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Phylogenetic signal in amphibian sensitivity to copper sulfate relative to experimental temperature

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Abstract. The release of large quantities of chemicals into the environment represents a major source of environmental disturbance. In recent years, the focus of ecotoxicology has shifted from describing the effects of chemical contaminants on individual species to developing more integrated approaches for predicting and evaluating long term effects of chemicals across species and ecosystems. Traditional ecotoxicology is typically based on data of sensitivity to a contaminant of a few surrogate species and often considers little variability in chemical sensitivity within and among taxonomic groups. This approach assumes that evolutionary history and phylogenetic relatedness among species have little or no impact on species' sensitivity to chemical compounds. Few studies have tested this assumption. Using phylogenetic comparative methods and published data for amphibians, we show that sensitivity to copper sulfate, a commonly used pesticide, exhibits a strong phylogenetic signal when controlling for experimental temperature. Our results indicate that evolutionary history needs to be accounted for to make accurate predictions of amphibian sensitivity to this contaminant under different temperature scenarios. Since physiological and metabolic traits showing high phylogenetic signal likely underlie variation in species sensitivity to chemical stressors, future studies should evaluate and predict species vulnerability to pollutants using evolutionarily informed approaches.

Key words: *Anura; ecotoxicology; evolutionary biology; heavy metal contamination; LC50; phylogeny; temperature.*

Introduction

Pollutants are one of the major causes of the current biodiversity loss (Novacek and Cleland 2001). Chemical contaminants pose a high risk to biodiversity in nearly 80% of inhabited areas (Vörösmarty et al. 2010), and pollution is among the main causes of environmental stress (e.g., Gibbs et al. 2009). Among pollutants, some pesticides threaten wildlife by causing increased mortality, population declines, and by changing communities and altering ecosystem function (reviewed in Köhler and Triebskorn 2013).

A repeatable quantitative measure of the relationship between exposure and toxicity is essential to evaluate the effects of chemicals on organisms. This relationship is normally characterized by two variables: the dose or concentration of the substance that the organism is exposed to, and the response, which may be mortality, growth, reproductive performance, or other phenotypic measures (Wright and Welbourn 2002). Experimentally obtaining the response to contaminant exposure can be time consuming, costly, and difficult to carry out on non-model species (e.g., species with long generation times or producing few offspring), especially if the species is endangered or of conservation interest. Traditional ecotoxicology and risk assessment have therefore mainly focused on a handful of model species that can be easily studied under laboratory conditions. The subsequent toxicity results from these surrogate species are then often extrapolated to larger taxonomic groups (e.g., invertebrates, fish, and amphibians) based on very few data points (e.g., EFSA 2013:169). It is therefore important to understand the degree to which the sensitivity to a contaminant is conserved between species at different taxonomic levels. Such an understanding could aid in the development of predictive tools to estimate the relationship between exposure and response for a large number of species without the need to test each one experimentally.

Some efforts to extrapolate chemical sensitivity among species have already been made using species sensitivity distributions (SSDs; e.g., Awkerman et al. 2008, Raimondo et al. 2013) and interspecies correlation estimation (ICE; Raimondo et al. 2010). ICE relies on

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pairwise comparisons of toxicity data for multiple chemicals (Raimondo et al. 2010), and the results of this approach indicate that toxicity predictions are more accurate for more closely related species (Raimondo et al. 2010). Predictions obtained with such approaches, however, are based on comparisons of species within broad taxonomic relationships (e.g., genus, family), and do not account for phylogenetic relatedness among species, which more accurately reflects the evolutionary history of species and the processes which have shaped their biology. Specifically, unlike phylogeny, taxonomy may arbitrarily define the nested ranks to which a species or a group of species belongs to and considers, incorrectly, all species within such ranks to be equally related to each other (Harvey and Pagel 1991). Furthermore, shared evolutionary history between species leads to phenotypic similarity in proportion to their degree of relatedness, so that closely related species resemble each other phenotypically more than expected by chance ("phylogenetic signal"; Harvey and Pagel 1991, Blomberg and Garland 2002, Freckleton et al. 2002). It is well established that ignoring phylogenetic signal in comparative data often leads to erroneous conclusions when employing standard statistical methods (such as correlation estimations) or taxonomically informed methods (Harvey and Pagel 1991). Thus, phylogenetic comparative approaches that explicitly estimate the strength of phylogenetic signal in the data should instead be used (e.g., Harvey and Pagel 1991, Freckleton et al. 2002, Capellini et al. 2010).

The importance of integrating concepts and knowledge from evolutionary biology into ecological risk assessment to uncover inter- and intraspecific patterns and mechanisms of tolerance and instances of local adaptation to pollutants has recently been recognized (Coutellec and Barata 2011, Dallinger and Höckner 2013), but is still seldom applied, especially in vertebrate ecotoxicology. In this context, phylogenetic relationships among species provide more reliable indications of species or family sensitivity to pollutants. Previous studies have found that the phylogenetic signal of body mass, life history traits, and physiological and morphological traits is very high (Blomberg and Garland 2002, Freckleton et al. 2002, Kamilar and Cooper 2013). Because the ability to eliminate or deactivate a toxic compound is controlled by physiological processes and pathways, it is likely that a strong phylogentic signal is also present in species sensitivity to pollutants.

Sensitivity and bioaccumulation of cadmium appear to be more similar among phylogenetically closer (as compared to more distantly related) aquatic insects (Buchwalter et al. 2008, Poteat and Buchwalter 2013), although the strength of the phylogenetic signal is not always quantified in these studies. Similar results are obtained for sensitivity in fish and aquatic invertebrates to four different pesticides (Guénard et al. 2011). Finally, phylogenetic signal in tolerance to zinc in sediment is shown to be variable across a phylogeny of non-biting midges (Chrironmidae), and independent of phylogenetic relationships above the genus level (Carew et al. 2011). By contrast, in vertebrates other than fish, the analysis of phylogenetic patterns in species sensitivity to pesticides has been very limited. Sensitivity and mortality effects in response to endosulfan, a common insecticide, vary among three anuran families, showing some, but not high, levels of phylogenetic signal (Hammond et al. 2012).

Amphibians (see Plate 1) are declining worldwide and pesticides have been indicated as one of the major contributing causes of this decline (e.g., Boone and Bridges 2003, Davidson 2004, see also Kerby et al. 2009). Thus, amphibians are an important group for investigating the effects of pollutants on biodiversity and for studying patterns of sensitivity to environmental contaminants across species. However, comparative data on pesticide impacts on a diversity of amphibian species are still scarce. Therefore, studying existing ecotoxicological data for different species of amphibians and contaminants in a phylogenetic framework may help identify key mechanisms of response to pollutants among species. In this study, we used published comparative data and phylogenetic comparative methods to quantify the strength of the phylogenetic signal in sensitivity to copper sulfate in eleven amphibian species, while taking into account the influence of experimental temperature on toxicity measures. Understanding the influence of temperature on pesticide sensitivity in amphibians is also of special concern, as climatic projections estimate a global increase in environmental temperature, which may eventually interact with amphibian physiological processes for responding to contaminants, for example by accelerating their metabolism.

MATERIALS AND METHODS

Data on sensitivity to copper sulfate, measured as LC50 (median lethal concentration, 50%), in amphibians were extracted from published reviews (Fryday and Thompson 2012), the U.S. EPA ECOTOX database (last accessed on 7 September 2013; U.S. EPA 2013), and a literature search using "LC50," "copper sulfate," and "amphibians" together as keywords (last search 7 September 2013). Data from ECOTOX and Fryday and Thompson (2012) were double-checked against the original papers (Table 1). LC50 corresponds to the estimated concentration of a test substance at which half of the test population dies over a specific timeframe (Wright and Welbourn 2002) and represents one reliable and objective parameter commonly used as a measure of sensitivity of a species to a contaminant. Higher LC50 values indicate lower species sensitivity and vice versa.

The LC50 is dependent on both the concentration of the chemical used and on the duration of exposure. However, it may also be influenced by other factors such as the specific experimental conditions (e.g., temperature, salinity, hardness), the studied species, and the

TABLE 1. Data set used in this study.

Family and species	T (°C)	LC50 (mg/L)	Reference
Bufonidae			
Bufo boreas			
Data point 1	22	0.12	Dwyer et al. (1999)
Data point 2	22	0.12	Dwyer et al. (2005)
Mean/geometric mean	22	0.12	
Bufo bufo			
Data point 1	20 ± 0.5	0.08	García-Muñoz et al. (2010)
Data point 2	20 ± 0.5	0.09	García-Muñoz et al. (2010)
Mean/geometric mean	20	0.085	
Bufo calamita			
Data point 1	20 ± 0.5	0.08	García-Muñoz et al. (2010)
Data point 2	20 ± 0.5	0.1	García-Muñoz et al. (2010)
Mean/geometric mean	20	0.089	
Bufo melanostictus			
Data point 1	31.5	0.32	Khangarot and Ray (1987)
Mean/geometric mean	···	···	
Bufo viridis			
Data point 1	20.55	0.1	Gürkan and Hayretdağ (2012)
Mean/geometric mean	···	···	
Alytidae			
Discoglossus jeanneae			
Data point 1	20 ± 0.5	0.08	García-Muñoz et al. (2010)
Data point 2	20 ± 0.5	0.1	García-Muñoz et al. (2010)
Mean/geometric mean	20	0.089	
Ranidae			
Euphlyctis hexadactylus			
Data point 1	15	0.039	Khangarot et al. (1985)
Mean/geometric mean	···	···	
Hoplobatrachus tigerinus			
Data point 1	26.5	0.389	Khangarot et al. (1981)
Mean/geometric mean	···	···	
Rana perezi			
Data point 1	20 ± 0.5	0.36	García-Muñoz et al. (2010)
Data point 2	20 ± 0.5	0.57	García-Muñoz et al. (2010)
Mean/geometric mean	20	0.453	
Rana sphenocephala			
Data point 1	22	0.23	Bridges et al. (2002)
Mean/geometric mean	···	···	
Pelobatidae			
Pelobates cultripes			
Data point 1	20 ± 0.5	0.22	García-Muñoz et al. (2010)
Data point 2	20 ± 0.5	0.22	García-Muñoz et al. (2010)
Mean/geometric mean	20	0.22	

Notes: "Family" and "species" indicate the species considered in this study (nomenclature according to Pyron and Wiens 2013) and the families to which they belong. Temperature (T) at which the study was carried out and LC50 (median lethal concentration, 50%) values for copper sulfate are reported as in the source study, and as mean and geometric mean values, respectively, when more than one data point for these parameters was available. Ellipses indicate where a second set of data points was not available and the mean for the T and the geometric mean for the LC50 could not be calculated.

developmental stage of individuals tested. The species-specific LC50 values measured under the same experimental conditions indicate how sensitive different species may be to a contaminant. To ensure that the data set contained comparable data for different amphibian species tested with copper sulfate, we only used LC50 values from studies carried out under the same experimental conditions: static conditions without test medium renewal (i.e., the solution containing the contaminant is not renewed during the test), technical test material (i.e., does not contain other chemical active ingredients or inert compounds), tadpole (or larval) stage, 96-hour exposure, and observation duration. The only factors that differed across studies in our sample were species, experimental temperature, and LC50. Variation in experimental temperatures across studies was taken into account and statistically controlled for since effects of temperature on modifying the toxicity of compounds cannot be generalized easily (see *Results and discussion* section below). The final data set contained comparable data for 11 species belonging to four amphibian families (Table 1). We calculated the mean and geometric mean values of temperature and LC50, respectively, for species for which more than one data point was available. For LC50, we used the geometric mean instead of the mean to prevent bias against very large numbers that may be present in LC50 measurements.

We tested a phylogenetic generalized least squares model (PGLS; Pagel 1997, 1999, Freckleton et al. 2002) with LC50 as response variable and temperature as predictor, using the package "caper" (Orme 2012) in R (R version 3.0.1; R Development Core Team 2013) and the time-calibrated amphibian phylogenetic tree by Pyron and Wiens (2013). In PGLS, the phylogeny is represented by a variance–covariance matrix of species' degree of relatedness in which the diagonal elements of the matrix (the "variance") are the path lengths from the root to the tips of the tree for each species, while the off-diagonal values (the "covariances") are the shared path length from the root to the last common ancestor for each pair of species (Rohlf 2001, Freckleton et al. 2002, Orme 2012). The strength of the phylogenetic signal in the model is quantified through the parameter lambda (λ) that can vary between 0 (no phylogenetic signal; the species can be considered independent data points) and 1 (the pattern of similarity between species is proportional to the time of common evolution under a Brownian motion model of evolution; Pagel 1997, 1999, Freckleton et al. 2002). Therefore, a model with λ equal to 0 is equivalent to a model without phylogeny being accounted for. Estimating the phylogenetic signal of LC50 alone would not be informative given the strong confounding effects of temperature on sensitivity (see *Results and discussion*); therefore, we estimated the strength of the phylogenetic signal in the "relative sensitivity" to copper sulfate, i.e., while statistically controlling for experimental temperature. In PGLS regression models, the strength of the phylogenetic signal is estimated on the model residuals (Pagel 1997, 1999, Freckleton et al. 2002, Revell 2010). All model parameters in PGLS including λ were estimated with maximum likelihood (ML). In order to highlight differences between accounting for the effect of phylogeny in the LC50 data or not, we compared a linear model with λ estimated (λ_{ML}) to a model in which the phylogenetic signal in the data is ignored by forcing λ to be equal to 0 (λ_0). Alternative nested models such as these can be compared using a likelihood ratio test (LRT) against a chi-square distribution with 1 df (Rohlf 2001, Freckleton et al. 2002, Capellini et al. 2010, Orme 2012).

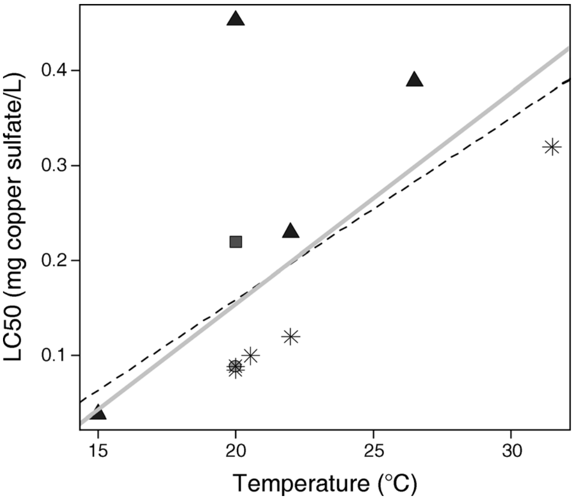

FIG. 1. Fit lines of the linear model of LC50 (median lethal concentration, 50%) values for copper sulfate on temperature. The dashed line indicates the linear model without phylogeny, while gray solid line refers to the linear model with the phylogenetic signal in the data accounted for (see *Materials and methods* for detailed explanations). Different symbols refer to different families: triangles represent ranids, stars represent bufonids, the square represents a pelobatid, and the circle represents an alytid.

RESULTS AND DISCUSSION

Higher temperature is associated with decreased sensitivity to copper sulfate (higher LC50 values; Fig. 1). When phylogenetic relatedness is taken into account and the strength of the phylogenetic signal in the data is estimated, temperature accounts for 68% of the variance in LC50 (model log-likelihood = 10.1, $t_2 = 4.39$, $P = 0.002$) and estimated $\lambda_{ML} = 1$, indicating that there is a strong phylogenetic signal in relative sensitivity to copper sulfate. Diagnostic plots indicate that all the assumptions of linear models with λ_{ML} (estimated) or forced to be equal to 0 are met. A PGLS model with λ fixed to 0 (phylogenetic relationships not taken into account) indicates that only a 32% of the variance in LC50 can be explained by temperature, and does not provide a statistically better fit to the data than the model with λ_{ML} (model log-likelihood = 8.7, $t_2 = 2.07$, $P = 0.068$; LRT_1, model $\lambda_{ML}/\lambda_0 = 2.9$, $P = 0.090$). Through simulations and analyses of empirical data, Freckleton et al. (2002) show that, for small sample sizes ($n < 20$ species), the estimated ML value of λ (λ_{ML}) may not be significantly different from 0 (or 1), even when λ_{ML} is close to 1 (or 0, i.e., its maximum or minimum possible value) because of the wide confidence intervals around λ_{ML}; however, incorrect inference for the strength of the phylogenetic signal is rare. The diagnostic plot of the log-likelihood surface for the estimation of λ shows that the log-likelihood surface increases rapidly with increas-

PLATE 1. *Discoglossus jeanneae*, one of the amphibian species in our study. Photo credit: M. A. Carretero.

ing λ values toward 1 in our analysis, suggesting that the phylogenetic signal in relative LC50 is indeed high in our sample, despite the low power due to the sample size ($n = 11$) when testing the difference against a model in which λ is forced to be equal to 0. We conclude that our results support the presence of a strong phylogenetic signal ($\lambda_{ML} = 1$) in relative LC50 after accounting for temperature, and indicate that when phylogenetic relatedness is not taken into account, the influence of temperature on sensitivity is underestimated. Furthermore, our analyses suggest that sensitivity to copper sulfate decreases when temperature increases and that this is particularly evident for bufonid frogs (Fig. 1).

Our results are in disagreement with previous studies showing that bioavailability and toxicity of contaminants on wildlife will generally increase with increasing temperatures (Noyes et al. 2009 and references therein). Studies on one or a few amphibian species show a contrasting pattern: With increasing temperature, toxicity is higher for endosulfan, carbaryl, and pyrethroid insecticides, but lower for atrazine (Materna et al. 1995, Boone and Bridges 1999, Broomhall 2002, 2004, Rohr et al. 2011).

Copper bioavailability, and therefore toxicity, is affected by changes in physicochemical characteristics of the water, such as temperature (reviewed in U.S. EPA 2007). Studies on the effects of higher temperature on copper toxicity indicate contrasting results in some species of mussels, crustaceans, and fish (e.g., Richards and Beitinger 1995, Rao and Khan 2000, Boeckman and Bidwell 2006, Carvalho and Fernandes 2006, Khan et al. 2006, Hansen et al. 2009). Our results indicate that, overall, an increase in temperature is associated with lower sensitivity to copper sulfate across a sample of 11 amphibian species.

Copper sulfate is one of the few compounds for which comparable toxicity data from multiple amphibian species are available and thus allows quantification of the possible presence of a phylogenetic signal in species sensitivity. In most cases, amphibian toxicity data for industrial chemicals and pesticides are limited to a small number of species, particularly *Xenopus*, which is only distantly related to native amphibian taxa in many countries where chemicals are used. Our study shows that relative sensitivity to copper sulfate, after accounting for experimental temperature, exhibits a strong phylogenetic signal and that diversity in sensitivity among families may exist. Therefore, the applicability of *Xenopus* as a model for amphibian sensitivity to a compound might lead to highly inaccurate estimates for the whole lineage and effort should instead be focused on quantifying sensitivity for a more diverse sample of species from across the whole amphibian radiation. Once more data become available, phylogenetic comparative approaches utilizing PGLS models in a Bayesian framework can be used to derive phylogenetically informed predictions (see Organ et al. 2007) of the sensitivity to pollutants.

Our approach highlights the importance of adopting an evolutionary framework and using phylogenetic comparative methods to gain key insights in ecotoxicology, such as identifying patterns of sensitivity to a common pesticide within a clade and quantifying the influence of environmental factors, like temperature, on interspecific sensitivity. This approach can be particularly helpful not only to investigate interspecific responses to other important pollutants, but especially to derive more accurate predictions of sensitivity to distinct pesticides in endangered species and/or species that are difficult to sample or raise under laboratory conditions. Furthermore, once more data are available for a larger sample of species, phylogenetic comparative methods can be used to pinpoint the underlying physiological causes for differences in sensitivity among species and families to the same contaminant, discover why these vary across life stages and environmental conditions, and uncover what makes some contaminants more toxic than others.

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