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RESEARCH ARTICLE

Ecology explains anhydrobiotic performance across tardigrades, but the shared evolutionary history matters more

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Abstract

1. Desiccation stress is lethal to most animals. However, some microinvertebrate groups have evolved coping strategies, such as the ability to undergo anhydrobiosis (i.e. survival despite the loss of almost all body water). Tardigrades are one such group, where the molecular mechanisms of anhydrobiosis have been more thoroughly studied. Despite the ecological, evolutionary and biotechnological importance of anhydrobiosis, little is known about its inter- and intra-specific variability nor its relationship with natural habitat conditions or phylogenetic history.
2. We developed a new index—anhydrobiotic recovery index (ARI)—to evaluate the anhydrobiotic performance of tardigrade populations from the family Macrobiotidae. Moreover, we compared the explanatory role of habitat humidity and phylogenetic history on this trait using a variance partitioning approach.
3. We found that ARI is correlated with both microhabitat humidity and yearly rainfall, but it is mostly driven by phylogenetic niche conservatism (i.e. a high portion of ARI variation is explained by phylogeny alone). Finally, we showed that anhydrobiotic performance is highly variable, even between closely related species, and that their response to local ecological conditions is tightly linked to their phylogenetic history.
4. This study not only presents key insights into an emerging model system, but also provides a new methodological approach for wider scale studies of the ecological and evolutionary implications of anhydrobiosis.

KEYWORDS

anhydrobiosis, anhydrobiotic recovery index, desiccation, Tardigrada, variance partitioning

1 | INTRODUCTION

The ability to survive extreme abiotic stress is known as cryptobiosis (Arakawa, 2022; Clegg, 2001; Møbjerg et al., 2011; Møbjerg & Neves, 2021; Rebecchi et al., 2020), and one of its most widespread and studied forms is anhydrobiosis, the adaptation to environmental

desiccation through a reversible state of suspended animation, found in many taxa, for example animals, fungi and plants (Guidetti et al., 2011; Kaczmarek et al., 2019; Morales-Sánchez et al., 2022; Rebecchi et al., 2020; Wełnicz et al., 2011; Wiemken, 1990). Desiccated individuals can survive without liquid water even for years (Fontaneto et al., 2012; Grewal, 2000; Rebecchi et al., 2006;

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Roszkowska et al., 2020) after which, upon rehydration, they are able to resume normal metabolic activity. Some organisms, termed as holoanhydrobionts, can undergo anhydrobiosis throughout their life cycle. These include some species in the phyla Rotifera, Nematoda and Tardigrada (Jönsson, 2005), whereas in others only some life stages are anhydrobiotic, such as eggs of the brine shrimp and other microcrustaceans (Clegg, 1965), or larvae of the African chironomid *Polypedilum vanderplanki* Hinton, 1951 (Watanabe et al., 2003).

Tardigrades are considered model animals for the study of survival under extreme stressors (e.g. Erdmann & Kaczmarek, 2017; Guidetti et al., 2011) and extensive research on the mechanisms underlying their resistance has been done in the recent years (reviewed in Arakawa, 2022; Krakowiak et al., 2023; Møbjerg & Neves, 2021; Rebecchi et al., 2020). In tardigrades, anhydrobiosis is achieved by morphological and biochemical processes. Morphologically, anhydrobiotic tardigrades form a so-called 'tun', in which the limbs and head are withdrawn and the body is compressed along the antero-posterior axis (Halberg et al., 2013). These morphological changes, essential for a successful anhydrobiosis, are actively produced by movements and muscle contraction (Halberg et al., 2013) and not by a passive reduction of body volume due to the loss of water. The biochemical processes responsible for anhydrobiotic survival are not fully understood, but several mechanisms are used and various bio-protectants are produced by tardigrades (Boothby & Pielak, 2017; Giovannini, Boothby, et al., 2022; Giovannini, Corsetto, et al., 2022; Murai et al., 2021; Wang et al., 2014; Wojciechowska et al., 2021; Yamaguchi et al., 2012), both constitutively and in response to environmental cues (Møbjerg & Neves, 2021). Despite decades of research on anhydrobiosis, most of the research effort has been devoted to the understanding of the physiological and molecular mechanisms that allow animals to survive almost complete desiccation (see any of the many reviews published on the topic, for example: Arakawa, 2022; Hibshman et al., 2020; Rebecchi et al., 2020; Sogame & Kikawada, 2017). In contrast, the interplay between anhydrobiosis and evolutionary processes or how evolutionary and ecological pressures lead to this astonishing adaptation are still poorly studied (Kaczmarek et al., 2019; McGill et al., 2015; Ricci & Caprioli, 2005; Roszkowska et al., 2023).

Desiccation tolerance, and anhydrobiotic capability in particular, can be seen as an evolutionary beneficial trait, as it allowed for the colonisation of terrestrial habitats by aquatic organisms. Jönsson et al. (2001) suggested (with a focus on tardigrades) that habitat-dependent selection could act on the anhydrobiotic ability, provided that three conditions are met: (i) existence of additive genetic variance (total effect on a trait stemming from one or more gene loci, each of which contributes to the trait in a measurable way; Singh & Singh, 2018) for this trait, (ii) within-species variation to desiccation exposure and (iii) costly anhydrobiotic adaptations (i.e. lower fitness in the absence of anhydrobiotic stress, for example due to lower reproductive output after diverting resources from the production of gametes, especially eggs, to the synthesis of anhydrobiosis-related proteins; Arakawa, 2022). Based on these assumptions, one can predict that (i) there should be a correlation between the desiccation

frequency of a habitat and the anhydrobiotic capabilities of anhydrobiotic animals inhabiting it, and that (ii) there could be intra-specific, interpopulation (adaptive) variability in anhydrobiotic performance. Comparative experimental data that address these two predictions are still scarce, as most of the studies focused on testing only one or two species/populations at the time. Moreover, it is not known how quickly desiccation resistance can respond to selection, but experimental data on nematodes showed a quick response to artificial selection for desiccation resistance (Anbesse et al., 2013), hinting at the possibility that the modulation of desiccation resistance in response to different selective forces in nature could evolve relatively quickly.

Among the studies involving multiple tardigrade species, almost all included only populations from terrestrial habitats (mosses, lichens, soil), although these taxa provide a representative sample across limnoterrestrial tardigrade families. In these, anhydrobiotic ability was measured as the proportion of individuals surviving experimental anhydrobiotic treatments. Importantly, regardless of the adopted methodology, each of these studies observed variability in anhydrobiotic performance between the analysed taxa, suggesting that there is considerable phylogenetic variation in this trait as well as in the colonised habitat. Some of these works only compared a couple of species (Erdmann et al., 2021; Rebecchi et al., 2006; Roszkowska, Gołdyn, et al., 2021), albeit multiple geographically separated populations of the same species were occasionally analysed too (Faurby et al., 2008; Jönsson et al., 2001). Other studies had a broader scope: seven species from seven families in (Wright, 1989), four species from two families in (Roszkowska et al., 2022) and three species from three families in (Sömme & Meier, 1995). A comparison of anhydrobiotic performance between freshwater and terrestrial taxa has only been performed once (Cesari et al., 2012), in a dataset of nine species from five different habitats (moss, lichen, leaf litter, gutter sediment and freshwater sediment).

Importantly, however, none of the studies published to date took the phylogenetic context into consideration, which is necessary when comparing data obtained from representatives of phyletic lineages that differ in the extent of their shared evolutionary history (Cornwell & Nakagawa, 2017; Felsenstein, 1985). Moreover, recovery from anhydrobiosis is a complex process, during which damages resulting from desiccation (Rebecchi et al., 2009) can be repaired (Giovannini, Corsetto, et al., 2022). Thus, a proportion of recovered individuals alone (a typical measure of anhydrobiotic survival) could conceal other important aspects of fitness, such as the recovery speed. Although some studies (e.g. Giovannini, Boothby, et al., 2022; Roszkowska et al., 2023) did record the proportion of motile animals at different time points, each time step was analysed separately (e.g. when comparing treatment groups), thus dismissing the temporal autocorrelation in this type of data.

To propose a standardised tool for quantifying anhydrobiotic performance that accounts for multiple anhydrobiotic recovery dimensions (survival and recovery speed) and provides uncertainty estimates, here, we analysed 29 populations representing 19 species of macrobiotid tardigrades. In addition, using phylogenetic models,

we tested the effect of two key ecological variables linked to desiccation intensity (microhabitat dryness and precipitation) on anhydrobiotic performance. Finally, using a variance partitioning approach (Desvignes et al., 2003), we discuss the relative roles of adaptation to the local ecological condition and of evolutionary history in explaining inter-specific variation in anhydrobiotic performance.

2 | MATERIALS AND METHODS

2.1 | Sampling and culturing

We used recently sampled populations and laboratory sourced cultures of 19 species of tardigrades from the family Macrobiotidae (Table S1). Therefore, to ensure comparable levels of laboratory acclimatisation and avoid confounding effects of phenotypic plasticity and maternal effects, we cultured all populations for at least 3 months (and at least two generations) under uniform conditions. Animals were kept in 5 cm diameter, ventilated, Petri dishes with a finely scratched bottom, filled with mineral water and fed *libitum* with algae (*Chlorococcum hypnosporum* Starr, 1955 and *Chlorella* sp.) and nematodes (*Panagrellus pycnus* Thorne, 1938). Cultures were kept in a climate chamber with a photoperiod 2:22 L:D and a daily temperature cycle of 14–16–18–16°C (each shift lasting 6 h). Half of the rearing medium was replaced every week.

2.2 | Ecological variables

We used two types of ecological data: micro- and macroclimate humidity. To account for microclimatic conditions, we classified the source substrate according to a humidity scale modified from (Ramazzotti & Maucci, 1983) (Table S2). The categories of this scale reflect the degree of hydration and range from one (wet substrates permanently submerged) to five (dry substrates exposed to the sun for a great part of the day). These data were obtained by us when sampling or provided by the original sample collectors. For the analysis, the categories were centred to the third class corresponding to the intermediate dryness level. To account for macroclimatic conditions, we extracted annual precipitation at the resolution of 10 min from the BIO12 WorldClim dataset (worldclim.org/data/bioclim.html) at each population sampling coordinates (Table S1). The annual precipitation data were scaled and inverted before data analysis (inverting the rainfall, results in the same direction of the substrate dryness categories, i.e. the higher the inverted rainfall values, the drier the habitat).

2.3 | Genotyping and phylogenetic reconstruction

We extracted DNA from individual animals by following a Chelex® 100 resin extraction method (BioRad) with modifications described in detail in (Stec et al., 2020). We sequenced four DNA fragments,

three nuclear (18S rRNA, 28S rRNA, ITS-2) and one mitochondrial (COI). All fragments were amplified and sequenced according to the protocols and primers described in (Stec et al., 2020). To read the sequencing products, we used an ABI 3130xl sequence analyser at the Department of Biological and Environmental Sciences of the University of Jyväskylä. Sequences were processed in MEGA7 and submitted to NCBI GenBank (Table S3). We conducted the phylogenetic analyses using concatenated 18S rRNA+28S rRNA+ITS-2+COI sequences from the populations utilised in this study. For some populations sequences were already available in GenBank and were downloaded (Table S3). To improve the accuracy of the phylogenetic reconstruction, we included additional taxa from Macrobiotidae (Table S3), that were trimmed from the tree in the data analysis phase. Newly generated sequences of *Bertolanius weglarskae* (Dastych, 1972) were used as outgroup (Table S3). For sequences alignment, model selection, and Bayesian inference phylogenetic reconstruction, we used the same protocol as in (Stec et al., 2021).

2.4 | Anhydrobiosis performance

For each population, we used from 13 to 123 actively moving individuals (hatchlings were excluded). We performed all desiccation and rehydration protocols at room temperature (20–22°C). We desiccated them in groups of up to 40 individuals (when more than 40 individuals per population were used, we divided them into separate groups). We placed these groups on 1 cm² pieces of chromatographic paper (Whatman Grade: 3MM CHR) with 50 µL of mineral water inside 3.5 cm diameter ventilated Petri dishes. Petri dishes were kept at 75% relative humidity (RH) for 24 h and then moved to 0% RH for 30 days. The 75% RH condition was achieved by placing a container with saturated NaCl solution in an airtight box, while an airtight box lined with silica gel was used for the 0% RH regime.

We rehydrated individuals by first adding 50 µL of mineral water to the piece of paper, waiting 15 min, and then submerging it with additional 4–5 mL of mineral water. We monitored the recovery from anhydrobiosis by counting and removing motile individuals (according to the 'first movement' criteria by Roszkowska et al., 2022; i.e. any visible sign of the movement of claws, legs, body, buccal tube, etc.) at 1, 4 and 24 h after the start of rehydration. The 24 h period was set as the maximum, as after this time no further individuals recovered (Giovannini, Boothby, et al., 2022).

We fitted an exponential cumulative distribution function (CDF) to the cumulative proportion of recovered animals over time. *Anhydrobiotic recovery index* (ARI) is defined as the relative area under the recovery curve (AUC) in a defined x-variable range (in our case from 0 to 24 h; Equation 1 and Figure 1): bound between 0 and 1, it is the overall survival success penalised by the recovery time (i.e. the slower the animals take to recover from anhydrobiosis, the lower the index will be). The exponential CDF function was reparametrised with p (overall survival to anhydrobiosis) and Q (time in hours for 90% of the surviving animals to recover motility; Equation 1). This parameterisation allows to specify biologically relevant priors and to

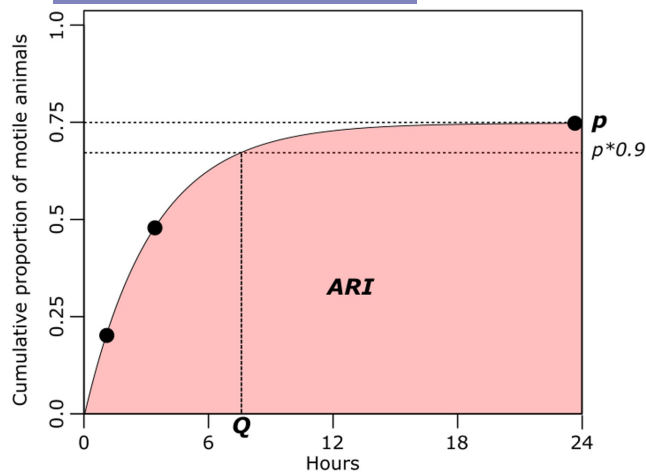


FIGURE 1 Example of cumulative exponential distribution function fitting an associate parameters and indexes. Solid dots represent example observed data points (at 1, 4 and 24 h as in this paper experiment). For the interpretation of p and Q see the main text.

obtain easy to interpret parameter estimates. For p we used a uniform prior between 0 and 1, whereas for Q we used a normal distribution with mean on 0 and standard deviation of 100 (but truncated to include only positive values).

We fitted the exponential CDF to the data for each population and obtained the ARI estimate (along with its 95% credible interval) through Bayesian methods with the function 'fit_model' from the R package 'anhydrofit' (Vecchi & Calhim, 2022).

Equation 1: Modified cumulative exponential distribution function. For the interpretation of p and Q see the main text. The X -axis indicates the time after the beginning of rehydration expressed in hours, whereas the Y -axis represents the cumulative proportion of motile animals.

$$y = p \times \left(1 - e^{-x \times \frac{\ln(1-0.9)}{Q}} \right). \quad (1)$$

2.5 | Comparative phylogenetic methods

To test and quantify the variance components explained by the partial effect of ecological predictors, phylogeny and their shared effect (i.e. phylogenetically structured functional variability), we used a modified version of the phylogenetic variance decomposition (PVR) technique developed by Desdevises et al. (2003). The original PVR approach used principal coordinate analysis (PCoA) to extract the eigenvectors of the phylogenetic distance matrix. The eigenvectors, which indicate phylogenetic differences among groups of species at different levels of the phylogeny, are then employed as predictor variables in the partial regressions (see as example Gonçalves-Souza et al., 2014). In our analyses, we account for phylogeny by including, when applicable, the phylogenetic covariance matrix as a random effect using generalised linear mixed models (GLMMs). The R^2 of these models are then used to calculate the explained variation in the response variable (ARI) between the ecological matrix

(component a : substrate dryness scale and rainfall), the phylogenetic variance-covariance matrix (component c), and the overlap of these effects (component b). In summary, the analysis follows these steps:

Step 1: Implementation of a GLMM on both ecological variables, and we included the phylogenetic covariance matrix as a random effect. The coefficient R^2_{tot} represents the sum of components a , b and c of the decomposition.

Step 2: Regression of the response variable against the ecological variables only, without accounting for phylogeny in a generalised linear model (GLM). The coefficient R^2_{eco} of this GLM is equal to components $a + b$ of the decomposition.

Step 3: Implementation of a GLMM only with the intercept and the phylogenetic covariance matrix as random effect. The coefficient R^2_{phy} of this model is equal to the components $b + c$ of the decomposition.

Step 4: Calculation of the individual value of each component following the subtraction proposed by (Desdevises et al., 2003) component $a = R^2_{tot} - R^2_{phy}$; component $b = R^2_{eco} + R^2_{phy} - R^2_{tot}$; component $c = R^2_{tot} - R^2_{eco}$. Additionally, the residual variance (component d) can be calculated as $d = 1 - (a + b + c)$.

Component a is the ecological component (in this case it would explain an adaptation to local ecological conditions), component b is the phylogenetically structured environmental variation (often known as 'phylogenetic niche conservatism') and component c is the pure phylogenetic component. We fitted all the above-mentioned (phylogenetic) generalised linear (mixed) models with the R package for Bayesian GLMM 'brms' (Bürkner, 2018) with the Binomial family and a logit link function for the likelihood calculation. The three model formulas used for the regressions are as follows:

Model_{tot}: $ARI \sim \text{Dryness category} + \text{Rainfall} + \text{Dryness category} \times \text{Rainfall} + (1 | \text{Phylogeny})$.

Model_{eco}: $ARI \sim \text{Dryness category} + \text{Rainfall} + \text{Dryness category} \times \text{Rainfall}$.

Model_{phy}: $ARI \sim (1 | \text{Phylogeny})$.

In all the models, as priors for all β parameters (Dryness category, Rainfall, Dryness category \times Rainfall), we used normal distributions $N(0, 10)$, whereas for all other parameters (Intercepts, sd) priors, we used student's t -distributions $t(3, 0, 2.5)$. The priors were chosen to be weakly informative and to improve sampling efficiency and convergence. Since the differences across populations in sample size resulted in different uncertainties in the ARI estimates, we included this population-specific uncertainty in the GLMMs. To achieve the latter, we transformed the posterior distributions of ARI values for each population to a binomial proportion of success/trials through the method-of-moments estimations of the parameters of a Beta distribution. With this transformation, a posterior distribution of ARI values between 0 and 1 is transformed into a success/trials proportion that will approximate almost perfectly the mean and variance of the original posterior distribution and it is easy to include in a

GLMM as a response variable with a Binomial distribution family. When appropriate, a phylogenetic variance–covariance matrix calculated under the assumption of Brownian motion was included as a random effect. For all the models we run four parallel chains, each of 100,000 generations sampled every 100 generations and with a burn-in of 10%. The convergence of the Bayesian regressions was assessed by inspection of effective sample size (ESS) and Rhat.

To exclude the effect of long-term culturing on anhydrobiotic performance, a preliminary model including all fixed and random effects described above, plus the estimated months of culturing before the *ARI* estimation was fitted as above (Data S1). As the culturing time had no effect (Bayesian p -value=0.908, Data S1), it was not considered in other analyses. All analysis scripts are available as Data S1.

3 | RESULTS

3.1 | Anhydrobiosis index in Macrobiotidae

We obtained anhydrobiosis recovery data for 29 populations belonging to 19 tardigrade species of the family Macrobiotidae (Figures 2 and 3; Table S4; Figure S1). The estimated *ARI* ranged from circa 0 in two populations of *Paramacrobiotus fairbanksi* Schill et al., 2010 (S09 and S85) to almost 1 in a population of *Macrobiotus cf. sapiens* (S1981). The anhydrobiotic survival (p) and time to reactivation of 90% of the surviving animals (Q) also showed high inter-specific variability. Finally, all three estimated parameters (*ARI*, p , Q) differed across populations of the same species (Figures 2 and 3; Table S4; Figure S1).

3.2 | Effect of ecological variables on *ARI*

The complete GLMM regression output (including both ecological fixed and phylogenetic random effects) (Table 1) showed that *ARI* is positively associated with the substrate dryness (Bayesian p -value=0.002) and lower precipitations (Bayesian p -value=0.002), with similar effect magnitude of one level difference in substrate dryness category as one standard deviation change in annual rainfall (which corresponds to 440mm of rain/year). These two ecological predictors have additive effects (i.e. the interaction term estimate has a large uncertainty range that includes zero).

3.3 | Phylogenetic variance decomposition

The complete regression including both fixed (ecology) and random (phylogeny) effects explained a substantial proportion of the variance (about 97%), and almost the same amount of variance was explained by the phylogeny-only model (about 96%). The model ignoring phylogeny explained about 20% of the variance. Plots showing the congruence between observed and predicted values, as well

as binned residual plots (following Gelman & Hill, 2007), and posterior predictive plots are provided in Figure S2.

The phylogenetic variance decomposition components (Table 2) show a very low (and compatible with 0) value for the ecology component (average 1.2%; Bayesian p -value=0.206). A high amount of variance was explained by the phylogeny alone (average 77.4%, Bayesian p -value<0.001), whereas the variance explained by the shared effect of ecology and phylogeny was intermediate (average 18.5%; Bayesian p -value<0.001).

4 | DISCUSSION

Our study quantifies anhydrobiotic ability in a novel way, and highlights the inter-specific variation in this trait within a single tardigrade family (Macrobiotidae). Furthermore, we show that shared evolutionary history, rather than micro- or macro-ecological factors alone, seems to be the main explanation for the observed diversity in this trait at the examined taxonomic scale.

4.1 | Anhydrobiotic performance quantification

Measuring and quantifying fitness is a challenging but a crucial task for our understanding of life (Keller et al., 2022). Since 'Drying to equilibrium with even moderately dry air is instantly lethal to most species of animals and plants' (Alpert, 2006), the ability to survive desiccation (anhydrobiosis) is an example of a complex physiological trait with profound consequences for fitness. The quantification of different facets of fitness (including recovery from anhydrobiosis) has been shown to be far from easy, even in comprehensively studied groups such as bryophytes (e.g. Morales-Sánchez et al., 2022). Anhydrobiotic recovery in tardigrades can be described by two components: the proportion of animals that survive desiccation (p) and the time it takes for them to recover after rehydration (Q). However, previous research has generally ignored the second aspect, with two noteworthy exceptions: Crowe and Higgins (1967), who tested how mean revival time is influenced by pH, dissolved oxygen and temperature in *P. aff. areolatus*; and Rebecchi et al. (2009), who tested Δ recovery (difference between motility at 1 and 24 h after rehydration) in a population then identified as '*P. richtersi*' (now known to represent as *P. spatialis* Guidetti et al., 2019) at different desiccation conditions. Two other studies (Roszkowska et al., 2023; Roszkowska, Gołdyn, et al., 2021) tested the recovery time (as time to movement after rehydration) against the anhydrobiosis duration. We found that the two components— p and Q —despite being generally correlated, can still show a non-negligible independence (Figure 3) and should therefore be considered jointly when analysing anhydrobiotic performance. On the one hand, some taxa had similar overall survival rates but very different recovery times (e.g. *M. annewintarsae* Vecchi & Stec, 2021 and *M. vladimiri* Bertolani et al., 2011 both had 88% survival, but the former took an average of 4.4 h to

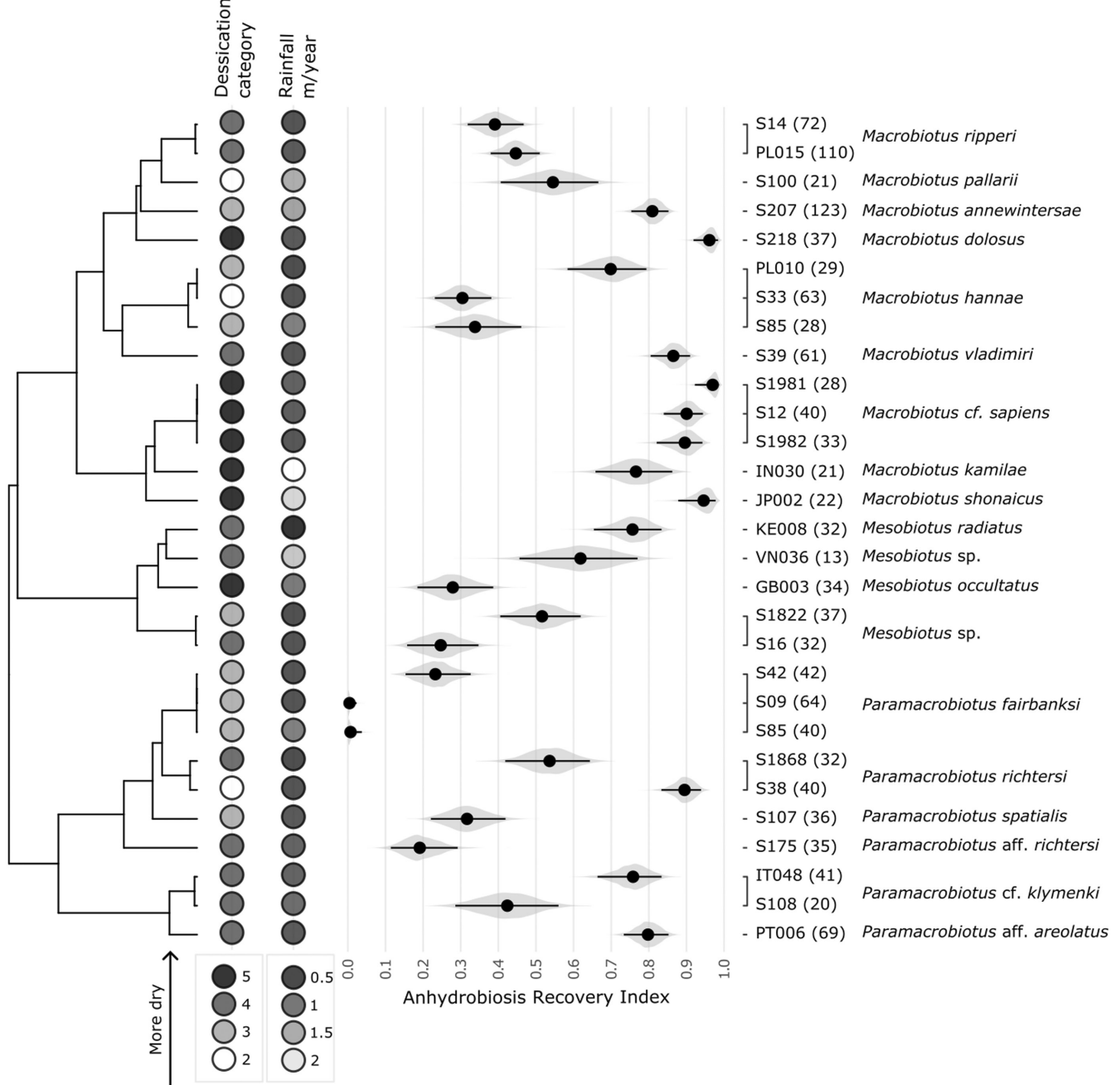


FIGURE 2 Anhydrobiotic recovery index (ARI) estimates for 29 populations representing 19 species of macrobiotid tardigrades in a phylogenetic context. Grey violins represent the posterior distributions densities for each mean (dots) and 95% HDI (horizontal black lines). The two ecological variables used in the analyses are shown as filled circles, where darker shades reflect dryer micro- (substrate desiccation category) and macro- (annual rainfall) environmental conditions. The numbers in parenthesis after the population codes indicate the number of animals tested.

recover, while the latter—only 1.7h; Table S4; Figure S1). On the other hand, some species had similar recovery times but vastly different survival rates (e.g. *M. hanna*e Nowak & Stec, 2018 S33 and *M. pallarii* Maucci, 1954 had an average recovery time of 15 h, but 41% and 75% survival respectively; Table S4; Figure S1). To address these complexities, we developed the ARI, which combines overall survival and recovery speed into a single and interpretable metric. The ARI provides a biologically meaningful measure

of anhydrobiotic recovery by representing the time at which individuals could start feeding and reproducing after natural occurring bouts of anhydrobiosis. Additionally, the Bayesian implementation of ARI presented in this study provides not only the punctual estimation of its value, but also the extent of uncertainty around it. One methodological caveat should be pointed out when measuring anhydrobiotic performances: the animals used should all have comparable energetic and nutritional conditions as they can

FIGURE 3 Relationship between p and Q estimates. Each dot represents a population. The dotted line represents the linear relationship between p and Q , obtained by fitting a linear model to the average p and Q estimates with the exclusion of two *Paramacrobiotus fairbanksi* populations (S09 and S85—white dots), as due to the model specification, estimates of Q when p is close to 0 are unreliable and biologically meaningless. In grey, ARI isolines are additionally shown.

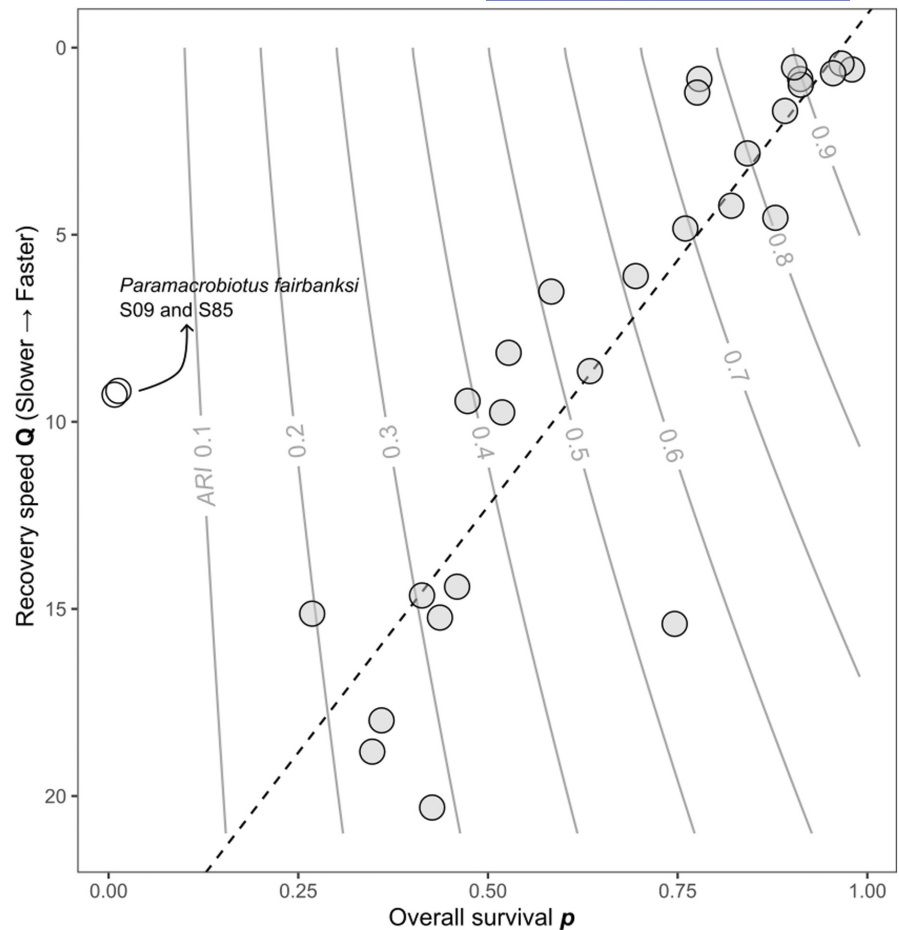


TABLE 1 Full model (including both ecology and phylogeny) fixed effects output.

Predictor	Posterior mean [95% HDI]	Bayesian p -value
Intercept	-0.998 [-5.701–3.512]	0.641
Substrate dryness category	1.322 [0.384–2.345]	0.002
Annual rainfall (inverted)	1.250 [0.477–2.082]	0.002
Substrate \times Rainfall	-0.784 [-1.978–0.232]	0.134

impact significantly anhydrobiotic performances (Czernekova & Jönsson, 2016). In this study we opted for using laboratory populations to take advantage of uniform culturing conditions (known as a ‘common garden experimental design’), which helps to minimise the effects of phenotypic plasticity and expose any genetic background-based differences. It could be argued, however, that using standardised culturing conditions for taxa sourced across different climatic areas, and thus different optima, could result in non-comparable energetic condition across the taxa used. As it is practically impossible to establish through multiple trials the best culturing conditions for each and every taxon, we limited our study to those species that did thrive in the conditions mentioned in the Methods section (i.e. our culturing conditions were close optimal for all).

TABLE 2 Phylogenetic variance decomposition analysis for the role of ecology and phylogeny in explaining the variation in anhydrobiosis recovery index (ARI).

Variance component	Posterior mean [95% HDI]	Bayesian p -value
(a) Ecology	0.012 [-0.016–0.045]	0.206
(b) Ecology + Phylogeny	0.185 [0.135–0.233]	<0.001
(c) Phylogeny	0.774 [0.734–0.818]	<0.001
(d) Residual (1 – (a + b + c))	0.029 [0.014–0.046]	<0.001

Abbreviation: HDI, high density interval.

4.2 | Phylogenetic signal in anhydrobiotic performance

Although trade-offs between tolerance to desiccation and other life history traits have been suggested (Alpert, 2006), they have never been directly tested. For example, trade-offs between desiccation tolerance and competitive ability in plants have been explained by niche partitioning across habitats with aridity gradients (Heilmeyer et al., 2005; Van Geest et al., 2005). In addition, trade-offs between fecundity and desiccation tolerance have been suggested based on empirical comparison of rotifer species (Ricci & Caprioli, 2005). In some tardigrade species, the time available

for the expression of anhydrobiotic-associated genes coding for some peculiar proteins (e.g. CAHS, SAHS, MAHS and LEAM) determines the ability to survive rapid and sudden desiccation (Arakawa, 2022). The constitutive expression of those abundant anhydrobiotic-related proteins most surely subtract resources that otherwise could be allocated to growth and reproduction, thus imposing a trade-off between anhydrobiotic resistance and competitiveness. Given such a strong premise for trade-offs and a clearly genetic base for anhydrobiotic capabilities, it would be expected to see a close matching between habitat desiccation and anhydrobiotic performances. We observed, however, a very large proportion of variance in *ARI* (77%) explained by the phylogenetic signal. A large phylogenetic component *c* is suggestive that phylogeny explains a substantial portion of the variation in *ARI*, which is independent of the analysed ecological variables. This could stem from a stochastic mechanism driving trait variation, such as the effects of past adaptations preserved by phylogenetic signal of other non-measured traits affecting tardigrade *ARI*. Potential pleiotropy of genes involved in anhydrobiosis could be constraining their evolutionary lability. For example, (Cesari et al., 2012) identified the *tps* gene, involved in the synthesis pathway of the sugar trehalose, a molecule known to be involved in desiccation resistance (Cornette & Kikawada, 2011; Jönsson & Persson, 2010; Watanabe, 2006; Wiemken, 1990), in the non-anhydrobiotic, freshwater tardigrade *Dactylobiotus parthenogeneticus* Bertolani, 1982. The authors therefore hypothesised that trehalose could be involved also in other biochemical processes other than anhydrobiosis. Data supporting this hypothesis for our dataset is still lacking, as we still do not have the genus or species-specific molecular characterisation to match this study's *ARI* diversity, despite already having some information of the molecular mechanisms (and their genetic basis) of anhydrobiosis in Macrobiotidae (Boothby et al., 2017; Giovannini, Boothby, et al., 2022; Giovannini, Corsetto, et al., 2022; Rebecchi et al., 2011; Rizzo et al., 2010; Savic et al., 2015). Moreover, the presence of eurytopic tardigrade species with high desiccation tolerance, such as some *P. aff. richtersi* spp., can be a confounding factor in our analysis.

Our results support the theoretically predicted association between tardigrade species anhydrobiotic performance and habitat dryness in which they were found (Jönsson, 2001) (Table 1; Figure S3). However, the shared evolutionary pattern between *ARI* and these environmental variables, renders establishing the causal direction between these variables impossible. Shared phylogenetic history accounts for a much larger proportion of variance in *ARI*. Methodological limitations could partially explain the latter. The substrate dryness scale attributed to each population is a micro-scale ecological proxy that assumes the sampled animals represent a thriving or a well-adapted population to those micro-climate conditions. Although the gold standard method to obtain this information would be through long-term monitoring of taxa persistence in the sampled habitat, more technically feasible proxies are possible. For instance, the density of both animal and eggs in a sample could be a good metric for assessing if

a population is thriving. In particular, the presence of hatchlings and eggs would be a good indicator that the population is actively reproducing and thus the abiotic and biotic conditions are optimal. This method, however, should account for seasonality in tardigrades populations too (see e.g. Guidetti et al., 1999; Kathman & Nelson, 1987; Morgan, 1977). Furthermore, a more quantitative measurement of the habitat dryness could be possible by implementing micro data-loggers (e.g. hygrometers in moss cushions by Jang & Viles, 2022). Also, moss substrates could be classified based on species-specific humidity preference databases (Morales-Sánchez et al., 2022).

4.3 | Interpopulation variability in anhydrobiotic performance

Intra-specific variability in anhydrobiotic performance has been rarely addressed in invertebrates. Notable exceptions are agriculturally relevant nematodes (e.g. see Grewal et al., 2002; Liu & Glazer, 2000) and one study on rotifers (Ricci & Caprioli, 2005). When dealing with microscopic invertebrates such as tardigrades, there are two main technical issues that must be taken into account when comparing anhydrobiotic performances between different populations. The first is the presence of cryptic species that would require genetic data to be taxonomically differentiated (Faurby et al., 2008). Given this premise, reports of intra-specific variability should be supported with reliable taxonomic identification (Gašiorek et al., 2021). The second issue regards potential confounds in the source of the observed trait variation. For instance, energetic conditions and body size affect the anhydrobiotic survival probabilities (Czernekova & Jönsson, 2016; Jönsson & Rebecchi, 2002). Therefore, experimental work on interpopulation variability in anhydrobiotic performance should account at least for the energetic and developmental status of the animals. However, the latter is largely impossible to standardise when individuals are extracted directly from the wild (e.g. see Roszkowska et al., 2022) and due to technical difficulties in maintaining tardigrades in culture (Altiero & Rebecchi, 2001; Roszkowska, Gołdyn, et al., 2021). Interestingly, even when nutritional state and age and sex structure are buffered by equivalent culturing methods and history, we still detected a considerable variation in interpopulation variability: from hardly any (e.g. *M. ripperi*, *M. cf. sapiens*) to clear differences (e.g. *ARI* range = 0.53–0.89 in *P. aff. richtersi* or *ARI* range = 0.42–0.76 in *P. cf. klymenki*). Assuming that the culturing reduced the impact of potential nutritional and age structure confounders, the observed variation could be explained by genetic differences. Given the possible multi-layered causes of intra-specific differences in anhydrobiotic recovery, future research on the (molecular) mechanisms behind desiccation tolerance could benefit from comparing populations of the same species that differ in *ARI*. Furthermore, the methodology behind the *ARI* estimation can be used to assess other aspects of cryptobiosis competence that use time-point monitoring of events, such as longevity and reproductive success.

5 | CONCLUSIONS

We provide a new standardised metric (ARI) that quantifies desiccation tolerance by combining both overall survival and recovery speed. Our analyses show that desiccation tolerance is highly variable even among closely related species and that its variability is mostly explained by the shared phylogenetic history, rather than adaptation to local ecological conditions. Moreover, there were often, but not always, considerable intra-specific differences. We showed that studies on fine-scale variation in desiccation tolerance in tardigrades can shed light on the evolutionary and ecological forces behind the evolution of this unique trait, as well as, on the physiological mechanisms behind this extraordinary adaptation.

AUTHOR CONTRIBUTIONS

M. Vecchi and S. Calhim conceived the ideas and designed the methodology; M. Vecchi, D. Stec, L. Rebecchi, Ł. Michalczyk and S. Calhim provided resources; M. Vecchi and S. Calhim collected the data; M. Vecchi and S. Calhim analysed the data; M. Vecchi led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

Data and analysis R script are freely available from figshare (Vecchi et al., 2023), and as supplementary material (Data S1).

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SUPPORTING INFORMATION

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

Table S1: Data on the populations used.

Table S2: Substrate dryness category definitions.

Table S3: GenBank accession numbers of the sequences used to reconstruct the phylogeny. Taxa marked with * were pruned from the tree after the phylogenetic reconstruction.

Table S4: Summary of *ARI* and cumulative Weibull fitting posterior estimates for the tested populations.

Data S1: R script, data and model results in tabular format.

Figure S1: Plot showing average p and Q estimates along with the 95% density hull of posterior predictions. Hull colours represent average *ARI*.

Figure S2: Diagnostic plots for the three GLMMs. First row: congruence between predicted and observed *ARI* for each model. Black dots indicate observed *ARI*, whereas violins indicate models' posterior predictions. Violin colour indicates average difference between the observed data points and the expected values; Second row: binned residual plots; Third row: posterior predictive plots.

Figure S3: Plot showing the relationship between substrate category, Yearly rainfall and *ARI*. Grey dashed lines represent the average of the variables observed in our dataset. A small amount of random noise was added on the x-axis (substrate category) to remove overlap between points and improve their visibility.

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