2019. *Rana huanrensis*. *The IUCN Red List of Threatened Species 2019*: e.T58619A63855773. https://doi.org/http://dx.doi.org/10.2305/IUCN.UK.2019-1.RLTS.T58619A63855773.en

Johansson, F., Lederer, B. & Lind, M.I. 2010. Trait performance correlations across life stages under environmental stress conditions in the common frog, *Rana temporaria*. *PLoS ONE* 5: e11680. https://doi.org/10.1371/journal.pone.0011680

Kearney, B.D., Byrne, P.G. & Reina, R.D. 2016. Short- and long- term consequences of developmental saline stress: Impacts on anuran respiration and behaviour. *Royal Society Open Science* 3. https://doi.org/10.1098/rsos.150640

Ki, K.S., Gim, J.Y. & Lee, J.Y. 2016. Water temperature and sound environment characteristics of Huanren Brown Frog oviposition sites. *Korean Journal Environmental Ecology* 30: 344–352.

Kim, J.B., Min, M.S., Yang, S.Y. & Matsui, M. 2002. Genetic relationships among Korean Brown Frog species (Anura, Ranidae), with special reference to evolutionary divergences between two allied species *Rana dybowskii* and *R. huanrenensis*. *Zoological Science* 19: 369–382. https://doi.org/10.2108/zsj.19.369

Knesl, O., Hart, B.L., Fine, A.H., Cooper, L., Patterson-Kane, E., Houlihan, K.E. & Anthony, R. 2017. Veterinarians and humane endings: When is it the right time to euthanize a companion animal? *Frontiers in Veterinary Science* 4: 1–7. https://doi.org/10.3389/fvets.2017.00045

Koch, N.M. & Wilcoxen, T.E. 2018. The effects of tail damage on tadpole development and leaping ability after metamorphosis in Cuban tree frogs (*Osteopilus septentrionalis*). *Bios* 89: 165–173.

Leyse, K. & Lind, A. 2003. Tissue collection protocol for genetic research. *University of California, Davis*: 1–9.

Maher, J.M., Werner, E.E. & Denver, R.J. 2013. Stress hormones mediate predator-induced phenotypic plasticity in amphibian tadpoles. *Proceedings of the Royal Society B: Biological Sciences* 280: 20123075. https://doi.org/10.1098/rspb.2012.3075

Mccollum, A.S.A. & Leimberger, J.D. 1997. Predator-induced morphological changes in an amphibian : predation by dragonflies affects tadpole shape and color. *Eecologia* 109: 615–621.

Mescher, A.L., Neff, A.W. & King, M.W. 2016. Inflammation and immunity in organ regeneration. *Developmental and Comparative Immunology* 66: 98–110. https://doi.org/10.1016/j.dci.2016.02.015

Müller, A.S., Lenhardt, P.P. & Theissinger, K. 2013. Pros and cons of external swabbing of amphibians for genetic analyses. *European Journal of Wildlife Research* 59: 609–612. https://doi.org/10.1007/s10344-013-0747-2

Na, S., Um, Y., Kim, H., An, C. & Yi, H. 2017. The Effect of Habitat Size on Huanren Brown Frog (*Rana huanrensis*) Larvae's Growth Rate in Mt. Surak. *Ecology and Resilient Infrastructure* 4: 75–82.

Nakai, Y., Nakajima, K. & Yaoita, Y. 2017. Mechanisms of tail resorption during anuran metamorphosis. *Biomolecular Concepts* 8: 179–183. https://doi.org/10.1515/bmc-2017-0022

Nakajima, K., Fujimoto, K. & Yaoita, Y. 2005. Programmed cell death during amphibian metamorphosis. *Seminars in Cell and Developmental Biology* 16: 271–280. https://doi.org/10.1016/j.semcdb.2004.12.006

Oielska, M. 2009. *Reproduction of Amphibians*. 4th ed. CRC Press. 436 pp.

Picazo, M.G. & García-Olmo, D.C. 2015. Dna from tissues of young mice is optimal for genotyping. *Electronic Journal of Biotechnology* 18: 83–87. https://doi.org/10.1016/j.ejbt.2014.12.002

Pichlmüller, F., Sträub, C. & Helfer, V. 2013. Skin swabbing of amphibian larvae yields sufficient DNA for efficient sequencing and reliable microsatellite genotyping. *Amphibia-Reptilia* 34: 517–523. https://doi.org/10.1163/15685381

Polo-Cavia, N. & Gomez-Mestre, I. 2014. Learned recognition of introduced predators determines survival of tadpole prey. *Functional Ecology* 28: 432–439. https://doi.org/10.1111/1365-2435.12175

Prunier, J., Kaufmann, B., Grolet, O., Picard, D., Pompanon, F. & Joly, P. 2012. Skin swabbing as a new efficient DNA sampling technique in amphibians, and 14 new microsatellite markers in the alpine newt (*Ichthyosaura alpestris*). *Molecular Ecology Resources* 12: 524–531. https://doi.org/10.1111/j.1755-0998.2012.03116.x

Saha, B. & Gupta, B. 2011. the Development & Metamorphosis of an Endangered frog, *Rana leptoglossa* (Cope, 1868). *International Journal of Advanced Biological Research* 1: 67–76.

Schoonjans, F. 2012. *Digimizer manual: Easy-to-use image analysis software*. 4th ed. MedCalc Software bvba. 107 pp.

Segev, O., Polevikove, A., Blank, L., Goedbloed, D., Küpfer, E., Gershberg, A., Koplovich, A. & Blaustein, L. 2015. Effects of tail clipping on larval performance and tail regeneration rates in the near eastern fire salamander, *Salamandra infraimmaculata*. *PLoS ONE* 10: 1–12. https://doi.org/10.1371/journal.pone.0128077

Simmons, A.M. & Horowitz, S.S. 2007. *Plasticity in the Auditory System across Metamorphosis. In: Narins P.M., Feng A.S., Fay R.R., Popper A.N. (eds) Hearing and Sound Communication in Amphibians.* Springer, New York, NY

Singh, U.A., Kumari, M. & Iyengar, S. 2018. Method for improving the quality of genomic DNA obtained from minute quantities of tissue and blood samples using Chelex 100 resin. *Biological Procedures Online* 20: 1–8. https://doi.org/10.1186/s12575-018-0077-6

Siwon Optical Technology Dimis-M optical camera.

Straube, D. & Juen, A. 2013. Storage and shipping of tissue samples for DNA analyses: A case study on earthworms. *European Journal of Soil Biology* 57: 13–18. https://doi.org/10.1016/j.ejsobi.2013.04.001

Sumida, M., Kondo, Y., Kanamori, Y. & Nishioka, M. 2002. Inter- and intraspecific evolutionary relationships of the rice frog *Rana limnocharis* and the allied species *R. cancrivora* inferred from crossing experiments and mitochondrial DNA sequences of the *12S* and *16S* rRNA genes. *Molecular Phylogenetics and Evolution* 25: 293–305. https://doi.org/10.1016/S1055-7903(02)00243-9

Tapia, V.S., Herrera-Rojas, M. & Larrain, J. 2017. JAK-STAT pathway activation in response to spinal cord injury in regenerative and non-regenerative stages of *Xenopus laevis*. *Regeneration* 4: 21–35. https://doi.org/10.1002/reg2.74

Urszán, T.J., Török, J., Hettyey, A., Garamszegi, L.Z. & Herczeg, G. 2015. Behavioural consistency and life history of *Rana dalmatina* tadpoles. *Oecologia* 178: 129–140. https://doi.org/10.1007/s00442-014-3207-0

Wong, P.B.Y., Wiley, E.O., Johnson, W.E., Ryder, O.A., O'Brien, S.J., Haussler, D., Koepfli, K.P., Houck, M.L., Perelman, P., Mastromonaco, G., Bentley, A.C., Venkatesh, B., Zhang, Y.P., Murphy, R.W. & G10KCOS. 2012. Tissue sampling methods and standards for vertebrate genomics. *GigaScience* 1: 1–12. https://doi.org/10.1186/2047-217X-1-8

Zamora-Camacho, F.J., Cortés-Manzaneque, S. & Aragón, P. 2018. Simulated predation pressure in Pelobates cultripes tadpoles modulates morphology at the metamorphic stage. *Current Zoology* 65: 651–656. https://doi.org/10.1093/cz/zoy097

Zemanova, M.A. 2019. Poor implementation of non-invasive sampling in wildlife genetics studies. *Rethinking Ecology* 4: 119–132. https://doi.org/10.3897/rethinkingecology.4.32751.