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Mercury contamination in the European green toad *Bufotes viridis* in Vienna, Austria.

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ABSTRACT

Mercury (Hg) contamination affects all ecosystems worldwide. Its deleterious effects on wildlife and humans encompass a diversity of impacts from individual to population levels. In the present study, we quantified Hg concentration across various tissues (blood, brain, muscle, and toe) of green toads (*Bufotes viridis*) and investigated the use of toe clips as a proxy of Hg concentration in internal tissues, including the brain. Our results show distinct patterns of Hg contamination across tissues, with the highest Hg concentration in the blood with 1.496 ± 0.772 $\mu g.g^{-1}$ dry weight (dw), followed by muscle tissue with 0.687 ± 0.376 $\mu g.g^{-1}$ dw, brain tissue with 0.542 ± 0.319 $\mu g.g^{-1}$ dw, and toes with 0.229 ± 0.143 $\mu g.g^{-1}$ dw. A strong relationship has been found between toe and brain Hg concentrations ($R^2 = 0.857$, $p < 0.001$). These results emphasize the potential of toe clipping as a reliable, non-lethal method for predicting brain Hg concentrations in the green toad. Further, results open the possibility of assessing the potential association between Hg contamination and the cognitive performance of amphibians.

1. Introduction

Environmental pollution is a global threat to humans and wildlife. Mercury (Hg) is one of the most concerning, prevalent contaminants found in ecosystems worldwide (Chen et al., 2018). While Hg can have natural origins, human activities such as gold mining, fossil fuel combustion, biomass burning, cement production, and agricultural practices have drastically elevated its levels in the environment, surpassing concentrations seen in preindustrial times by large margins (Driscoll et al., 2013; GMA, 2019). These anthropogenic activities have altered the global Hg cycle and have frequently overwhelmed natural cycling (Pirrone et al., 2010; Amos et al., 2013). In anoxic conditions, Hg can be methylated by microorganisms mainly via sulfate-reducing and iron-reducing bacteria into methylmercury (MeHg), the most toxic and bioavailable form of Hg (Compeau and Bartha, 1985; Benoit et al., 2003; Podar et al., 2015). MeHg bioaccumulates in tissues of living organisms during their lifetime and biomagnifies through trophic chains, resulting in elevated Hg contamination levels in top predators (Atwell et al., 1998; Lavoie et al., 2013; Eagles-Smith et al., 2018).

Toxic effects of Hg in humans and wildlife encompass neurological and neurobehavioral alteration, physiological and reproductive impairment, and endocrine disruption (Scheuhammer et al., 2007; Tan et al., 2009; Evers, 2018). While Hg toxicity is well documented in mammals, fishes, and birds, reptiles and amphibians remain understudied. In amphibians, Hg levels and impact remain, for most species, unknown, despite the fact that it has been shown that Hg contamination occurs in this group (Terhivuo et al., 1984; Hothem et al., 2010; Tornabene et al., 2023). Due to the complex life cycle of amphibians, exposure to aquatic contamination, as well as stressors in their terrestrial habitat, increases the risk of toxic effects (Salice et al., 2011). To date, our understanding of the deleterious effects of Hg on amphibians is limited to an increase in larvae mortality (Bergeron et al., 2011), an increase in larval development duration and prevalence of spinal malformation in larvae (Todd et al., 2011), behavioral disruptions (Burke et al., 2010), and reduction of tadpole body condition (Schlippe-Justicia et al., 2024). Notably, most studies have focused exclusively on aquatic habitats and associated Hg contamination. Amphibians are already one of the most endangered taxa worldwide (Stuart et al., 2004; Barnosky

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et al., 2011; IUCN, 2024); understanding the effects of environmental pollutants on physiology, behavior, and ultimately survival is therefore required for risk assessment and to set conservation measures.

The European green toad (*Bufotes viridis*) is one example of an amphibian species that used to be very common around Eastern and Central Europe and became locally highly endangered (Sachs et al., 2020; Hölglund et al., 2022). Land degradation and loss of primary habitat caused the green toad to increasingly inhabit urban and anthropogenically altered sites (Valkanova et al., 2009; Landler et al., 2023a; Vargová et al., 2023). Often, such habitats provide ponds with stable water levels, allowing regular reproduction. However, they potentially increase toxic loads and lead to physiological alterations as well as lower survival rates. Quantifying Hg concentrations in green toad populations is important to plan future conservation measures and evaluate environmental risk factors. Furthermore, toads, as other amphibians, are potentially reducing pest insects and increase native biodiversity (Wanger et al., 2010; Sathe and Patil, 2014), their decrease may result in increased pest insects in agricultural areas. Also, toads may act as bioindicators due to their permeable skin and complex life cycles, switching from aquatic to terrestrial habitats (Salice et al., 2011).

Most research on Hg contamination in amphibians relies on lethal sampling, which limits the potential to understand chronic Hg effects on adults and the next generations. Recently, studies have validated toe clips as a non-lethal sampling method to quantify Hg contamination (Bergeron et al., 2010; Todd et al., 2012; Pfleeger et al., 2016), which is primordial to evaluating Hg toxicity in threatened species. However, to the best of our knowledge, the use of toe clips as a proxy of Hg concentration in the brain of amphibians has not been yet evaluated. It would be important to understand mercury effects on behavior and neurobiology without sacrificing animals.

The goals of our study were to 1) quantify for the first time Hg concentrations found in green toad tissues, and 2) evaluate the use of toe clips as a proxy for Hg concentration in other tissues, including the brain.

2. Material and methods

2.1. Sample collection

The European green toad (*Bufotes viridis*) is a mid-sized amphibian well adapted to semi-arid to arid conditions. It is often referred to as a pioneer species, colonizing anthropogenic environments and human settlements up to larger cities (Mazgajska and Mazgajski, 2020; Konowalik et al., 2020). Austria's main distribution area is in the Eastern part of the country, including outskirts and even sites close to the center of Vienna (Sistani et al., 2021; Landler et al., 2023b). To date, the largest known population in this city is located at Simmeringer Haide, a traditional farmland south-east of Vienna. Since the twentieth century, however, this green toad habitat is dominated by greenhouses and polytunnels, while open cultivated land is limited to small patches. An industrial area and power plants border the limits of the current core area of this population. Further, a highway runs over parts of it. During seasonal breeding, mass mortality of migrating toads is moderated by a roadkill mitigation project (Staufer et al., 2023). Between May 5th, 2022, and June 4th, 2022, we collected 11 green toad carcasses (all adult individuals, 10 from the Simmeringer Haide and one from Donaufeld, Fig. 1) in Vienna, Austria, which were as soon as possible frozen at −20°C. Toads were killed by road traffic or of unknown cause. However, this study only used freshly collected carcasses to allow blood and brain tissue analysis. Individual dissections were performed in the laboratory where we extracted brain and muscle tissues, drew blood from the heart, and clipped toes. Toad sampling was performed under permit MA22–230917–2020 attributed by the authority of Vienna, Austria, and collected as part of the roadkill mitigation project n°1509826–2021–25 on behalf of the Vienna Environmental Protection Department – MA 22.

2.2. Mercury analysis

Whole blood, brain, muscle, and toes of each individual were freeze-dried for 48 h, grounded, and homogenized. Total Hg (THg) concentrations were determined by direct measurement using an atomic absorption spectrometer AMA-254 (Advanced Mercury Analyzer-254; Altec®) at La Rochelle University, France, following the instrumental method from Lemaire et al. (2021). For each sample (0.12–1.98 mg dw) at least two replicates were analyzed, and the reproducibility for replicate samples was approved when the relative standard deviation was below 10 %. Certified Reference Material (CRM) TORT-3 (lobster hepatopancreas; certified Hg concentration: 0.292 ± 0.022 $\mu g.g^{-1}$ dry weight (dw), NRCC) was analyzed at the beginning and at the end of the analytical cycle to validate the method. Measured values for CRM TORT-3 were 0.297 ± 0.005 $\mu g.g^{-1}$ dw (n = 7), with a recovery of 101.9 ± 1.5 %. Blanks were included at the beginning of each analytical run and the limit of quantification of the AMA was 0.05 ng. Hg concentrations are further expressed in $\mu g.g^{-1}$ dw.

In parallel to carcass collection, we collected water samples (June

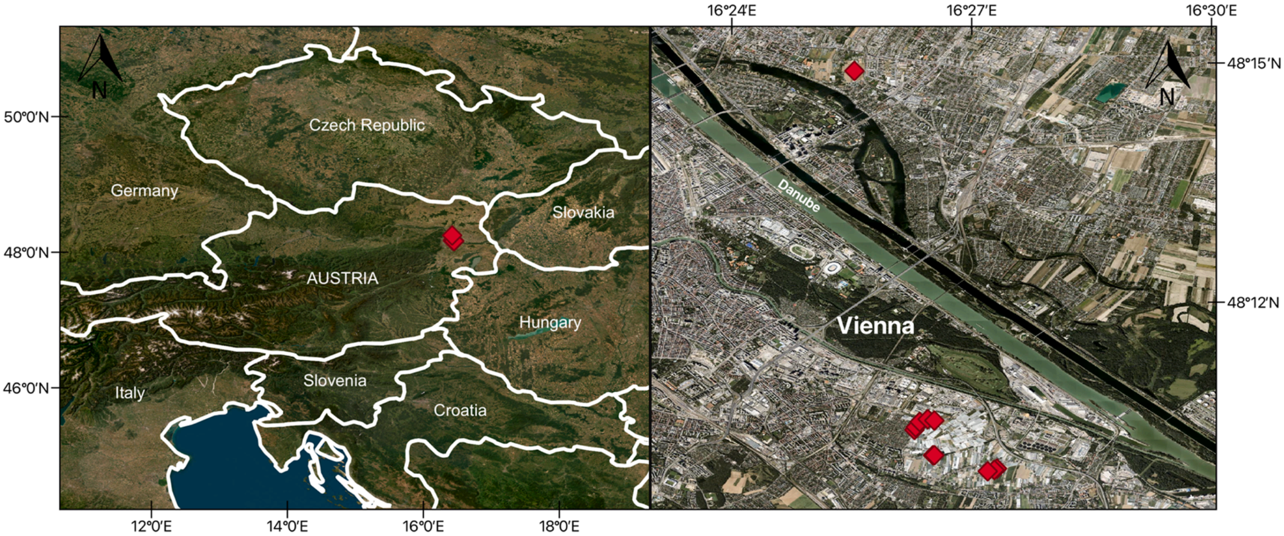

Fig. 1. Geographical location of the 11 green toad carcasses collected (red dots) between May 5th 2022 and June 4th 2022. Ten adults from the Simmeringer Haide and one from Donaufeld, Vienna, Austria.

2nd, 2022) from two breeding ponds of green toads in Simmeringer Haide and sent them to the commercial lab GEOtest in Brno, Czech Republic, to measure mercury content in the water (limit of detection $< 0.50\ \mu g.l^{-1}$).

2.3. Statistical analysis

Statistical analysis was performed using the Software R v.4.2.2 (R Core Team, 2022).

All data were first checked for normality and homogeneity of variances. Analyses of Hg concentrations were performed on log-transformed values. The difference of Hg concentrations between tissues was assessed by ANOVA and Pairwise *t*-test. The relationships between Hg concentrations in different tissues were assessed via linear regressions. The significance level for statistical analyses was always set at $p < 0.05$.

3. Results

Mercury concentrations ranged from 0.109 to $3.549\ \mu g.g^{-1}$ and differed between tissues (ANOVA, $p < 0.001$). Mean Hg concentrations were the highest in whole blood with $1.496 \pm 0.772\ \mu g.g^{-1}$, followed by muscle tissue with $0.687 \pm 0.376\ \mu g.g^{-1}$, brain tissue with $0.542 \pm 0.319\ \mu g.g^{-1}$, and toes with $0.229 \pm 0.143\ \mu g.g^{-1}$ (Table 1).

All tissues showed significant positive relationships between them (Table 2). The strongest relationships were between muscle and brain ($R^2 = 0.913$, $p < 0.001$), muscle and toes ($R^2 = 0.876$, $p < 0.001$), and brain and toes ($R^2 = 0.857$, $p < 0.001$) (Fig. 2).

Mercury concentrations in the water samples from breeding sites were below the limit of detection ($< 0.50\ \mu g.L^{-1}$).

4. Discussion

In the present study, we demonstrated a strong correlation between Hg concentrations quantified in toe clippings and those found in the brain and muscles of green toad. Environmental contaminants are considered to contribute to the global decline of amphibians significantly (Orton et al., 2023); however, knowledge of bioaccumulation and the specific impacts of Hg on amphibians still lags behind those observed in other vertebrates. Mercury contamination has been identified to occur in most amphibians, which have been evaluated so far, and to date, understanding of its effects on individuals and populations remains largely unknown (but see Schlippe-Justicia et al., 2024). Developing non-lethal sampling methods to quantify Hg concentrations is pivotal to understanding its chronic effects, especially in threatened species. These findings are particularly valuable as they open up new possibilities for understanding the impact of this potent neurotoxin on amphibian cognition.

In this study, we detected Hg contamination in all individuals. The highest Hg concentration in green toad was found in the blood with $1.496 \pm 0.772\ \mu g.g^{-1}$ dw (ranging from 0.681 to $3.549\ \mu g.g^{-1}$ dw) and the lowest in toes clips with $0.229 \pm 0.143\ \mu g.g^{-1}$ dw (ranging from 0.109 to $0.617\ \mu g.g^{-1}$ dw). Blood concentrations are in the range of those found in the American toad *Anaxyrus americanus*, with blood Hg ranging from 0.082 to $4.235\ \mu g.g^{-1}$ ww, and Hg concentrations in toe clippings ranging from 0.032 to $0.602\ \mu g.g^{-1}$ dw (Todd et al., 2012).

Table 1
Mercury (Hg) concentrations (in $\mu g.g^{-1}$ dry weight) in different tissues of the European green toad *Bufotes viridis* from Vienna, Austria.

Tissues	n	Hg Mean ± SD	Hg Min – Max
Brain	11	0.542 ± 0.319	0.273 – 1.341
Muscle	11	0.687 ± 0.376	0.326 – 1.706
Toes	11	0.229 ± 0.143	0.109 – 0.617
Whole Blood	11	1.496 ± 0.772	0.681 – 3.549

Table 2
Relationships between tissue mercury concentrations of the European green toad *Bufotes viridis* from Vienna, Austria. Significant relationships are in bold ($p < 0.001$).

	Brain	Muscle	Toe
Brain	-	**0.913**	**0.857**
Muscle	-	-	**0.876**
Whole Blood	**0.546**	**0.696**	**0.530**

Nevertheless, minimum Hg concentrations in our study were surprisingly high and particularly worrying. Blood is known to represent the recent Hg exposure, equal to the lifetime of erythrocytes (Rodnan et al., 1957; Monteiro and Furness, 2001). In predators, diet is the main route of Hg exposure (Chumchal et al., 2011; McGrew et al., 2014; Rumbold et al., 2018; Jackson et al., 2021). In contrast, muscles and the brain are storage tissues known to reflect the long-term assimilation of Hg. However, a potential Hg exposure route through the skin cannot be excluded in amphibians as it allows water and ionic exchange, and the contribution this may represent to the Hg burden is still unknown. The most likely source of mercury contamination in this study are legacy effects from earlier pollution. We did not detect concerning Hg levels in breeding waters, suggesting dietary contamination by contaminated prey. Further studies on Hg contamination throughout the entire trophic chain are necessary to understand better the risks associated with the prey and predators of the green toad in the area.

Methylmercury (MeHg) is the predominant Hg form in blood, brain, and muscles (Faccio et al., 2019; Henny et al., 2002; Oliveira Ribeiro et al., 1999). The elimination rate of MeHg is relatively slow and varies between species and tissues, ranging from 5 days to 3 years for whole-body MeHg burden (reviewed in Chételat et al., 2020). To the best of our knowledge, it has not yet been investigated in amphibians. The high Hg levels in muscles, which represent long-term exposure, are concerning, considering that toads collected in this study are assumed not to be older than 5 years. In addition, the results from the present study are particularly high as they are among the highest found in amphibians studied so far. A recent assessment of MeHg by Tornabene et al. (2023) shows lower concentrations in 14 amphibian species throughout the USA.

All tissues analyzed in the present study have been correlated (Fig. 1, Table 2). Generally, muscles, blood, brain, liver, and kidneys are known to be correlated as they are metabolically active tissues (Cizdziel et al., 2003). The fact that toe clips were positively correlated to internal tissues validates the potential to use this non-lethal sampling method to access internal values, which offers an alternative approach to Hg evaluation in living amphibians. Our application promotes an alternative to lethal sampling for a taxon severely affected by contaminants requiring long-term monitoring without consequences for wild populations and conservation goals.

Mercury concentrations in the brain of green toad were relatively high, $0.542 \pm 0.319\ \mu g.g^{-1}$ dw (ranging from 0.273 to $1.341\ \mu g.g^{-1}$ dw, Table 1). In the Everglades, an ecosystem known to be impacted by Hg contamination, Hg concentration in the brain of pig frog, *Lithobates grylio*, was two-fold lower, with $0.357 \pm 0.08\ \mu g.g^{-1}$ dw (Ugarte et al., 2005; ww converted to dw assuming 83 % of water in the brain according to Churchill and Storey 1995). For comparison, concentrations we have found in green toad brains exceed Hg concentrations quantified in top predators' brain such as the American alligator, *Alligator mississippiensis*, in Georgia, USA, where values were $0.450 \pm 0.12\ \mu g.g^{-1}$ dw (Arnold, 2000), and $0.270 \pm 0.043\ \mu g.g^{-1}$ dw in Louisiana, USA (Moore et al., 2022), or in the polar bear *Ursus maritimus* where brain concentrations were $0.28 \pm 0.07\ \mu g.g^{-1}$ dw (Krey et al., 2012). However, these values are much lower than those reported in the brains of giant petrels from the Southern Indian Ocean, which ranged between 1.58 and $13.23\ \mu g.g^{-1}$ dw (Renedo et al., 2021).

Methylmercury, the most toxic form of Hg, generally accounts for

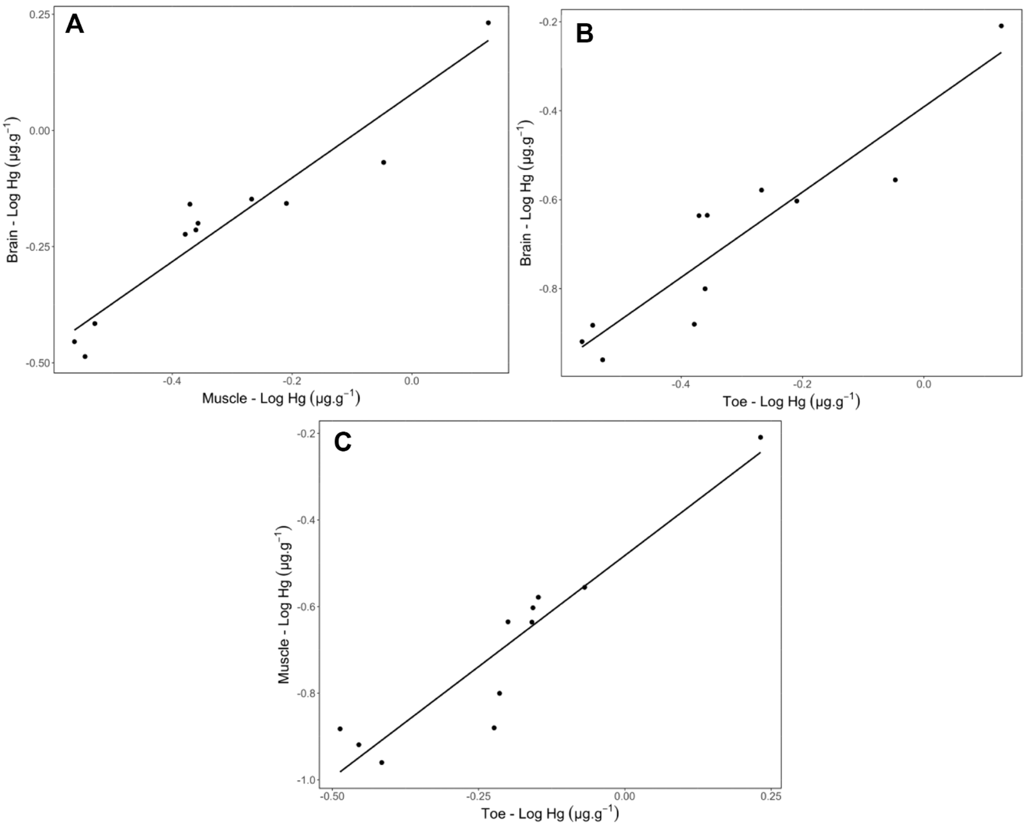

Fig. 2. Linear regressions between mercury (Hg) concentrations (in µg.g^{-1} dry weight) in **A**: Brain and Muscle ($R^2 = 0.913$, $p < 0.001$), **B**: Brain and Toe ($R^2 = 0.857$, $p < 0.001$), **C**: Muscle and Toe ($R^2 = 0.876$, $p < 0.001$) of the European green toad *Bufotes viridis* in Vienna, Austria.

most but not all Hg found in the brain (Basu et al., 2009; Renedo et al., 2021; Manceau et al., 2021). Therefore, high brain Hg levels of green toad raise concern regarding potential neurotoxic effects and cognitive impairment. In amphibians, Hg has been identified to be responsible for the alteration of behavior and performance in the two-lined salamander, *Eurycea bislineata* (Burke et al., 2010). While the brain is a known target organ for Hg accumulation, studies evaluating Hg concentrations in wildlife brains are rare despite the apparent importance of such data. Basu et al. (2009) reported that even at low Hg levels in the brain (approximately 0.1–1.0 µg.g^{-1} dw), polar bears showed a relationship between brain Hg concentration and NMDA (N-methyl-D-aspartic acid) receptor concentration, indicating neurochemical effects. It is important to emphasize that recent studies on brain toxicity have revealed that levels of Hg in the brain and its impact are species-dependent, and that coprecipitation of Hg with selenium (Se) into tiemannite (Hg:Se) can drastically reduce toxicity of Hg (Manceau et al., 2021). Goutte et al. (2014) have shown that low Hg concentrations (>2 µg.g^{-1}) have strong effects on population dynamics of South Polar Skuas (*Catharacta maccormicki*) from Adélie Land, while no effects were observed at high Hg concentrations (12 µg.g^{-1}) in Brown Skuas (*Catharacta lonnbergi*) from Kergelen Archipelago, highlighting that a direct relationship between high Hg concentrations and toxicity cannot be referred to without assessing the significance of cofactors such as Se. Future studies on Hg contamination in amphibians need to consider Se:Hg ratio to understand potential detoxification processes that can protect populations from Hg toxicity, additionally repercussions of chronic Hg concentration in the brain of amphibians require further evaluation.

Results of the present study show a positive relationship between toe and brain Hg concentrations ($R^2 = 0.857$, $p < 0.001$, Fig. 1). While toe clipping is a common sampling method in amphibian genetic studies with relatively little impact on the fitness of the individual (Ursprung et al., 2011; Fisher et al., 2013; Ginnan et al., 2014), it has further been used as an appropriate predictor of blood and muscle Hg concentrations in amphibians (Todd et al., 2012), and the results of the present study reinforce these finding (Fig. 1, Table 2).

One of the main results of this study is the confirmation that toe clips are reliable in assessing brain Hg concentrations in green toads. While quantification of Hg concentration in amphibian brain tissue was limited to sampling in dead individuals, the relationship between toes and brain Hg concentrations allows future studies to extend the understanding of Hg neurotoxicity and potential cognitive repercussions on amphibians without lethal sampling. However, future studies should continue investigating species-specific relationships between tissues to facilitate field-based studies providing a non-lethal approach.

5. Conclusion

In the present study, high Hg concentrations have been quantified in different tissues of the green toad, which is worrying in regard of the known deleterious effects of this contaminant on wildlife. These results raise questions about potential sources of Hg at study sites and the extent of trace element contamination in residing wildlife.

Green toad brain Hg concentrations are concerning, especially given the neurotoxicity of Hg. However, the strong relationship between internal organs and toe clippings, where the latter can be collected through non-lethal sampling methods, opens new possibilities to study the potential effects of Hg contamination on the cognitive performance of amphibians, as toe and brain Hg concentrations show a particularly strong relationship.

On a final note, we recommend future studies to consider a potential protective effect of selenium when investigating the effect of Hg at an individual and population level.

CRediT authorship contribution statement

Mangione Rosanna: Writing – original draft, Methodology, Conceptualization. **Spießberger Magdalena:** Methodology. **Burgstaller Stephan:** Methodology. **Staufer Martina:** Methodology. **Landler Lukas:** Writing – original draft, Methodology, Conceptualization. **Jérémy Lemaire:** Writing – original draft, Visualization, Methodology, Data curation, Conceptualization. **Bustamante Paco:** Writing – original draft, Resources. **Gruber Edith:** Methodology. **Zaller Johann:** Methodology.

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.

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Data Availability

Data will be made available on request.

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