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Effects of synthetic acid rain and organic and inorganic acids on survival and $CaCO_3$ piercing stylets in tardigrades

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Abstract

Long-term environment acidifications due to decrease pH of the rainwaters affect both soils and water bodies. The organisms most likely to be affected by acid rain are the ones that possess vital organs made of calcium carbonate; among them are tardigrades, presenting aragonite piercing stylets in feeding apparatuses. A positive relationship between acidic rainfall and loss of tardigrades diversity has been already shown, but there is lack of knowledge of its lethal and sublethal effects. This study quantifies the effects of the acute exposure of three eutardigrade, *Acutuncus antarcticus*, *Hypsibius exemplaris*, and *Macrobiotus* cf. *hufelandi*, to synthetic acid rains and to organic and inorganic acids (hydrochloric, acetic, sulfuric, and nitric acids) naturally occurring in the environment. The cumulative proportion of dead animals in respect of exposition time was fitted to cumulative Weibull Distribution using a Bayesian framework. At the end of the experiments, animals were observed to investigate damages to their piercing stylets. Besides, stylets were finely morphologically described with Scanning Electron Microscopy. This study shows that acid rains and the other tested acids negatively affect tardigrades accordingly with pH, time of exposure, and tardigrade species. Freshwater species show a better resistance to acidity than the moss dwelling species, which can better acclimate over the time to low pH. The stylets resulted unaltered in almost all of the alive specimens. The results suggest that the tested tardigrades taxa have the ability to buffer the environmental proton change and the negative effect on their populations could be counteracted.

KEYWORDS
aragonite, climate change, ecophysiology, environmental acidification, pollution, tardigrada

1 | INTRODUCTION

Back in the 60s of the past century, scientists started to warn about of the use of fossil fuels being the cause of one of the main threats for environment and health, that is, the increasing of acid rainwater depositions. Sulfur and nitrogen oxides were detected as the main hazardous pollutants causing acidic rains (Grennfelt et al., 2020). These oxides lower the pH of the rainwater to below 5.6 (Qu & Han, 2021) with recorded rainfalls that reached, for example, pH 4.5 in Italy (Pirretti, G., Agenzia Prevenzione Ambiente Energia Emilia-Romagna, pers. comm.), pH 4.3 in New Hampshire, U.S.A. (Buso et al., 2000), or pH 4.4 in China (Qu & Han, 2021). These pollutants are powerfully

corrosives if in mixture of one to another (i.e., sulfonitric mixture) or with other atmospheric chemicals (Rothschild, 2019). At the dawn of the 21st century, the strategies for pollution control led to the deflections of the menace of acid rains in the occidental industrialized countries and only sporadically, sulfur and nitrogen oxides are now detectable above safety limits (Guerreiro et al., 2014). Nevertheless, the long-term effects of the environment acidifications are not a thing of the past (Vuorenmaa et al., 2017) as the pollutants emitted by countries characterized by increasing population (Mohajan, 2018; Mwaanga et al., 2019; Qu & Han, 2021) are nullifying the past efforts, due to the ability of these pollutants to quick move and disperse in the atmosphere (Forsius et al., 2021).

The effects of acid rains on the environments, especially concerning forestry, agricultural settings, and aquatic systems, have been largely studied (for reviews see Fatima et al., 2020; Fowler et al., 2020; Lovett et al., 2009). Soils and water bodies, the final receivers of the atmospheric acid deposition, tend to lose nutrients and accumulate toxic cations (Lovett et al., 2009). Plants, animals, and microorganisms suffer from both direct effects of the acidity and indirect effects of their environment quality depletion (Liu et al., 2019). Moreover, acidic environments lead to a prior dissolution of calcium carbonates that is followed by the release of carbon dioxide gas and precipitation of cation calcium as sulfur or ammonium salts, thus becoming unavailable for organisms (Benton, 2018). As a result, the organisms with vital organs based on calcium mineral (in form of calcite or aragonite) are most likely to be affected by this depletion (Clapham & Payne, 2011), unless they evolve strategies to buffer against this environmental acidification (Ewald et al., 2009).

Tardigrades constitute a phylum of aquatic micrometazoans spread worldwide (Guidetti et al., 2011), and owning piercing stylets made of aragonite in their feeding apparatus (Bird & McClure, 1997; Guidetti et al., 2012, 2013, 2015; Massa et al., in press), they fall into the category of organisms potentially affected by acid rains. All terrestrial (i.e., living in moss, lichen, and soil) and aquatic (marine and freshwater) dwelling tardigrades, require a layer of liquid water to be active. Despite this, if in their substrate the water becomes unavailable, for example, by freezing or evaporation, many tardigrade species can enter a suspended cryptobiotic life state or form cysts (Bertolani et al., 2019; Guidetti et al., 2011; Janelt & Poprawa, 2020; Møbjerg et al., 2011; Rebecchi et al., 2007, 2020; Roszkowska et al., 2020, 2023; Møbjerg & Nives, 2021). Tardigrades can withstand many harsh environmental stressors (such as radiations, pollutants, and others), both in their hydrated state, as well as in their cryptobiotic state (e.g., Altiero et al., 2011; Giovannini et al., 2018; Horikawa et al., 2006; Hygum et al., 2017; for a review see Møbjerg & Neves, 2021). Séméria, (1981, 1982) found a negative relationship between air quality (decreased by a high concentration of sulfur oxides) and tardigrades diversity, while Meininger et al. (1985) found a negative correlation between tardigrades abundance and acidification of the pH in arctic mosses. Since then, the effects of pollution and pH on tardigrade species abundance and presence have been further investigated with consistent outcomes (e.g., Steiner, 1994a, 1994b; de Peluffo et al., 2006; Johansson et al., 2011; Nelson et al., 2020): that is, a decrease of tardigrades populations as consequence of lower air quality. Due to these results, tardigrade communities have suggested to be potential indicators for air pollution (Steiner, 1994b).

To implement the use of tardigrades as a suitable tool for environmental pollution biomonitoring, it is needed to enrich the knowledge of the ecophysiological responses of tardigrades species inhabiting different habitats. This study aims to investigate the lethal and sublethal effects of different concentrations of acids (synthetic acid rains [SAR] and other inorganic and organic acids found in the natural environment) on three species of tardigrades, collected in different environments and in different climatic and biogeographic regions. We further investigated the capability of tardigrades to preserve the integrity of the calcium carbonate structures in their buccal apparatuses despite environmental acidification.

2 | MATERIALS AND METHODS

2.1 | Tardigrade taxa

Three cultured populations from three eutardigrades taxa were used in the experiments (*Acutuncus antarcticus* (Richters, 1904), *Hypsibius exemplaris* Gąsiorek et al., 2018; and *Macrobiotus* cf. *hufelandi*) differ for phyletic lineages and inhabited substrate. Two of them, *A. antarcticus* and *H. exemplaris*, are widely used as model species in stress tolerance studies (e.g., Giovannini et al., 2018, 2022; Goldstein, 2018; Kondo et al., 2020; Poprawa et al., 2022; Wojciechowska et al., 2021). These three species are cultured at the Laboratory of Evolutionary Zoology of the Department of Life Sciences (University of Modena and Reggio Emilia [UNIMORE]) as follows:

i. *A. antarcticus* (Acutuncidae) is cultured in culture water (pH 8.2, measured on culture medium) within 250 mL glass flasks at 14°C, with a photoperiod of 12 h dark/12 h light, and fed with the green algae *Chlorococcum* sp. *ad libitum* (Altiero et al., 2015). The starting population was collected in 2018 from a microbial mat in a freshwater temporary pond (74°4'4"S, 164°0'2"E, 70 m a.s.l.; near the "Mario Zucchelli" Station, Victoria Land, Antarctica; polar climate);
ii. *H. exemplaris* (Hypsibiidae) is cultured in distilled water (pH 8.0, measured on culture medium) in 8 cm plastic petri dishes, layered with Agar (1.2% w/v in distilled water), at 14°C with a photoperiod of 12 h dark/12 h light, and fed with *Chlorococcum* sp. *ad libitum*. The population was started from a commercial isogenic culture (Sciento strain Z151; derived from a single female collected from the leaves rotting in a stable pond; 53°3'3"N, 2°2'4"W, 75 m a.s.l.; Darcy Lever, Grater Manchester, United Kingdom; temperate oceanic climate);
iii. *M.* cf. *hufelandi* (Macrobiotidae) is cultured in an indoor mesocosm consisting of 8 cm plastic petri dishes containing a piece of the original moss inhabited by the species, misted once a

week with distilled water. The water squeezed from the soaked moss was at pH 8.0. The petri dish is kept closed with a plastic cover and at room temperature with natural light cycle; additional food sources are unnecessary. The moss was collected in November 2020 from the top of a travertine sill of a boundary wall at the Campus of UNIMORE (44°3'5"N, 10°5'3"E, 40 m a.s.l.; Modena, Italy; continental climate).

Tardigrades of the three taxa were extracted from the cultures using a glass pipette under a dissecting microscope and washed two times in distilled water to avoid the presence of food sources and/or sediment, then starved in distilled water at 12°C overnight before the experiments.

2.2 | Acids solutions and SAR preparation

The following acids at pH of 4.50 ± 0.01 were used in the experiments: hydrochloric acid [HCl], acetic acid [CH_3COOH], sulfuric acid [H_2SO_4], nitric acid [HNO_3], other than SAR solution. The SAR was prepared from a mother solution of H_2SO_4 and HNO_3 in a ratio 3:1 by volume (Moiseenko, 2005); SAR solutions were prepared with four different pH: 4.00 ± 0.01, 4.50 ± 0.01, 5.00 ± 0.01, and 5.50 ± 0.01 by mixing distilled water and SAR mother solution. Information relative to the molar concentration of each solution together with information on the acids nature and chemical behaviors are presented in the Supporting Information: Table S1.

Each acid and SAR solutions, along with a bottle containing 250 mL of distilled water to use as control treatment were prepared the day before each experiment and stored at 12°C overnight to bring them to the same temperature of the experiment. The distilled water had a measured pH of 6.20 ± 0.01 after overnight air exposure due to the absorption on carbonate oxides from the air.

2.3 | SAR acute toxicity tests

The experiments to test the effect of an acute exposure to SAR were conducted on the three taxa. Experiments were carried out in 24-wells cell culture plates at 12°C in the dark. Using a glass pipette, tardigrades were placed individually in each well with a small drop (about 300 µL) of distilled water to avoid desiccation of the animal during the set of the experiments, then 1.5 mL of the SAR solution was added. For each taxon, sixty specimens for each of the four SAR concentrations, that is, pH 4.0, 4.5, 5.0, and 5.5, and the control were used for a total of 300 specimens. The experiment was conducted in a static condition keeping the animals in the initial solution during all the experiment.

The mobility (i.e., movements of the body and/or legs) of each specimen was determined using a stereomicroscope (magnification X400) by observing the animal for a maximum of 5 s. The animals were checked for mobility at different interval of time from the beginning of the experiment: initially, they were checked every 45 min, for a total of 11 times (t_1–t_{11}), then after 12 h (t_{12}), then it was checked every 2 h from 24 to 32 h (t_{24}–t_{32}), and finally at 36 h (t_{36}), and at 48 h (t_{48}) for a total of 19 checks. Based on preliminary trials, the endpoint of the experiment was a priori fixed at 48 h.

2.4 | Acids acute toxicity test

The acids static acute toxicity test was performed on the three experimental species.

Experiments were carried on 24-wells cell culture plates at 12°C in the dark. Using a glass pipette, tardigrades were placed individually in each well with a small drop (about 300 µL) of distilled water to avoid desiccation of the animal during the set of the experiment, then 1.5 mL of the selected acid (pH 4.5) was added in each well. For each species and each treatment (hydrochloric acid, acetic acid, sulfuric acid, nitric acid and control), 24 tardigrades were used, for a total of 360 individuals (120 for each species). The experiment was conducted in a static condition keeping the animals in the initial solution during all the experiment.

The mobility (i.e., movements of the body and/or legs) of each specimen was determined using a stereomicroscope (magnification X400) by observing the animal for a maximum of 5 s. The animals were checked for mobility every 30 min for 13 times (t_1–t_{13}; total of 6 h) from the beginning of the experiment. Based on preliminary trials, the endpoint of the experiment was a priori fixed at 6 h.

2.5 | Observation of the animals after the exposure to SAR and acids

After the experiments with SAR and acids, each specimen (showing or not movements) was placed in distilled water for 2 h in a clean well of a 24-wells cellular plate. Then, each animal was mounted on a temporary slide with distilled water and observed at X400 (with a light microscope [LM], with differential interference [DIC] and phase [PhC] contrasts, Leica Leitz BMRD equipped with an Amscope MU1803 camera). The specimens' observations allowed to check if animals who lost the mobility during the experiments were actually dead (the correspondence between immobility and death was confirmed for all immobile animals), to detect possible signs of molting phase, and to observe the morphology of the aragonite stylets in the feeding apparatus. To evaluate the stylets regeneration ability during inter-molting phase, specimens showing damaged (broken, blunt, or without condyles portion) or dissolved stylets and not in molting phase were recovered and kept in distilled water at 12°C in the dark and observed with the microscope every 24 h until death or molting process.

2.6 | Statistical analyses

Data were analyzed considering each tardigrade as a single replicate (Supporting Information: Materials and Methods 1–2). Tardigrades

that started the molting process during the experiment were not considered for the statistical analyses.

To evaluate the effect of the different pH of SAR solutions on tardigrade species, we fitted a cumulative Weibull distribution function (CDFW) model to the cumulative number of dead animals in respect to time (in h). The CDFW was reparametrized to its median (*M*) and the shape parameter (*k*). The median of the CDFW represents the timepoint at which 50% of the animals were dead, namely LD_{50}, whereas the shape parameter describes how the probability of death changes with time (decreases over time with $k < 1$, is constant with $k = 1$, increases over time with $k > 1$). Both *M* and k are described by a linear regression with log link, with species, and with interaction of species and pH change from controls with the formulae: log(M) ~ species + species*pH and log(k) ~ species + species*pH. To evaluate the effect of different acids on tardigrade species we used a similar approach as above, but with the formula of the regression being: log (M) ~ species + species*acid and log(k) ~ species + species*acid.

The models were fitted in a Bayesian framework with the software JAGS (Plummer, 2004) through the R (R Core Team, 2016) package "R2jags" (Su et al., 2015).

The R code for the data analysis including more detailed information on the model and the reparameterization of the CDFW, the computing formula of the Bayesian *p*-value (Makowski et al., 2019), the raw data, and the total statistical results are in the Supplementary Materials and Methods 1–2.

2.7 | Effects of environment acidification on the $CaCO_3$ stylets

M. cf. *hufelandi* were exposed to hydrochloric acid [HCl] at pH 1.3 and 2.0 (3 specimens for each pH) and distilled water (pH = 6.2; 20 specimens) to observe possible dissolution of the $CaCO_3$ stylets of the tardigrade feeding apparatus.

To form a space between the slide and the coverslip and allow the tardigrades to be alive during the experiment, slides were covered with alimentary plastic film, and a square hole was made in it with a razor blade. *Macrobi.* cf. *hufelandi* individuals were singularly placed in the square hole within a drop of water and covered with a coverslip, which was secured with transparent tape on its two opposite sides. Then a drop (500 µL) of acid solution was added on one of the free sides of the coverslip so that it could penetrate under the coverslip and reach the animal, while in treatment with only distilled water the coverslip was completely sealed with tape. A movie of the feeding apparatus of the treated animal on slide was shot (with the microscope and camera mentioned above) to record the changes in the stylets.

2.8 | Analyses of the piercing stylets composition and morphology

To extract the piercing stylets from the three studied species, five animals for each species were placed individually on a fragment of a glass coverslip with 200 µL of distilled water and burned at short breaks with a blowtorch lighter until the organic matter was completely oxidated and only the inorganic rests remained. The stylets were then cleaned with distilled water and eventual animal remains were removed with a gentle passage of a brush. The piece of coverslips carrying the stylets were mounted on a stub with a carbon tab and sputter-coated with gold. Elements composing the stylet were detected using energy-dispersive X-ray spectroscopy (EDX), by X-ray mapping and X-ray area-analysis methods, performed with the X-EDS QUANTAX-200 (Bruker Corporation) mounted on the Scanning Electron Microscope [SEM] Nova NanoSEM 450 (Fei Company) available at the "Centro Interdipartimentale Grandi Strumenti" of UNIMORE. The SEM was also used to examine the morphology of the stylets.

3 | RESULTS

3.1 | Exposure to SAR and acids

The sensibility to the experimental condition, that is, probability of death in the control treatment significantly decreases over time ($k < 1$; Bayesian $p < 0.001$) in all the tested species (Figure 1c; Table 1 and 2) and there are no differences among them.

The exposure of the three tardigrade species to SAR shows that the survival of the specimens of all species is strongly negatively affected by the pH decrease (i.e., increase of acidity) (LD_{50} multiplier < 0.1; Bayesian $p < 0.001$; Figure 1b; Table 1; Supporting Information: Materials and Methods 1). Therefore, the species result sensitive to SAR solutions.

The three different tardigrade species showed differences in the LD_{50} (i.e., the time needed to reach the 50% of death individuals) when exposed to the control condition (distilled water). In particular, the LD_{50} of the controls of *M.* cf. *hufelandi* (moss-inhabiting species) is significantly lower (Bayesian $p < 0.001$) from those of *A. antarcticus* and *H. exemplaris* (freshwater species; Figure 1a; Tables 1 and 2; Supporting Information: Materials and Methods 1)

At pH 4.5, *M.* cf. *hufelandi* and *H. exemplaris* result more sensitive (mean $LD_{50} = 7.9$ and 13.1, respectively; Tab. 1; Supplementary Materials and Methods 1), while *A. antarcticus* is the least affected by SAR (mean $LD_{50} = 15.6$; Table 1; Supporting Information: Materials and Methods 1). For each pH point lowering, LD_{50} significantly (Bayesian *p*-value < 0.001) decreases to the 9.4% (mean of 8.1% −10.7%, min−max of 95% confidence interval) in *M.* cf. *hufelandi*, to 7.7% (6.3%–9.3%) in *H. exemplaris*, and to 9.3% (7.9%–11.0%) in *A. antarcticus* (Figures 1b and 2). The sensibility to acidity increases over time only for *A. antarcticus* ($k > 1$; Bayesian $p < 0.001$; Figure 1d and Table 1; Supporting Information: Materials and Methods 1) and significantly differs from the other species (Bayesian $p < 0.001$; Figure 1d; Supporting Information: Materials and Methods 1) in which it doesn't change.

The effects on survival of the different acids (HCl, CH_3COOH, H_2SO_4, HNO_3, and SAR; all at a pH = 4.5) tested on tardigrades are

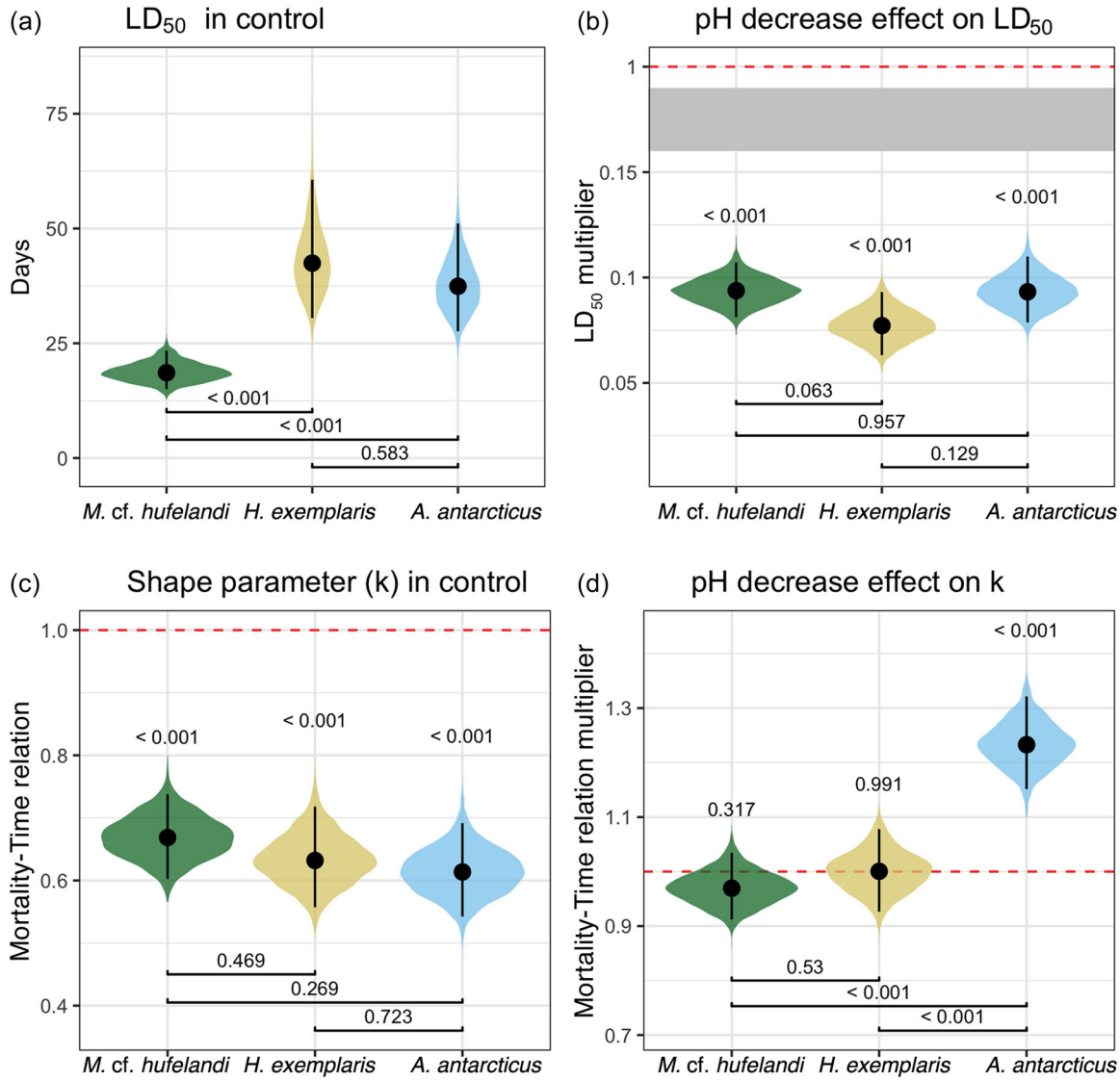

FIGURE 1 Estimated parameters of the modified Weibull CDFW fitted on the SAR mortality data for three species of eutardigrades. (a) LD_{50} in days in the control treatment (distilled water); (b) Effect of pH decrease on LD_{50} (horizontal dashed red line indicated the value at which there is no effect of the pH change on LD_{50}); (c) Shape parameter *k* (indicating if positive or negative time-mortality relation) for the control treatment (horizontal dashed red line indicated the value at which the mortality is constant in time); (d) Effect of pH decrease on *k* (horizontal dashed red line indicated the value at which there is no effect of the pH change on *k*). CDFW: cumulative Weibull distribution function; SAR: synthetic acid rains; LD_{50}: Time to death of 50% of individuals; violin: posterior density; dot: mean; line: 95% Confidence Interval; numbers above violins: Bayesian *p*-values for the comparison with the null effect (red dashed lines); numbers upon horizontal bars: Bayesian p-value for the difference of parameters between species.

species-specific. Acetic acid (CH_3COOH) has a significant effect that differs among the three species, with *A. antarcticus* having the highest LD_{50} and *M.* cf. *hufelandi* the lowest (Bayesian $p < 0.001$; Supporting Information: Materials and Methods 2). Comparing the distribution of the LD_{50}, all the species react similarly to the exposition to H_2SO_4 and HNO_3 and there are no statistical differences among them, even if by comparing the mean LD_{50}, *M.* cf. *hufelandi* is more affected than the aquatic species (*H. exemplaris*, *A. antarcticus*). The highest sensibility of *M.* cf. *hufelandi* for these two acids is also evidenced after SAR exposition (Bayesian $p < 0.001$;Supporting Information: Materials and Methods 2) that instead has similar effects on the two aquatic species. *Macrobiotus* cf. *hufelandi* and *H. exemplaris* have a similar LD_{50} pattern during exposition to SAR (and SAR effects are comparable with its constitutive compounds, namely H_2SO_4 and HNO_3), while the two species are more sensitive to HCl and CH_3COOH exposure (i.e., these acids are more lethal) with LD_{50} significantly reduced compared with the LD_{50} of SAR (Bayesian $p < 0.001$; Figure 3; Supporting Information: Materials and Methods 2). In *A. antarcticus* there are no statistical differences among the LD_{50} of the tested acids. Nevertheless, it is worth noting that *A. antarcticus* does not show for all acids the same pattern of the other two tested species. In fact, whilst the effects on *A. antarcticus* are

TABLE 1 Estimated parameters of the modified Weibull CDF fitted on the SAR mortality data for three species of eutardigrades.

Species	Upper 95% CI	Lower 95% CI	Median
	LD_{50} in control (days)		
Acutuncus antarcticus	27.615	51.116	37.418
Hypsibius exemplaris	30.491	60.615	42.457
Macrobiotus cf. *hufelandi*	15.001	23.411	18.592
	pH decrease effect on LD_{50} (LD_{50} multiplier)		
Acutuncus antarcticus	0.079	0.110	0.093
Hypsibius exemplaris	0.063	0.093	0.077
Macrobiotus cf. *hufelandi*	0.081	0.107	0.094
	Shape parameter [K] in control		
Acutuncus antarcticus	0.542	0.692	0.614
Hypsibius exemplaris	0.557	0.718	0.632
Macrobiotus cf. *hufelandi*	0.603	0.738	0.669
	pH decrease effect on shape parameter [K]		
Acutuncus antarcticus	1.151	1.321	1.233
Hypsibius exemplaris	0.926	1.078	1.000
Macrobiotus cf. *hufelandi*	0.912	1.034	0.969

Note: LD_{50} expressed in days.

Abbreviations: 95% CI, 95% confidence interval; CDFW, cumulative Weibull distribution function; SAR, synthetic acid rains.

TABLE 2 LD_{50} calculated form the fitting of Weibull CDF function to mortality data for three different eutardigrade species at different SAR pH values.

Species	pH	Upper 95% CI	Lower 95% CI	Median
		LD_{50} (minute)		
Macrobiotus cf. *hufelandi*	4.0	2.1	2.7	2.5
	4.5	7.3	8.6	7.9
	5.0	23.6	28.4	25.9
	5.5	74.8	96.9	84.3
	6.2	369.3	566.9	438.0
Hypsibius exemplaris	4.0	3.3	4.3	3.6
	4.5	12.1	14.4	13.2
	5.0	41.5	51.9	47.1
	5.5	140.4	200.2	169.4
	6.2	744.9	1334.5	1013.4
Acutuncus antarcticus	4.0	4.4	5.4	4.8
	4.5	14.8	17.1	16.0
	5.0	45.4	59.4	52.5
	5.5	137.1	208.2	172.0
	6.2	640.7	1249.4	916.6

Note: LD_{50} expressed in minutes.

Abbreviations: 95% CI, 95% confidence interval; CDFW, cumulative Weibull distribution function; SAR, synthetic acid rains.

similar for H_2SO_4, HNO_3, and SAR, similarly to other species, the HCl and CH_3COOH exposure results slightly less deleterious than SAR, contrarily to what we observe in *M*. cf. *hufelandi* and *H. exemplaris*. Moreover, the LD_{50} calculated for *A. antarcticus* exposed to HCl and CH_3COOH are the highest compared with those of both *M*. cf. *hufelandi* and *H. exemplaris* (Bayesian $p < 0.001$; Supporting Information: Materials and Methods 2).

3.2 | Stylet shape and composition

The SEM/EDX analyses on the stylets of each species obtained burning the organic part of the animals showed that they were formed by a single anatomical structure composed of calcium, carbonium, and oxygen, confirming their calcium carbonate nature as previously stated by Guidetti et al. (2015). The stylets of *A. antarcticus*, *H. exemplaris*, and *M*. cf. *hufelandi* had a similar shape (Figure 4a–c), For descriptive purposes, we propose to subdivide the stylet in three parts: fibula (*Lat*. pin), phlexum (*Lat*. bend), and epiphysis (*Gr*. bonehead; Figure 4d). The descriptions of these parts, valid for all the three species, will follow the arrows in Figure 4.

The fibula is the anterior part of the stylet and represents about 2/3 of the stylet total length, it resembles the anterior portion of a knife with a "plain" blade (in its internal portion) and "straight back" point with a sharp tip (see also Guidetti et al., 2012, 2013). The fibula is symmetrical in lateral view (i.e., from external to internal), while in dorsoventral view it is internally curved and externally straight. Its transverse section is approximately triangular and regularly increases in its section size moving posteriorly. The phlexum and the epiphysis are together about 1/3 of the stylet length.

The phlexum is the middle-posterior section of the stylet including the abrupt curvature of the stylet. Phlexum transverse section is almost circular. In *A. antarcticus*, it shows a dorsoventral depression in the external side. In *M*. cf. *hufelandi*, the depression is absent, while a striation is visible (Figure 4, empty arrowheads).

The epiphysis, resembling a bonehead, is composed of: a *corpus* (*Lat*. body), that in anterolateral view is approximately triangular bearing the *bulla* (*Lat*. button, stud); a median protuberance in its external side; two posterior symmetrical *apophyses*, enlargements connected with the corpus by branches.

The epiphyses are the portion of the stylets lying in the condyles of the stylet furca and differ between the analyzed species: in *M*. cf. *hufelandi*, on the external side it is possible to see two bulges departing laterally to the prominent bulla and joining the roundish apophyses, the branches are absent, but the bulges could derive from vestigial shortened and enlarged branches (Figure 4a); in *H. exemplaris* the corpus is laterally (i.e., from external to internal) flattened and dorsoventrally wider than the phlexum, the branches are absent, and

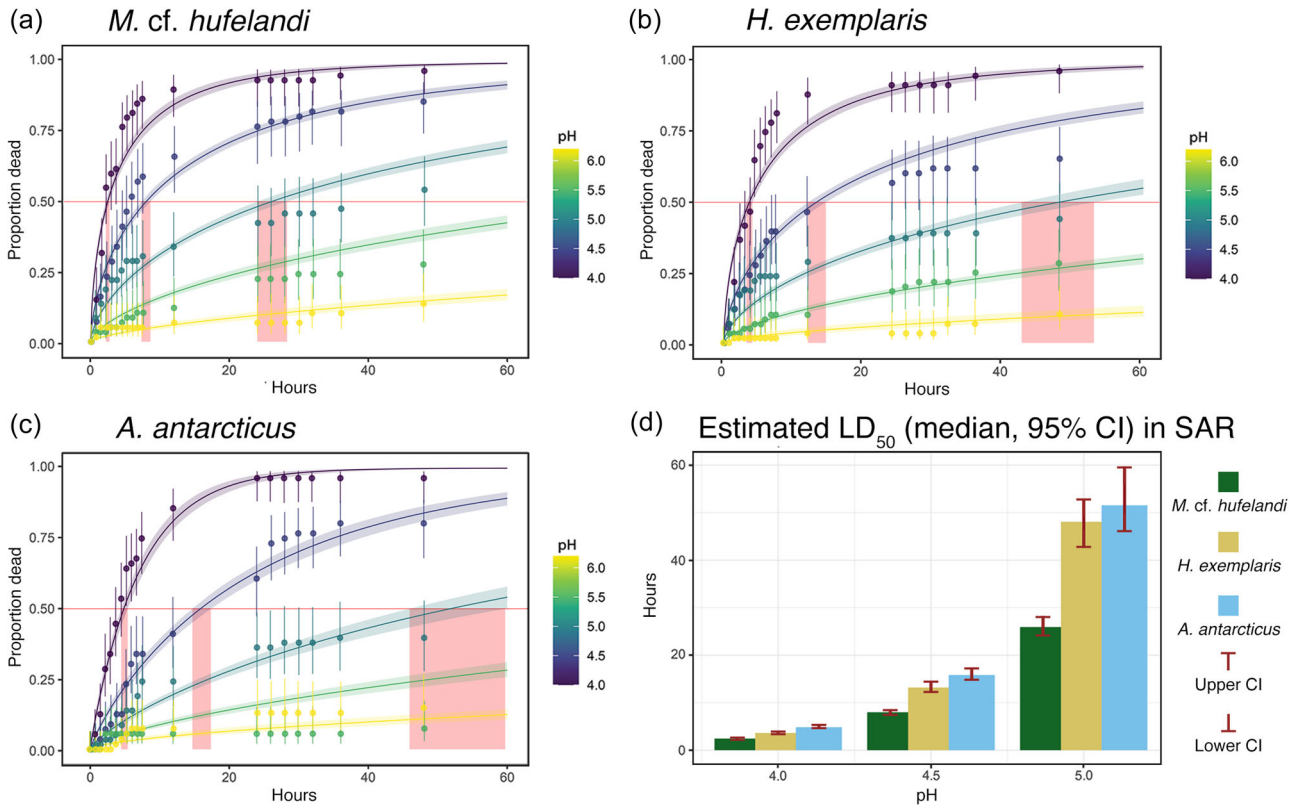

FIGURE 2 Fitting of Weibull CDFW function to mortality data for three different eutardigrade species at different SAR pH values over time (a–c). Estimated LD_{50} at different pH fitting in the 48 h observation range (d). (a–c) Circles: individual datapoints; vertical bars: 95% Confidence Interval (CI); shaded pink box: time intervals where the 95% CI of LD_{50} falls for each pH treatment in the observation range (48 h). CDFW: cumulative Weibull distribution function; SAR: synthetic acid rains.

the condyles are dorsoventrally flattened (Figure 4b); in *A. antarcticus* the corpus has the same size of the phlexum, the branches are absent, and the apophyses are small and roundish (Figure 4c).

3.3 | Effects of acid environment on the $CaCO_3$ stylets

The exposure of *M.* cf. *hufelandi* to high levels of acidity (HCl, pH 1.3) leads to an immediate death of the specimens. The complete dissolution of the stylets is reached in about 2 min after death with HCl pH 1.3, and in about 30 min with HCl pH 2 (in this case, death occurs in about 1 min). The first reaction of the animals after came in contact with acids was a retraction of the head and legs. The dissolution of the stylets starts, after death, from the tips of the fibula and from the phlexum (i.e., their mid-posterior portion), thus the two dissolution fronts move towards the center of the fibula and the posterior end of the epiphysis until its complete dissolution (Movie on YouTube https://youtu.be/sJo_0MLXYTI). Besides, we observed that *M.* cf. *hufelandi* in the sealed slide with distilled water becomes anoxic and distends after 3 h without any alteration in the morphology of the stylets.

More than 1260 animals have been placed on slide and photographed after the exposure to acids. We found in only two cases that acids disrupted the aragonite stylets of the feeding apparatus of alive specimens. In a specimen of *H. exemplaris* exposed to HNO_3 at pH 4.5, we found that the tips of both stylets were dissolved and the middle portion (corresponding to the phlexum) was less refractive (i.e., sign of ongoing dissolution) but it recovered its original morphology after about 24 h without molting. Another possible case concerns a specimen of *A. antarcticus* exposed to SAR at pH 4.0, in which the stylet fibulae were broken in half, but recovered their integrity in 12 days, we did not observe any molting events but due to this long interval considered, we cannot exclude that molting occurred and was not observed.

4 | DISCUSSION

According to our results, acid rains negatively affect tardigrade populations, and the impact of such effects can vary according to the pH and the time of exposition. The freshwater species *A. antarcticus* and *H. exemplaris* show a better resistance to acidity than the moss dwelling taxon *M.* cf. *hufelandi*. *Acutuncus antarcticus* has a high resistance to short exposure to acid rains but accumulates negative effects during time, increasing its sensibility to acidity; *H. exemplaris* reacts similarly to *A. antarcticus* in withstanding SAR, but its mortality remains stable over time during the exposure, resulting in a higher

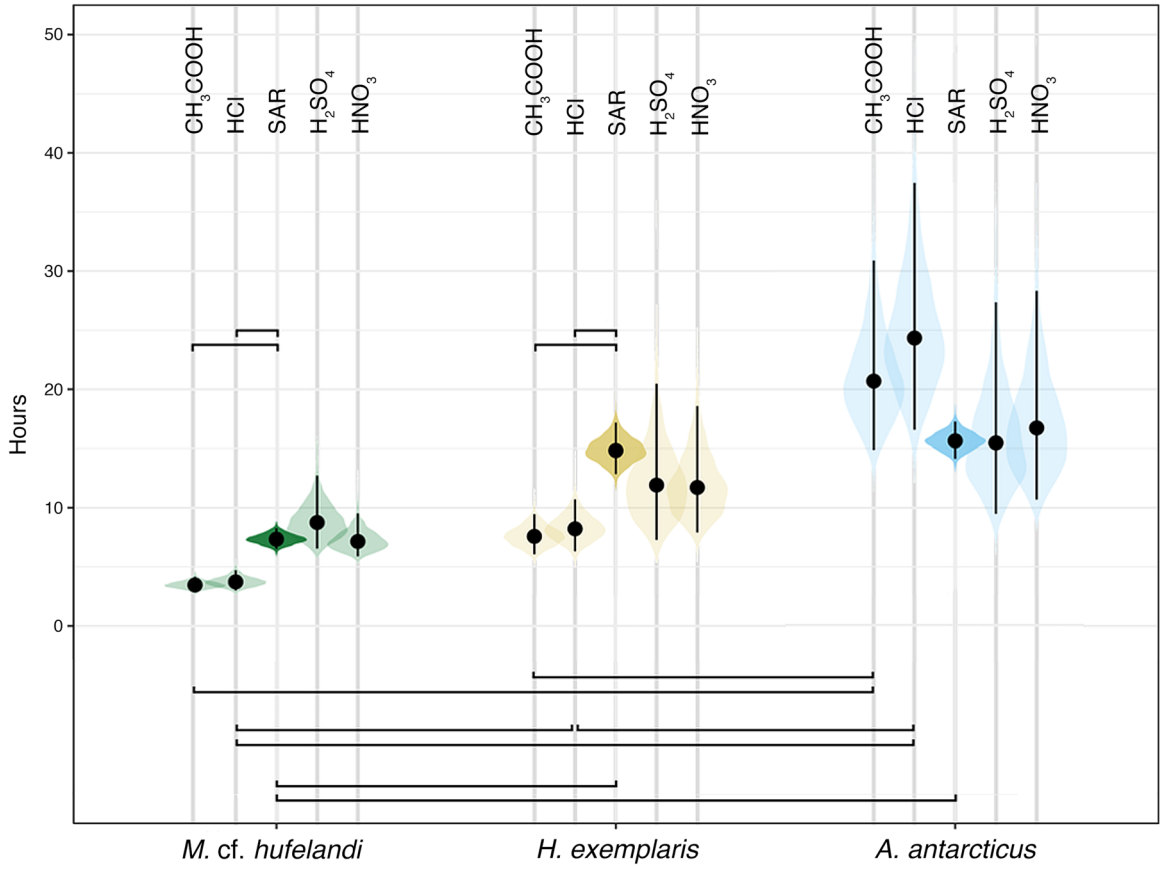

FIGURE 3 Estimated LD_{50} for five acids on three eutardigrade species at pH 4.5. Comparison between treatment was only tested between SAR and the other acids inside each species, and between the same acid across the different species. Violin: posterior density; dot: mean; line: 95% Confidence Interval; horizontal bars: treatment groups with difference with Bayesian $p < 0.05$. HCl: hydrochloric acid; CH_3COOH: acetic acid; SAR: synthetic acid rain; H_2SO_4: sulfuric acid; HNO_3: nitric acid.

LD_{50}. Therefore, the exposition to chronic or frequent acidification of the environment at pH < 5.0 could result in population decrease in *A. antarcticus* and *H. exemplaris* due to accumulation of damages or their inability to acclimatize. *Macrobiotus* cf. *hufelandi* seems to react differently to the exposure to acidity. Although it is the least resistant species, showing the lowest LD_{50}, the animals that survive to the first acidic shock slightly acclimate over the time.

The phenotypic variability of a population is the key for the survival of a species to the environmental stressors. The acid environment negatively affects the three tardigrade species, nevertheless, several specimens of each taxon (about 10%; see Figure 2) can withstand even very high levels of acidity (low pH down to 4.0) for a considerable time (certainly more than 48 h). These specimens could allow the survival of the population even after severely stressful events related to a sudden increase in environmental acidity.

Our experiments with SAR demonstrate the direct relationship between increasing in the acidity of the environment (pH lowering of the rains and water bodies) and viability of three species of tardigrades. However, pH is not the only factor affecting tardigrades mortality. In fact, the different tested acids (SAR, HCl, CH_3COOH, H_2SO_4, and HNO_3) also differently affects the survival of the tardigrade species in a species-specific way. SAR and its components, H_2SO_4 and HNO_3, resulted to be less lethal than acetic and hydrochloric acid for *H. exemplaris* and *M.* cf. *hufelandi*. While *A. antarcticus* responds differently resulting less negatively affected by HCl and CH_3COOH. Therefore, the nature of the acids does have a species-specific influence on the survival of the tardigrades. Specific metabolism of counterions could have a role in the ability of tardigrades to detoxify the pollutants (i.e., H^+ and counterions).

The difference in the species reaction to the tested acids could be explained by addressing the adaptation of the species to specific habitats. For example, the Antarctic semipermanent ponds inhabited by *A. antarcticus* collect water from melting ice rich in HCl and CH_3COOH. In fact, if compared with temperate areas, Antarctica is characterized by a higher concentration of HCl due to the greater persistence in the atmosphere of Cl^-, mostly derived by marine aerosol that is turned into HCl during the precipitation and trapped in the ice until summer melting (Legrand & Delmas, 1988). A higher concentration of CH_3COOH is instead due to the breakdown of the penguin guano during the Antarctic summer that persist on the semipermanent pond (Legrand et al., 2012). Moreover, *Acutuncus* species are adapted to ephemeral habitats, as temporary ponds (Cesari et al., 2016; Vecchi et al., 2023), that can be subject major fluctuation in pH values (Brendonck et al., 2000).

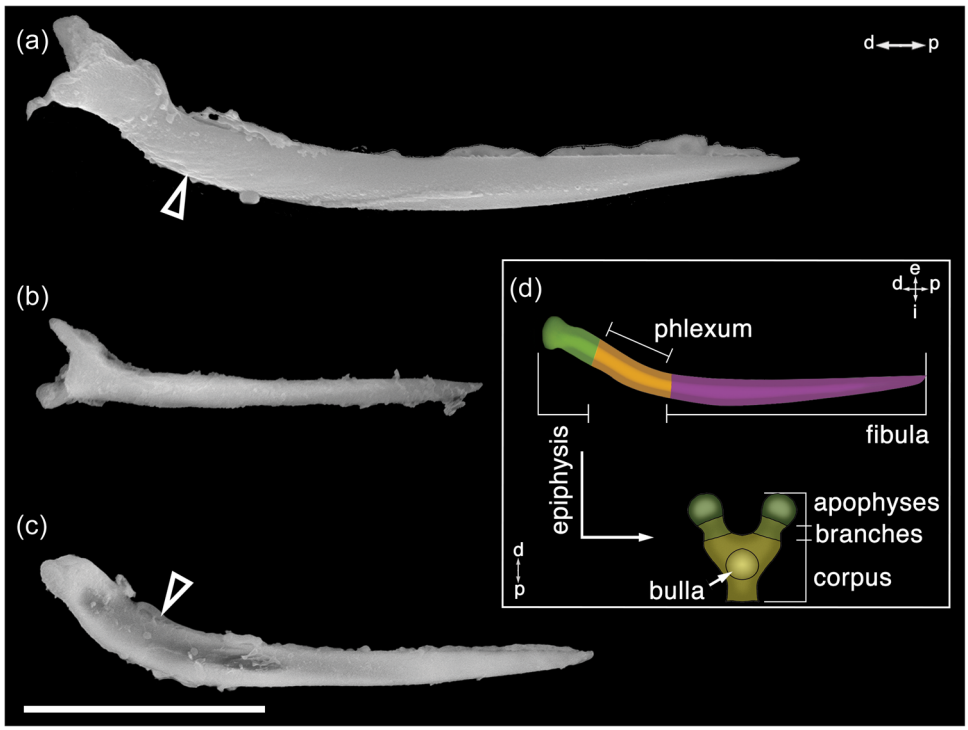

FIGURE 4 Aragonite piercing stylets extracted (a–c; SEM) and their schematic representation (d). (a) *Macrobiotus* cf. *hufelandi*, dorsal external view. (b) *Hypsibius exemplaris*, lateral internal view. (c) *Acutuncus antarcticus*, dorsal view. Empty arrowheads: striations and depression in the phlexum. d: distal to the anterior end of the animal; p: proximal to the anterior end of the animal; e: external side, that is, facing the lateral side of the animal; i: internal side, that is, facing the lateral side of the buccal tube wall and opposite to the external side. Scale bar 10 µm.

To understand the composition of the tardigrade stylets, Bird and McClure (1997) kept specimens of *Macrobiotus* cf. *pseudohufelandi* (*Xerobiotus* cf. *pseudohufelandi* according to Bertolani & Biserov, 1996; Massa et al., 2021) in water under a sealed coverslip on slide. According to their experiment, in this condition, the stylets of the specimens dissolved. Differently, we found that in *M.* cf. *hufelandi* the stylets remained intact. Furthermore, differently from Bird and McClure (1997) experiments, stylets of *M.* cf. *hufelandi* exposed to HCl 0.05 M (pH 1.3, same of Bird and McClure's experiment) dissolve faster (about 2 min after the animal death), than in *X.* cf. *pseudohufelandi* (50–90 min according to Bird & McClure, 1997) exposed to the same acid and molar concentration.

During the dissolution of the aragonite stylets, the phlexum is damaged first (altogether with the fibula tips), as shown in the movie of *M.* cf. *hufelandi* exposed to high acidity and and in the specimen of *H. exemplaris* in which the stylets have been reformed after the exposure to pH 4.5. Considering that the so-called salivary glands seem to be connected to the phlexum (visible in Gross et al., 2019), we suspect a role of these glands in the excretion of the exceeding protons to withstand high acidity.

Since the tips of the stylets are the first to dissolve when the animals are placed at low pH, we can speculate that the major uptake of H^+ ions is due to active and/or passive ingestion through the mouth that in the test species remains always open. It is worth to report that in the preliminary experiments, the stylets of animals in the advanced molting process (i.e., with the mouth sealed by cuticular folds, e.g., see Figure 2 in Guidetti et al., 2011) were not affected during the exposition to low pH.

In all experiments, the tested tardigrades maintaining an unaltered morphology of the $CaCO_3$ stylets, shown high tolerance of animals to moderate/high acidity, and their capabilities in buffering the proton charge penetrated from the environment. Despite this, we cannot exclude that in other tardigrade species a partial or total dissolution of the stylets occurs in alive specimens, as reported for *X.* cf. *pseudohufelandi* by Bird and McClure (1997). In this case, the dissolution of the stylets can be induced by the increased level of acidity and/or induced to buffer the environmental pH changes as happens in the snail *Elimia flava* (Lea, 1862) that uses internal stores of $CaCO_3$ to buffer hemolymph acidification when exposed to pH 4 (Ewald et al., 2009).

5 | CONCLUSION

This study demonstrates that various acids, including acid rain, have detrimental effects on tardigrades in a pH, time of exposure, and species-dependent manner. Freshwater tardigrade species, *A. antarcticus* and *H. exemplaris*, exhibit higher resistance to acidity compared to the moss-dwelling *M.* cf. *hufelandi*, which demonstrate better acclimation to low pH over time. The stylets of most surviving specimens were unaffected. These findings suggest that the studied tardigrade taxa possess the ability to buffer environmental pH

changes, and that their populations may be able to recover from the negative effects of acidity.

The ecophysiological responses of tardigrades to environmental stresses, like environmental acidification, are fundamental to assess the impact of anthropogenic stressors on tardigrades and the potential of these organisms as ecological indicator for biomonitoring. Crucial in our understanding of their ecophysiology is the metabolic destiny of the ions during and after the exposure. This is a valuable focus for future investigations.

As the tested species belong different phyletic lineages, it is reasonable to extend the ability to protect the stylets to at least all Parachela (Eutardigrada).

The three species selected for our experiments come from different biogeographic regions and have a wide distribution in their respective inhabited bioregions or, as species complex, around the world. For such reasons we suggest them as models for more stress resistance studies to validate them as possible bioindicator for air and freshwater quality.

AUTHORS CONTRIBUTION

Edoardo Massa and Lorena Rebecchi conceived the study. Edoardo Massa and Roberto Guidetti designed the experiments. Edoardo Massa performed the experiments, acquired the SEM images and EDX spectra, analyzed data, and wrote the first draft of the manuscript. Roberto Guidetti and Lorena Rebecchi supervised the research and provided financial support. All authors contributed to the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Html files containing the R script, the raw data, the statistical results, and the relative functions plottings are available on FigShare following the links:

Supporting Information: Materials and Methods 1 https://figshare.com/s/dddfaa236f76d3eb8117

Supporting Information: Materials and Methods 2 https://figshare.com/s/fe56ae64a60fcb23a64a

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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