

Aquatic insect ecophysiological traits reveal phylogenetically based differences in dissolved cadmium susceptibility

David B. Buchwalter^{*†}, Daniel J. Cain[‡], Caitrin A. Martin^{*}, Lingtian Xie^{*}, Samuel N. Luoma[‡], and Theodore Garland, Jr.[§]

^{*}Department of Environmental and Molecular Toxicology, Campus Box 7633, North Carolina State University, Raleigh, NC 27604; [†]Water Resources Division, U.S. Geological Survey, 345 Middlefield Road, MS 465, Menlo Park, CA 94025; and [§]Department of Biology, University of California, Riverside, CA 92521

Edited by George N. Somero, Stanford University, Pacific Grove, CA, and approved April 28, 2008 (received for review February 20, 2008)

We used a phylogenetically based comparative approach to evaluate the potential for physiological studies to reveal patterns of diversity in traits related to susceptibility to an environmental stressor, the trace metal cadmium (Cd). Physiological traits related to Cd bioaccumulation, compartmentalization, and ultimately susceptibility were measured in 21 aquatic insect species representing the orders Ephemeroptera, Plecoptera, and Trichoptera. We mapped these experimentally derived physiological traits onto a phylogeny and quantified the tendency for related species to be similar (phylogenetic signal). All traits related to Cd bioaccumulation and susceptibility exhibited statistically significant phylogenetic signal, although the signal strength varied among traits. Conventional and phylogenetically based regression models were compared, revealing great variability within orders but consistent, strong differences among insect families. Uptake and elimination rate constants were positively correlated among species, but only when effects of body size and phylogeny were incorporated in the analysis. Together, uptake and elimination rates predicted dramatic Cd bioaccumulation differences among species that agreed with field-based measurements. We discovered a potential tradeoff between the ability to eliminate Cd and the ability to detoxify it across species, particularly mayflies. The best-fit regression models were driven by phylogenetic parameters (especially differences among families) rather than functional traits, suggesting that it may eventually be possible to predict a taxon's physiological performance based on its phylogenetic position, provided adequate physiological information is available for close relatives. There appears to be great potential for evolutionary physiological approaches to augment our understanding of insect responses to environmental stressors in nature.

comparative methods | evolutionary physiology | bioaccumulation | phylogeny | tradeoff

With $\approx 6,500$ species described to date in North America (1), aquatic insects are a diverse and ecologically important group (2), particularly in rivers and streams. For example, the orders Ephemeroptera, Plecoptera, and Trichoptera (EPT taxa) include 58 recognized families and $\approx 2,700$ species (1). Among these many lineages, great diversity exists in morphology, life history characteristics, and physiology stemming from a long and complex evolutionary history. Although the origins of the Ephemeroptera are unknown (3), a general paradigm of the terrestrial ancestry of aquatic insects is widely accepted, with numerous invasions of freshwater habitats hypothesized throughout evolutionary history (4). Many of these invasions have entailed adaptive “solutions” that involve complex suites of traits that in combination determine the range of environmental conditions that a given taxon can tolerate.

Some traits that arose in response to past environmental challenges may now render certain species relatively more susceptible to modern anthropogenic pollutants. These pollutants may be either entirely novel (e.g., organophosphate insecticides) (see ref. 5) or were historically present at much lower concentrations in natural environments than they are in many

ecosystems today (e.g., trace metals) (6). This variation in susceptibility has practical implications, because the ecological structure of aquatic insect communities is often used to indicate the ecological conditions in freshwater systems (7–9). Differences among species' responses to environmental stressors can be profound, but it is uncertain whether the cause is related to functional ecology [usually the assumption (10, 11)] or physiological traits (5, 12–14), which have received considerably less attention. To the degree that either is involved, their link to phylogeny and evolutionary history remains poorly understood.

Here, we ask whether the tendency for related species to be similar (i.e., phylogenetic signal) (15, 16) extends to physiological traits that contribute to sensitivity to the stressor: cadmium. Generalizations about phylogenetic linkages to stress responses have been hindered to date by the limited number of species that can be studied. We overcome this hurdle by using highly developed methodologies for efficiently quantifying critical processes that control sensitivity to dissolved cadmium (see refs. 13, 17, and 18) (Fig. 1). These physiological processes have previously been considered in a conceptual model of metal toxicity (19) that explicitly assumes that chronic toxicity in nature is the manifestation of metal accumulation at target sites (i.e., metal-sensitive sites) that ensues when the rate of metal influx exceeds the combined rates of metal excretion and detoxification. In practice, the model can be tested by combining bioaccumulation kinetics (see ref. 17) and subcellular fractionation (e.g., refs. 18 and 20). We have used this integrated approach to reconcile apparent discrepancies between insect responses to trace metals in toxicity assays and in nature (13), to infer Cd sensitivity differences among predaceous stoneflies (14), and to understand the mechanisms underlying the metal tolerance of a caddis fly (21).

We used phylogenetic analyses (22–30) to explore physiological processes related to dissolved Cd susceptibility in 21 field-collected aquatic insect species representing eight EPT families [supporting information (SI) Table S1]. We tested for correlations and possible trade-offs among traits and used traits in combination to predict the more emergent property (sensitivity) in each of these species. We asked whether phylogenetic approaches (15, 16, 22–30) are potentially more powerful than traditional functional guild approaches (e.g., ref. 31) for predicting sensitivity differences among species. We compared statistical models that include body weight, feeding strategy, and lineage as independent variables by using both conventional and

Author contributions: D.B.B. and S.N.L. designed research; D.B.B., D.J.C., C.A.M., and L.X. performed research; D.B.B. and T.G. analyzed data; and D.B.B. and T.G. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[†]To whom correspondence should be addressed. E-mail: david.buchwalter@ncsu.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0801686105/DCSupplemental.

© 2008 by The National Academy of Sciences of the USA

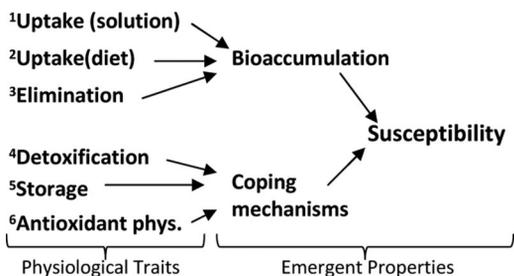


Fig. 1. Conceptual model of metal toxicity, adapted in part from ref. 19. Here we consider uptake (solution), elimination, and detoxification in the prediction of dissolved Cd susceptibility among species.

phylogenetically based statistical tools. Finally, we determined how phylogenetically based approaches can increase our understanding and prediction of interspecific susceptibility differences and augment the interpretive power of conventional stream assessments that use insects as ecological indicators.

Results

Cd Uptake from Solution (k_u). Under identical water chemistry conditions, cadmium uptake rate constants (k_u) varied 65-fold among the 21 species we studied (Fig. 2A). We used Blomberg *et al.*'s (15) K statistic to quantify the tendency for related species to be similar to each other (phylogenetic signal) in k_u measurements. In general, the level of phylogenetic signal present in our Cd uptake rate constants ($K = 0.488$; $P = 0.031$) was typical for physiological traits quantified in other studies. Blomberg *et al.* report that the average K value taken from 21 comparative physiology data sets was 0.53 (95% confidence interval 0.40–0.72). Because many physiological traits are expected to scale allometrically, we also determined K after removing the influence of log body weight ($K = 0.482$; $P = 0.041$).

To explore the role of phylogeny in explaining Cd uptake rate differences among species, we compared 18 regression models

that included combinations of body weight, clade (families or orders), and feeding type (Table S2). The best-fit regression model for log k_u measurements based on the Akaike Information Criterion (AIC) included log body weight (not significant) and family categories ($P = 0.012$) on a star phylogeny, thus demonstrating a significant phylogenetic component to dissolved Cd uptake. (Note: A star phylogeny assumes all taxa are equally related as in traditional statistical methods, whereas a tree includes hierarchical structure.) This pattern indicates that the phylogenetic component of variation for uptake is associated primarily with differences among families, the lowest phylogenetic level for which we have adequate sample size and meaningful hierarchical structure in our dataset. Body weight [which also exhibited a statistically significant phylogenetic signal for log values ($K = 0.532$; $P = 0.011$)] was negatively correlated with log k_u , but the statistical significance of family was not affected by the inclusion of log weight in regression models. The superiority of models including family as a factor over models including orders demonstrates that, within orders, there is considerable variation in uptake rate constants, which is apparent when the raw data are plotted onto the phylogeny of our tested species (Fig. 2).

Within orders, k_u values ranged 20-fold among 7 mayfly taxa, 25-fold among 10 stonefly taxa, and 38-fold among 4 caddis fly taxa. Among mayflies, ephemereids had consistently faster Cd uptake than both isonychiids and heptageniids, and species in any given family tended to be more similar to their family members than they were to species in other families. Among stoneflies, perlodids had uniformly slow Cd uptake relative to perlids, with the latter group exhibiting a 7-fold range in k_u values. Among caddis flies, *Rhyacophila* congeners were similarly slow in their Cd uptake. Interestingly, the two hydropsychids we examined were radically different with uptake in *Hydropsyche californica* ($0.42 \text{ liter} \cdot \text{g}^{-1} \cdot \text{d}^{-1}$) >10-fold faster than in *Cheumatopsyche* sp. ($0.04 \text{ liter} \cdot \text{g}^{-1} \cdot \text{d}^{-1}$). This pattern serves as a counterpoint to the general pattern for related species to be somewhat similar. Further examination of physiological variability is clearly warranted in this large family. We also explored

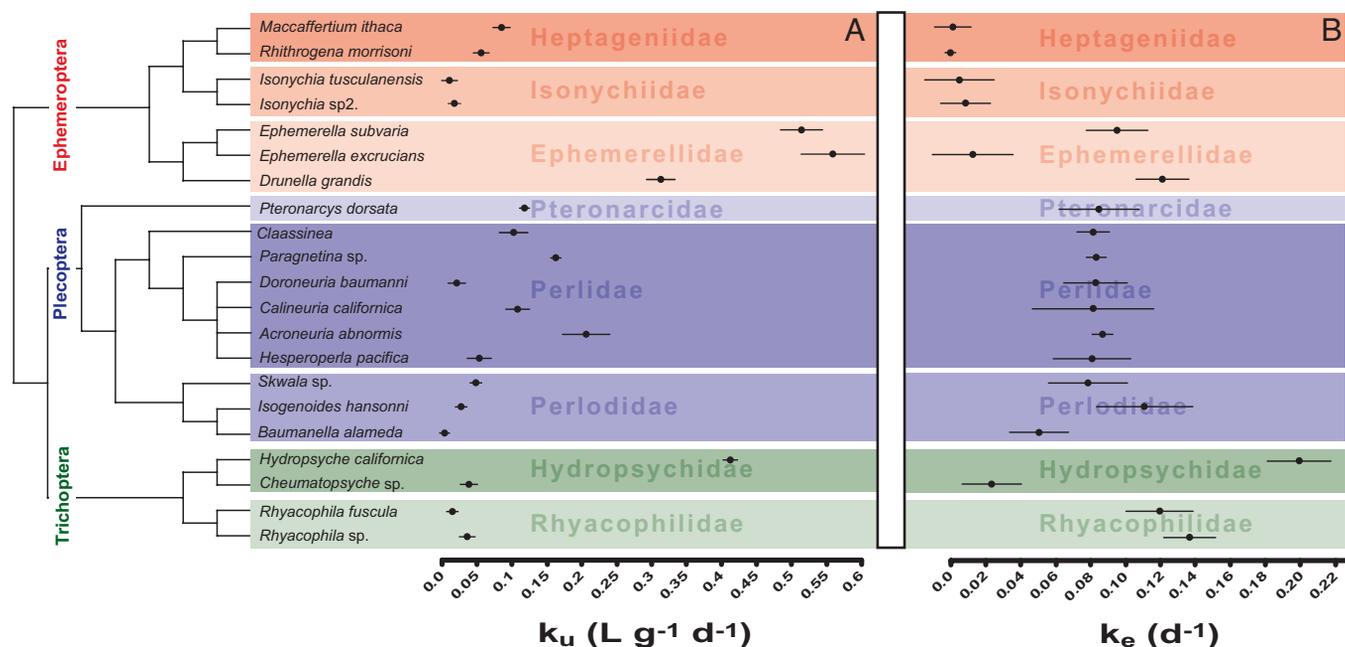


Fig. 2. Cadmium uptake rate constants ($k_u \pm \text{SE}$) (A) and elimination rate constants ($k_e \pm \text{SE}$) (B) derived from radiotracer experiments with 21 aquatic insect taxa representing the orders Ephemeroptera, Plecoptera, and Trichoptera. Pagel's arbitrary branch lengths (depicted) were used as input phylogenies in statistical analyses.

feeding in relation to Cd uptake, but this trait was not significant in any k_u models (Table S2).

Cadmium Elimination (k_e). In general, uptake rate constants alone were not necessarily strong predictors of bioaccumulation, because rates of elimination are very important in this process. Elimination rate constants (k_e) are perhaps the most important determinant of its overall bioaccumulation in nature, because they apply to both dissolved and dietary accumulation (17). The k_e values we present here represent a daily proportional loss of Cd from the body during the slow phase of efflux, which is thought to be more representative of loss rates under chronic exposure conditions found in nature (32). Efflux rate constants ranged ≈ 25 -fold among species in which we could measure loss (Fig. 2B). Log k_e estimates exhibited a much stronger phylogenetic signal ($K = 0.595$; $P = 0.013$) than did log k_u . This difference was accentuated by removing the influence of log body weight ($K = 0.753$; $P < 0.001$). Thus, relative to other physiological traits (15), Cd k_e values exhibit a very strong phylogenetic signal. Furthermore, k_e is strongly influenced by body size.

To explore the role of phylogeny in explaining Cd uptake rate differences among species, we compared regression models as described above (Table S3). The best-fit regression model based on AIC included log (body weight) ($P < 0.01$) and family categories ($P < 0.01$), demonstrating a significant phylogenetic component to Cd elimination. One model containing only feeding and weight in the absence of any phylogenetic information showed feeding to be statistically significant. However, feeding was not significant when family categories were included in the model, perhaps because feeding type tended to be uniform within families.

In our studies, taxa exhibiting the extremes of k_e estimates are instructive, because they represent some of the few groups for which generalizations of metal susceptibility in the field are available. For example, both heptageniid mayflies we examined did not exhibit any significant loss of Cd from their tissues over 10 days of efflux (Fig. 2B). Heptageniids have been described as particularly sensitive to trace metal exposures in the field, and are often extirpated from contaminated systems (33, 34). On the other hand, *H. californica* exhibited one of the highest efflux rates measured yet in any invertebrate (0.20) (21), and this genus is often described as being metal tolerant (33). Interestingly, the k_e value for *H. californica* is very close to that measured by Evans *et al.* (35) in *Hydropsyche bettini*, yet we also see “phylogenetic antisignal” (in the sense of ref. 15) when we compare *H. californica* to *Cheumatopsyche* sp., despite the fact that they are in the same family (Fig. 2B).

Subcellular Cd Compartmentalization. Because bioaccumulation alone is not necessarily a good predictor of sensitivity differences among species (Fig. 1; refs. 18, 19), it is important that bioaccumulation models are linked to other approaches to better understand the potential biological consequences of accumulated metals in a given species. The subcellular compartmentalization of accumulated metals gives some insight into the potential for toxicity (18), because species vary in their abilities to protect cells with metal-binding proteins such as metallothionein-like proteins (13), glutathione, and metal-rich granules (33). In our studies, the subcellular compartmentalization of Cd varied markedly among taxa (Table S4). We were particularly interested in Cd associated with microsomes, organelles, and heat-labile cytosolic proteins, because those compartments represent greater potential for Cd to interfere with vital physiological processes. Cd associated with the heat-stable protein (HSP) compartment has previously been assumed to be detoxified via metallothionein-like proteins (36, 37) and glutathione. Cd as-

sociated with cell debris was not considered to have toxicological significance to the larvae.

On average, there were no major differences among orders in terms of the proportion of Cd accumulated in potentially sensitive compartments (microsomes, organelles, and heat-labile cytosolic proteins). For all taxa tested, the average percentage (\pm SD) of accumulated Cd in these compartments was $36 \pm 14\%$. The perlid stonefly *Paragnetina* sp. had the smallest percentage (12%) of its body burden in these compartments, whereas the perlotid stonefly *Isogenoides hansonii* had the largest (64%). There was considerable variation within orders, families, and among genera, and no apparent trends in Cd compartmentalization into sensitive compartments was observed. Although there was an apparent difference in the proportion of Cd associated with the HSP compartment among orders (conventional one-way ANOVA: $F = 4.21$; $df = 2, 18$; $P = 0.032$) and families ($F = 3.03$; $df = 7, 13$; $P = 0.040$), these differences were not even close to significant in the equivalent phylogenetic analyses [phylogenetic generalized least-squares (PGLS): $F = 0.15$, $df = 2, 18$, $P = 0.860$; $F = 0.37$, $df = 7, 13$, $P = 0.901$, respectively]. Of possible biological significance is the observation that mayflies tended to accumulate higher percentages ($\approx 39\%$) of Cd in this compartment than did stoneflies ($\approx 18\%$) and caddis flies (17%). Phylogenetic signal in this trait ($K = 0.475$; $P = 0.057$) was typical for physiological traits (see above).

Correlations/Linkages Among Traits. The nonphylogenetic (star) correlation between cadmium uptake (log k_u) and elimination (log k_e) was 0.141 [ln maximum likelihood (ML) = -16.2238 ; $P = 0.5435$], and this correlation remained nonsignificant with the effects of log body weight controlled (ln ML for model = -14.1105 ; partial $r = 0.225$; $P = 0.3395$). However, the phylogenetic correlation (tree) between log k_u and log k_e was larger, 0.357 (ln ML = -13.6871 , $P = 0.1120$) and became statistically significant when the effects of log body weight were controlled (ln ML for model = -8.05635 ; partial $r = 0.505$; $P = 0.0232$). Comparison of the likelihoods indicates that the phylogenetic models better fit the data, and that the phylogenetic model including log body weight is the best, significantly better than the version without log body weight (ln likelihood ratio test, χ^2 with 1 $df = 11.26$; $P = 0.0008$). Therefore, size-independent log k_u and log k_e appear to have evolved together in a correlated fashion.

Among all species, elimination rate constants (k_e) and the proportion of Cd bound to the HSP (detoxified) compartment were negatively correlated regardless of whether k_e was log-transformed or phylogeny was considered. The best models based on AIC used a star phylogeny for log k_e ($r = -0.80$; $P < 0.0001$). This relationship was driven by the strong tendency within the Ephemeroptera ($r = -0.89$; $P < 0.01$), suggesting that mayfly taxa that do not efflux Cd effectively, instead allocate considerable resources toward detoxifying Cd (Fig. 3). No other relationships between biokinetic parameters and the proportional intracellular distribution of Cd were found.

Estimating Susceptibility Differences. As described (13), we predicted interspecific differences in dissolved Cd sensitivity by integrating kinetic parameters (annual Cd bioconcentration estimates) with estimates of detoxification capacity (the proportion of accumulated Cd that was measured in potentially sensitive subcellular fractions) (Fig. 4). This approach is aligned with a critical residue approach for regulating trace metal inputs to surface waters. These values for predicted metal concentrations at potentially sensitive sites ranged over three orders of magnitude among all taxa. This integrated estimate had a strong phylogenetic signal ($K = 0.718$; $P < 0.01$), which was even stronger after removing the influence of body weight ($K = 0.767$; $P < 0.01$).

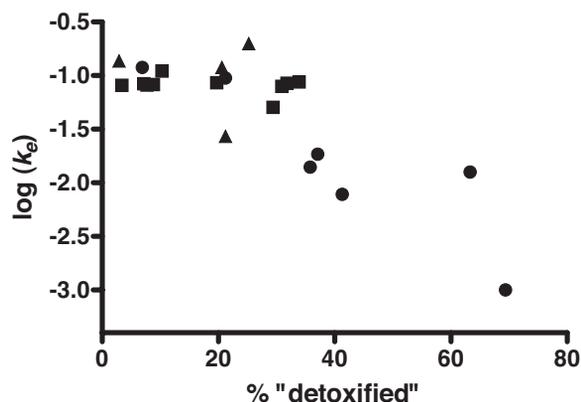


Fig. 3. Inverse relationship between the ability to eliminate Cd from tissues ($\log k_e$) and the association of Cd with HSPs. ●, Ephemeroptera; ■, Plecoptera; ▲, Trichoptera.

To explore the role of phylogeny in explaining susceptibility differences among species to dissolved Cd, we compared regression models as described above (Table S5). The best-fit regression model based on AIC included weight ($P = 0.017$) and family ($P < 0.001$), indicating a strong phylogenetic component to Cd susceptibility. In addition, it appears that within a given family, smaller-bodied species tend to be more sensitive to Cd than larger ones.

Discussion

Our primary purpose was to evaluate the potential for comparative studies to reveal patterns of diversity in traits related to susceptibility to a stressor. Our approach differs from traditional trait-based work (10, 11, 38) in insects in two major ways. First, we focus on physiological traits rather than ecological traits. We show that variability among taxa in susceptibility to a given level of stress, as defined by bioaccumulation into potentially toxic sites, can be strongly driven by phylogenetically linked physiological traits. Functional traits such as choice of food or seasonal aspects of development could result in different exposures in the

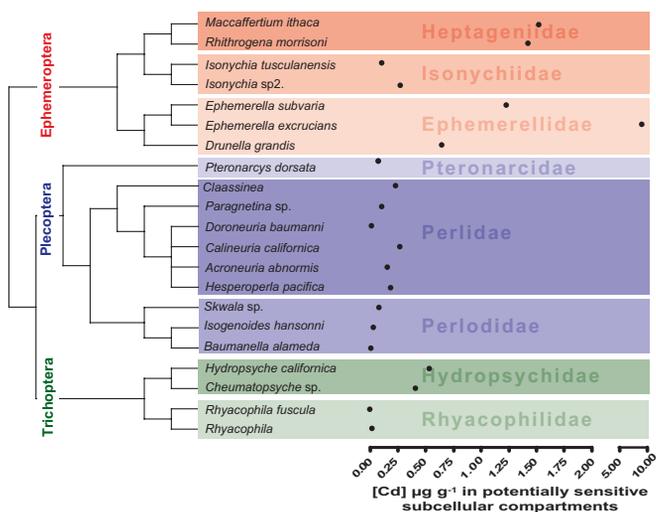


Fig. 4. Identification of Cd-susceptible taxa. Uptake and elimination rate constants were used to model Cd accumulation from exposure to dissolved Cd ($0.50 \mu\text{g}/\text{liter}$) for 1 year. The bioaccumulation estimates were modified to only consider the proportion of accumulated Cd associated with microsomes, organelles, and heat-labile cytosolic proteins measured in subcellular compartmentalization studies.

same habitat or the presence of sensitive life stages during periods of elevated metal exposure (spring run-off for example). However, here we show that the mechanistic linkage between physiology and a taxon's performance is powerful in the widely divergent aquatic insects we studied. Second, we adopt a phylogenetic perspective rather than a functional guild approach, because the tendency for related organisms to be similar to one another is pervasive (15, 16). Although we were specifically interested in testing whether this phylogenetic signal holds true for physiological traits related to Cd susceptibility, the approach may be applicable to susceptibility to other stressors as well.

Individual traits related to Cd bioaccumulation exhibited phylogenetic signal (elimination more so than uptake). This tendency for related animals to be physiologically similar has practical implications. If widespread, it may be used to overcome the inherent limitation in the number of species that can be studied in physiological experiments. It may eventually be possible to predict physiological performance in a taxon that has yet to be studied on the basis of its phylogenetic position, provided sufficient information is available for close relatives (e.g., see refs. 15 and 26). The current dataset is probably insufficient for making predictions beyond the family level (because of limited sample size and hierarchical structure within families). However, it is tempting to think that relatively fast uptake in ephemerellid mayflies or poor elimination in heptageniid mayflies could be hallmarks of those families. It is possible that more phylogenetically focused comparative studies, within a given family, for example, might allow for reasonable predictions for all members of that family (see ref. 26).

In combination, traits related to bioaccumulation and detoxification appeared to identify metal-sensitive species. Our studies identified the genus *Ephemerella* and two heptageniid mayflies as being particularly susceptible to dissolved Cd. These findings give mechanistic support to observations that these taxa are consistently absent from metal-contaminated habitats in nature (40) and are consistently more sensitive to metals in sophisticated toxicity tests (39–41). The presence of a relatively strong phylogenetic signal associated with the integrated trait of “sensitivity to dissolved Cd” suggests that sensitivity to metals may eventually be predictable on the basis of phylogenetic position, as described above. Given that this design was not a “common garden” experimental design (42), and species came from various locations in the United States, the degree of phylogenetic signal associated with these data is encouraging.

We found a positive correlation between the bioaccumulation parameters k_u and k_e , but only after controlling for the influence of body weight and considering phylogeny. We offer two potential explanations, not mutually exclusive (23), for why these two traits may have evolved in a correlated fashion. First, they may be positively genetically correlated, perhaps because some genes pleiotropically affect transporter densities in parallel in some tissues or organs. Quantitative-genetic analyses could test this hypothesis. Second, selection may have favored their correlated evolution. Studies of selection in laboratory mesocosms could test this idea. We also found a potential tradeoff between the ability to eliminate Cd and the ability to detoxify it and speculate that in insects an enhanced ability to eliminate may be a better strategy than expressing proteins or peptides for detoxification. The storage of metals in extracellular granules could be an efficient means of evading toxicity (43, 44), but we were unable to differentiate Cd associated with chitin with granules in our studies. Species that do well at eliminating, detoxifying, and/or storing metals, such as *H. californica*, are able to thrive in metal-rich environments (21).

The most reliable estimates of metal susceptibility still come from field-based observational approaches, where metals are usually present in mixtures, and dietary pathways of accumulation may in fact predominate in predatory insects (e.g., refs. 14,

45, and 46). In light of these complexities, it is somewhat surprising that our predictions of susceptibility to dissolved Cd so closely match field-based observations. We offer a few possible explanations for this. First, the efflux rate constant k_e applies to both dissolved and dietary exposure routes (14) and is arguably the most important determinant of bioaccumulation (17). Second, we have already established that uptake rates of Cd and zinc (Zn) covary strongly among species (12), so our rate constants of Cd uptake are likely also reasonable approximations for Zn. Third, recent work on mayflies (Family Heptageniidae and Ephemerellidae) using stable Cu and Cd isotopes as tracers suggest that transport rates of these elements covary (D.J.C., unpublished work). It will obviously be important to better understand the contributions of dietary metal bioaccumulation and improve on our ability to assess coping mechanisms and toxicity (Fig. 1).

Knowing which taxa are likely to be susceptible to specific anthropogenic disturbances would greatly enhance the interpretive power of biomonitoring and bioassessment programs, because it may allow species presence/absence or density to diagnose the causes of ecological impairment in aquatic systems. For many common environmental stressors, particularly those that directly affect insect physiological processes, such understanding appears possible through comparative physiological approaches (30, 47). Developing predictive capabilities based on the underlying physiological processes that determine species' responses to water quality would nicely complement other strategies that have traditionally focused on population and community-level responses.

Materials and Methods

Insect Collecting and Handling. All of the insect larvae used in these studies were field-collected from streams in Northern California, Colorado, Oregon, and North Carolina by using a D-frame kick net (Table S1). Species were selected based on phylogenetic considerations and local availability. Sites were chosen to minimize the potentially confounding influence of metal exposure history (21) (high-quality streams supporting a diversity of insects). Insects were held in an environmentally controlled room with a light/dark photoperiod of 16:8 h and a constant temperature of 15°C. Soft artificial river water (48 mg/liter NaHCO_3 , 30 mg/liter $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$, 30 mg/liter MgSO_4 , and 2 mg/liter KCl, adjusted to pH 6.85 by additions of 0.1 N NaOH) was used for acclimation (at least 4 days) to laboratory conditions and experiments. During acclimation, insects were fed a diet consisting of alfalfa, *Spirulina* aquarium flake food (O.S.I. Marine Lab), TetraMin fish food, or *Lumbriculus variegatus* ad libitum depending on the dietary requirements of each taxon. Insects were not fed for 1 day before experiments to reduce fecal material output and metal sorption onto fecal material during aqueous exposures. Only apparently healthy, intact, active animals were used in these experiments. A phylogeny of test species was compiled based on trees assembled on the Tree of Life web site (www.tolweb.org/tree). Physiological measures were mapped onto this phylogeny.

Physiological Experiments. The methods used in these physiological studies have been reported (13, 21). Larvae were exposed to an environmentally relevant concentration of Cd (≈ 4.6 nM) in artificial soft water. The gamma-emitting isotope ^{109}Cd was used to follow Cd accumulation and efflux kinetics *in vivo* by using a PerkinElmer Wallac Wizard 3-inch gamma counter. The accumulation phase of the experiment lasted 5 days for all taxa except for experiments with *Rhyacophila* sp. and *H. californica* (6 days). Approximately half of the larvae were used to conduct elimination experiments lasting 9–12 days. Briefly, insects were maintained in Cd-free water and assayed daily to monitor the loss of radioactivity from each larva. Efflux of Cd generally followed a biphasic pattern, and we focused on the slow phase of efflux as it more likely represents loss during long-term exposure scenarios (32, 48). Therefore, loss constants (k_e) were estimated by fitting efflux data after the first day of loss.

Subcellular Fractionation. After the accumulation phase of the experiment, approximately half of the larvae were assayed for ^{109}Cd and homogenized in

refrigerated, N_2 -saturated, 0.05 M Tris-HCl buffer (pH 7.4). The homogenate was subsequently separated by differential centrifugation and chemical and heat treatments into five operationally defined subcellular fractions following described procedures (20, 21, 33, 49). These fractions included cell debris, cellular organelles, microsomes, cytosolic proteins denatured by heat treatment (heat-denatured proteins, HDPs), and HSPs. In previous papers, we have referred to these cytosolic protein fractions as metallothionein-like proteins and nonmetallothionein-like proteins (13, 14, 21). Here, we have changed our nomenclature because our recent work has failed to demonstrate the presence of cysteine-rich, metallothionein-like proteins in the HSP fractions of various aquatic insects. Rather, it seems that this fraction is dominated by glutathione (L.X. and D.B.B., unpublished work). We maintain the convention of assuming that Cd in the HSP fraction represents detoxified Cd (36, 50). The heat-denatured fraction represents a variety of larger cytosolic proteins (20). Data from the subcellular fractions were summed into operationally defined metal-sensitive and detoxified compartments. The metal-sensitive compartment comprised the HDP, microsomal, and organellar fractions, each containing sites potentially vulnerable to Cd binding. The detoxified metal compartment was Cd associated with HSP. Cd in the cell debris fraction was interpreted as being associated with metal-rich granules or biologically inert tissue such as chiton and was not considered toxicologically important.

Statistics. For a univariate measure of phylogenetic signal, we computed the K statistic of Blomberg *et al.* (15) and used their randomization test for statistical significance, based on the mean-squared error (their Matlab PHYSIG.m program). We also applied these procedures after removing correlations with log body weight as described (15).

To elucidate whether phylogenetic signal was pervasive (distributed throughout the phylogenetic tree for the species under study) or occurred mainly in relation to disjunctions among orders or families, we compared regression models that included log body weight and either order or family (both coded as $K - 1$ dummy variables, where K is the number of orders or families) as independent variables. We included body weight because, on first principles, many physiological traits are expected to scale allometrically. For consistency among traits we retained body weight in models even when it was not statistically significant (i.e., $P > 0.05$). Alternative models were also computed for either a star phylogeny [no hierarchical structure, as is assumed by conventional statistical analyses (26, 48)] or the specified phylogenetic tree (PGLS), using Pagel's arbitrary branch lengths as implemented in the DOS PDTREE program (27). In addition, for the hierarchical phylogenies, we allowed branch lengths to vary between those indicated by the original input tree and a star phylogeny, in a way consistent with an Ornstein-Uhlenbeck (OU) model of (residual) trait evolution. Finally, we ran models that included feeding strategy as a quantitative trait, scored as 0 = nonpredator, 0.5 = intermediate, 1 = predator (see Table S1). All models were analyzed by using the Regressionv2.m Matlab program of Lavin *et al.* (30).

As an indicator of the relative support of models, we examined the AIC, using the smaller-is-better formulation [$\text{AIC} = (-2 \times \ln \text{ML}) + (2 \times \text{no. of parameters})$]. When comparing a series of models, the one with the lowest AIC is considered to provide the best fit to the data. As a rule of thumb, models whose AIC is ≤ 2 units larger can also be said to have substantial support (29, 30, 51). Note that MLs are used for computing the AIC, whereas restricted maximum likelihood (REML) is used for estimating coefficients in the model, such as the allometric scaling exponent and the OU transformation parameter, d .

To test for correlations between traits, we computed phylogenetically independent contrasts in the DOS PDTREE program (26, 27). To control for the effects of log body weight, we computed partial correlations (through the origin) with the contrasts using the REGRESSION procedure of SPSS version 11.5. We compared ln MLs (from Regressionv2.m) to determine which models (phylogenetic or not, with or without log body weight) best fit the data.

ACKNOWLEDGMENTS. We thank Phil Rainbow, Leah Bêche, and Gerald LeBlanc for commenting on early drafts of this manuscript; anonymous reviewers for improving the clarity of its presentation; Will Clements (Colorado State University, Fort Collins) for *C. sabulosa*; Dave Penrose (North Carolina State University) and Steve Fend (U.S. Geological Survey) for taxonomic support; and Ashley Dale and Jen Flippin for contributions to graphics. This work was supported by a National Research Council postdoctoral fellowship (to D.B.B.) and start-up funds provided by North Carolina State University (to D.B.B.). T.G. was supported by National Science Foundation Grant DEB-0416085.

1. Merritt RW, Cummins KW, Berg MB, eds (2008) *An Introduction to the Aquatic Insects of North America* (Kendall-Hunt, Dubuque, IA).
2. Wallace JB, Webster JR (1996) The role of macroinvertebrates in stream ecosystem function. *Annu Rev Ent* 41:115–139.

3. Barber-James HM, Gattolliat J-L, Sartori M, Hubbard MD (2008) Global diversity of mayflies (Ephemeroptera, Insecta) in freshwater. *Hydrobiologia* 595:339–350.
4. Kristensen NP (1981) Phylogeny of insect orders. *Annu Rev Entomol* 26:135–157.

5. Buchwalter DB, Jenkins JJ, Curtis LR (2002) Respiratory strategy is a major determinant of [³H]water and [¹⁴C]chlorpyrifos uptake in aquatic insects. *Can J Fish Aquat Sci* 59:1315–1322.
6. Nriagu JO, Pacyna JM (1988) Quantitative assessment of worldwide contamination of air, water, and soils by trace metals. *Nature* 333:134–139.
7. Barbour MT, Gerritsen J, Snyder BD, Stribling JB (1999) *Rapid Bioassessment Protocols for Use in Wadeable Streams and Rivers: Periphyton, Benthic Macroinvertebrates, and Fish* (Environmental Protection Agency, Washington, DC), EPA Publ No 841-B-99-002.
8. Rosenberg DM, Resh VH, King RS (2008) Use of aquatic insects in biomonitoring. *An Introduction to the Aquatic Insects of North America*, eds Merritt RW, Cummins KW, Berg MB (Kendall/Hunt, Dubuque, IA), pp 123–138.
9. Wright JF (2000) An introduction to RIVPACS. *Assessing the Biological Quality of Fresh Waters: RIVPACS and Other Techniques*, eds Wright JF, Sutcliffe DW, Furse MT (Freshwater Biological Association, Ableside, UK), pp 1–24.
10. Poff NL, et al. (2006) Functional trait niches of North American lotic insects: Trait-based ecological applications in light of phylogenetic relationships. *J North Am Benthol Soc* 25:730–755.
11. Statzner B, Hildrew AG, Resh VH (2001) Species traits and environmental constraints: Entomological research and the history of ecological theory. *Annu Rev Ent* 46:291–316.
12. Buchwalter DB, Luoma SN (2005) Differences in dissolved cadmium and zinc uptake among stream insects: Mechanistic explanations. *Environ Sci Technol* 39:498–504.
13. Buchwalter DB, Cain DJ, Clements WH, Luoma SN (2007) Using biodynamic models to reconcile differences between laboratory toxicity tests and field biomonitoring with aquatic insects. *Environ Sci Technol* 41:4821–4828.
14. Martin CA, Cain DJ, Luoma SN, Buchwalter DB (2007) Cadmium ecophysiology in seven stonefly (Plecoptera) species: Delineating sources and susceptibility. *Environ Sci Technol* 41:7171–7177.
15. Blomberg SP, Garland T, Jr, Ives AR (2003) Testing for phylogenetic signal in comparative data: Behavioral traits are more labile. *Evolution (Lawrence, Kans)* 57:717–745.
16. Freckleton RP, Harvey PH, Pagel MD (2002) Phylogenetic analysis and comparative data: A test and review of evidence. *Am Nat* 160:712–726.
17. Luoma SN, Rainbow PS (2005) Why is metal bioaccumulation so variable? Biodynamics as a unifying concept. *Environ Sci Technol* 39:1921–1931.
18. Wang WX, Rainbow PS (2006) Subcellular partitioning and the prediction of cadmium toxicity to aquatic organisms. *Environ Chem* 3:395–399.
19. Rainbow PS (2002) Trace metal concentrations in aquatic invertebrates: Why and so what? *Environ Pollut* 120:497–507.
20. Wallace WG, Lee B-G, Luoma SN (2003) The subcellular compartmentalization of Cd and Zn in two bivalves. I. The significance of metal-sensitive fractions (MSF) and biologically detoxified metal (BDM). *Mar Ecol Prog Ser* 249:183–197.
21. Cain DJ, Buchwalter DB, Luoma SN (2006) The influence of exposure history on the bioaccumulation and subcellular distribution of aqueous cadmium in the insect *Hydropsyche californica*. *Environ Toxicol Chem* 25:1042–1049.
22. Avise JC (2006) *Evolutionary Pathways in Nature: A Phylogenetic Approach* (Cambridge Univ Press, Cambridge, UK).
23. Garland T, Jr, Carter PA (1994) Evolutionary physiology. *Annu Rev Physiol* 56:579–621.
24. Harvey PH, Pagel MD (1991) *The Comparative Method in Evolutionary Biology* (Oxford Univ Press, Oxford).
25. Hochachka PW, Somero GN (2002) *Biochemical Adaptation: Mechanism and Process in Physiological Evolution* (Oxford Univ Press, Oxford).
26. Garland T, Jr, Ives AR (2000) Using the past to predict the present: Confidence intervals for regression equations in phylogenetic comparative methods. *Am Nat* 155:346–364.
27. Garland T, Jr, Midford PE, Ives AR (1999) An introduction to phylogenetically based statistical methods, with a new method for confidence intervals on ancestral states. *Am Zool* 39:374–388.
28. Brashares J, Garland T, Jr, Arcese P (2000) Phylogenetic analysis of coadaptation in behavior, diet, and body size in the African antelope. *Behav Ecol* 11:452–463.
29. Duncan RP, Forsyth DM, Hone J (2007) Testing the metabolic theory of ecology: Allometric scaling exponents in mammals. *Ecology* 88:324–333.
30. Lavin SR, Karasov WH, Ives AR, Middleton KM, Garland T, Jr (2008) Morphometrics of the avian small intestine, compared with nonflying mammals: A phylogenetic approach. *Physiol Biochem Zool*, in press.
31. Cummins KW, Klug MJ (1979) Feeding ecology of stream invertebrates. *Annu Rev Ecol System* 10:147–172.
32. Cutshall N (1974) Turnover of zinc-65 in oysters. *Health Phys* 26:327–331.
33. Cain DJ, Luoma SN, Wallace WG (2004) Linking metal bioaccumulation of aquatic insects to their distribution patterns in a mining-impacted river. *Environ Toxicol Chem* 23:1463–1473.
34. Clements WH (1991) Community responses of stream organisms to heavy metals: A review of observational and experimental approaches. *Metal Ecotoxicology Concepts and Applications*, eds Newman MC, McIntosh AW (Lewis, Chelsea, MI), pp 363–386.
35. Evans DR, Balch GC, Evans HE, Welbourn PM (2002) Simultaneous measurement of uptake and elimination of cadmium by caddisfly (Trichoptera:Hydropsychidae) larvae using stable isotope tracers. *Environ Toxicol Chem* 21:1032–1039.
36. Ritterhoff J, Zauke G-P (1998) Potential role of metal-binding proteins in cadmium detoxification in *Themisto libellula* (Mandt) and *Themisto abyssorum* Boeck from the Greenland Sea. *Mar Environ Res* 45:179–191.
37. Suzuki KT, Sunaga H, Hatakeyama S, Sumi Y, Suzuki T (1989) Differential binding of cadmium and copper to the same protein in a heavy metal tolerant species of mayfly (*Baetis thermicus*) larvae. *Comp Biochem Physiol* 94C:99–103.
38. Statzner B, Doledec S, Huguency B (2004) Biological trait composition of European stream invertebrate communities: Assessing the effects of various trait filter types. *Ecography* 27:470–488.
39. Clements WH (2004) Small-scale experiments support causal relationships between metal contamination and macroinvertebrate community responses. *Ecol Appl* 14:954–967.
40. Clark JL, Clements WH (2006) The use of *in situ* and stream microcosm experiments to assess population- and community-level responses to metals. *Environ Toxicol Chem* 25:2306–2312.
41. Clements WH, Carlisle DM, Courtney LA, Harrahy EA (2002) Integrating observational and experimental approaches to demonstrate causation in stream biomonitoring studies. *Environ Toxicol Chem* 21:1138–1146.
42. Garland T, Jr, Adolph SC (1991) Physiological differentiation of vertebrate populations. *Annu Rev Ecol Syst* 22:193–228.
43. Rainbow PS (1985) Accumulation of Zn, Cu, and Cd by crabs and barnacles. *Estuarine Coastal Shelf Sci* 21:669–686.
44. Rainbow PS, White SL (1989) Comparative strategies of heavy metal accumulation by crustaceans: Zinc, copper, and cadmium in a decapod, amphipod, and a barnacle. *Hydrobiologia* 174:245–262.
45. Croteau M-N, Hare L, Tessier A (2003) Difficulties in relating Cd concentrations in the predatory insect *Chaoborus* to those of its prey in nature. *Can J Fish Aquat Sci* 60:800–808.
46. Munger C, Hare L, Tessier A (1999) Cadmium sources and exchange rates for *Chaoborus* larvae in nature. *Limnol Oceanogr* 44:1763–1771.
47. Garland T, Jr, Bennett AF, Rezende EL (2005) Phylogenetic approaches in comparative physiology. *J Exp Biol* 208:3015–3035.
48. Croteau M-N, Luoma SN, Topping BR, Lopez CB (2004) Stable metal isotopes reveal copper accumulation and loss dynamics in the freshwater bivalve *Corbicula*. *Environ Sci Technol* 38:5002–5009.
49. Wallace WG, Lopez GR (1997) Bioavailability of biologically sequestered cadmium and the implications of metal toxicity. *Mar Ecol Prog Ser* 147:149–157.
50. Suzuki KT, et al. (1988) Binding of cadmium and copper in the mayfly *Baetis thermicus* larvae that inhabit a river polluted with heavy metals. *Comp Biochem Physiol* 19C:487–492.
51. Burnham KP, Anderson DR (2002) *Model Selection and Multimodel Inference: A Practical Information-Theoretic Approach* (Springer, New York).

Supporting Information

Buchwalter *et al.* 10.1073/pnas.0801686105

Table S1. Taxa, sampling locations, weights and number of individuals used in comparative studies of cadmium bioaccumulation and detoxification

Order	Family	Genus species	Sampling location	Body Weight \pm SD	<i>n</i>	Feeding
Ephemeroptera	Heptageniidae	<i>Mcaffertium ithaca</i>	36°22'10"N, 80°59'30"W	76.9 \pm 4.5	40	0
Ephemeroptera	Heptageniidae	<i>Rhithrogena morrisoni</i>	40°39'30"N, 122°55'30"W	27.4 \pm 1.3	22	0
Ephemeroptera	Isonychiidae	<i>Isonychia tusculanensis</i>	36°05'00"N, 79°01'00"W	51.5 \pm 1.5	20	0
Ephemeroptera	Isonychiidae	<i>Isonychia sp.</i>	36°22'10"N, 80°59'30"W	48.2 \pm 2.1	9	0
Ephemeroptera	Ephemerellidae	<i>Ephemerella subvaria</i>	36°22'10"N, 80°59'30"W	29.3 \pm 1.8	40	0
Ephemeroptera	Ephemerellidae	<i>E. excrucians</i>	40°39'30"N, 122°55'30"W	6.6 \pm 1.1	30	0
Ephemeroptera	Ephemerellidae	<i>Drunella grandis</i>	40°39'30"N, 122°55'30"W	157.4 \pm 9.4	30	0.5
Plecoptera	Perlidae	<i>Claassinea sabulosa</i>	40°40'01"N, 105°13'32"W	96.7 \pm 9.4	20	1
Plecoptera	Perlidae	<i>Paragnetina sp.</i>	36°22'10"N, 80°59'30"W	92.9 \pm 7.9	14	1
Plecoptera	Perlidae	<i>Doroneuria baumanni</i>	44°27'23"N, 121°38'41"W	335.6 \pm 142	12	1
Plecoptera	Perlidae	<i>Calineuria californica</i>	37°17'20"N, 122°04'20"W	105.1 \pm 10.1	20	1
Plecoptera	Perlidae	<i>Acroneuria abnormis</i>	36°22'10"N, 80°59'30"W	195.0 \pm 23.0	16	1
Plecoptera	Perlidae	<i>Hesperoperla pacifica</i>	37°17'20"N, 122°04'20"W	144.3 \pm 17.4	10	1
Plecoptera	Perlodidae	<i>Skwala sp.</i>	37°17'20"N, 122°04'20"W	78.8 \pm 6.8	10	1
Plecoptera	Perlodidae	<i>Isogenoides hansonii</i>	36°22'10"N, 80°59'30"W	108.5 \pm 13.2	6	1
Plecoptera	Perlodidae	<i>Baumanella alameda</i>	37°04'35"N, 121°28'02"W	34.9 \pm 1.6	9	1
Plecoptera	Pteronarcyidae	<i>Pteronarcys dorsata</i>	36°22'10"N, 80°59'30"W	139.0 \pm 5.4	18	0
Trichoptera	Hydropsychidae	<i>H. californica</i>	40°23'58"N, 122°07'44"W	24.4 \pm 1.8	40	0
Trichoptera	Hydropsychidae	<i>Cheumatopsyche sp.</i>	36°04'42"N, 79°00'31"W	18.9 \pm 1.2	40	0
Trichoptera	Rhyacophilidae	<i>Rhyacophila fuscula</i>	36°22'10"N, 80°59'30"W	69.7 \pm 4.3	23	1
Trichoptera	Rhyacophilidae	<i>Rhyacophila sp.</i>	37°16'07"N, 122°18'51"W	34.2 \pm 1.9	30	1

Feeding strategies were coded as predators (1), nonpredators (0), and intermediate (0.5).

Table S2. k_e models

Model	Parameters	d	P , wt	P , clade	P , feeding	ln ML	AIC
Star	Log (wt)		0.0768			-14.6576	35.3152
PGLS	Log (wt)		0.0081			-11.1458	28.2916
OU	Log (wt)	0.7007	0.0136			-10.4368	28.8736
Star	Log (wt) and family		0.0082	0.0004		5.22548	9.54905
PGLS	Log (wt) and family		0.0082	0.3227		-5.1685	30.3370
OU	Log (wt) and family	7.63×10^{-9}	0.0082	0.0004		5.2254	11.5491
Star	Log (wt) and order		0.1867	0.0172		-9.6366	29.2733
PGLS	Log (wt) and order		0.0104	0.6465		-10.607	31.2139
OU	Log (wt) and order	0.7650	0.0143	0.4855		-8.47348	28.9470
Star	Log (wt) and feeding		0.5916		0.0551	-12.453	32.9059
PGLS	Log (wt) and feeding		0.0174		0.7730	-11.0959	30.1918
OU	Log (wt) and feeding	0.6654	0.0352		0.5734	-10.1292	30.2584
Star	Log (wt) family and feeding		0.0192	0.0020	0.4531	5.78766	10.4247
PGLS	Log (wt) family and feeding		0.0120	0.3155	0.4470	-4.5910	31.1820
OU	Log (wt) family and feeding	7.63×10^{-9}	0.0192	0.0020	0.4531	5.78764	12.4247
Star	Log (wt) order and feeding		0.2855	0.0933	0.5081	-9.34013	30.6803
PGLS	Log (wt) order and feeding		0.0193	0.6814	0.8835	-10.5924	33.1849
OU	Log (wt) order and feeding	0.8304	0.0236	0.5866	0.8395	-8.33224	30.6645

Best-fit model is indicated in bold.

Table S3. Subcellular Cd compartmentalization after exposure to 0.52 $\mu\text{g}\cdot\text{liter}^{-1}\cdot\text{Cd}$

Taxon	Fractions				
	Detoxified HSP	Metal sensitive			Cell debris
		HDP	Organelles	Microsomes	
<i>Mccaffertium ithaca</i>	41.3 ± 2.4	15.6 ± 1.6	5.8 ± 0.4	8.6 ± 1.2	28.8 ± 2.6
<i>Rhithrogena morrisoni</i>	69.4	6.8	4.1	5.0	14.7
<i>Isonychia tusculanensis</i>	63.3 ± 0.5	6.8 ± 2.5	5.5 ± 0.8	6.0	18.3 ± 2.2
<i>Isonychia sp.</i>	37.1 ± 5.4	11.3 ± 3.5	10.2 ± 2.4	14.1 ± 3.6	27.2 ± 4.4
<i>Ephemerella subvaria</i>	21.2 ± 0.6	27.9 ± 0.3	7.0 ± 0.9	11.1 ± 0.1	32.8 ± 0.1
<i>Ephemerella excrucians</i>	35.8	33.0	5.2	10.5	15.4
<i>Drunella grandis</i>	6.9 ± 0.9	14.2 ± 1.2	16.8 ± 0.2	20.4 ± 4.2	41.6 ± 7.8
<i>Claassinea sabulosa</i>	8.8 ± 3.6	15.5 ± 3.0	14.1 ± 5.4	6.7 ± 1.2	52.9 ± 4.0
<i>Paragnetina sp.</i>	31.8 ± 0.7	2.6 ± 0.2	6.9 ± 3.7	2.4 ± 0.4	56.2 ± 3.4
<i>Doroneuria baumanni</i>	7.2 ± 1.1	24.7 ± 7.1	7.6 ± 0.8	11.1 ± 0.0	48.4 ± 6.8
<i>Calineuria californica</i>	3.4 ± 0.7	21.9 ± 2.4	7.9 ± 1.6	11.5 ± 0.4	55.4 ± 6.7
<i>Acroneuria abnormis</i>	33.9 ± 1.2	2.5 ± 0.1	4.7 ± 0.6	5.8 ± 0.3	53.2 ± 1.7
<i>Hesperoperla pacifica</i>	7.7 ± 4.8	32.7 ± 5.6	8.8 ± 0.0	8.8 ± 1.5	41.9 ± 3.9
<i>Skwala sp.</i>	30.9 ± 2.8	14.7 ± 1?	7.9 ± 0.7	8.1 ± 0.1	38.4 ± 4.6
<i>Isogenoides hansonii</i>	10.3 ± 0.3	48.7 ± 0.3	7.2 ± 0.3	7.9 ± 0.5	25.9 ± 0.5
<i>Baumanella alameda</i>	29.4 ± 3.1	15.4 ± 2.8	10.2 ± 0.8	10.8 ± 4.3	34.3 ± 3.8
<i>Pteronarcys dorsata</i>	19.7 ± 5.1	34.3 ± 3.1	9.2 ± 1.7	7.1 ± 1.2	28.5 ± 5.4
<i>Hydropsyche californica</i>	25.2 ± 0.7	16.9 ± 0.5	10.8 ± 3.3	9.8 ± 0.3	37.3 ± 4.8
<i>Cheumatopsyche sp.</i>	21.2 ± 4.7	18.8 ± 4.7	11.5 ± 1.3	19.3 ± 2.7	29.2 ± 10.0
<i>Rhyacophila fuscula</i>	20.6 ± 8.4	5.6 ± 2.5	8.4 ± 0.8	4.6 ± 1.2	60.7 ± 5.4
<i>Rhyacophila sp.</i>	2.9	20.4	2.3	10	64.4

Table S4. k_u models

Model	Parameters	d	P, wt	$P, clade$	$P, feeding$	In ML	AIC
Star	Log (wt)		0.4837			-15.2757	36.5515
PGLS	Log (wt)		0.6535			-16.2628	38.5255
OU	Log (wt)	0.3816	0.6099			-14.0830	36.1660
Star	Log (wt) and family		0.6635	0.0124		-1.95539	23.9108
PGLS	Log (wt) and family		0.8382	0.5763		-12.0801	44.1602
OU	Log (wt) and family	7.63×10^{-9}	0.6635	0.0124		-1.9554	25.9108
Star	Log (wt) and order		0.6071	0.6020		-14.6487	39.2975
PGLS	Log (wt) and order		0.6614	0.9395		-16.1857	42.3714
OU	Log (wt) and order	0.6360	0.6527	0.2615		-13.6433	39.2866
Star	Log (wt) and feeding		0.9079		0.2089	-14.3287	36.6574
PGLS	Log (wt) and feeding		0.9556		0.2783	-15.558	39.1160
OU	Log (wt) and feeding	0.3987	0.9750		0.2358	-13.1847	36.3693
Star	Log (wt) family and feeding		0.9165	0.0296	0.7889	-1.88376	25.7675
PGLS	Log (wt) family and feeding		0.9955	0.7213	0.7861	-12.0066	46.0131
OU	Log (wt) family and feeding	7.63×10^{-9}	0.9165	0.0296	0.9324	-1.88378	27.7676
Star	Log (wt) order and feeding		0.8310	0.7835	0.3308	-14.0085	40.0169
PGLS	Log (wt) order and feeding		0.9250	0.9663	0.3188	-15.5130	43.0259
OU	Log (wt) order and feeding	0.7086	0.9021	0.9385	0.3157	-12.9766	39.9532

Best-fit model is indicated in bold.

Table S5. Composite susceptibility models

Model	Parameters	<i>d</i>	<i>P</i> , wt	<i>P</i> , clade	<i>P</i> , feeding	ln ML	AIC
Star	Log (wt)		0.0440			-18.5129	43.0258
PGLS	Log (wt)		0.0210			-14.5544	35.1089
OU	Log (wt)	0.7906	0.0255			-14.1148	36.2297
Star	Log (wt) and family		0.0172	0.0001		3.77389	12.4522
PGLS	Log (wt) and family		0.0281	0.1233		-6.1503	32.3006
OU	Log (wt) and family	7.63×10^{-9}	0.0172	0.0001		3.77387	14.4523
Star	Log (wt) and order		0.0823	0.0125		-13.0972	36.1944
PGLS	Log (wt) and order		0.0237	0.5656		-13.8504	37.7009
OU	Log (wt) and order	0.8417	0.0261	0.4533		-11.8134	35.6268
Star	Log (wt) and feeding		0.7345		0.0030	-13.2269	34.4539
PGLS	Log (wt) and feeding		0.0808		0.0330	-11.8294	31.6589
OU	Log (wt) and feeding	0.6403	0.1247		0.0203	-10.7431	31.4863
Star	Log (wt) family and feeding		0.1183	0.0035	0.8107	3.83119	14.3376
PGLS	Log (wt) family and feeding		0.0810	0.4112	0.9429	-6.14518	34.2904
OU	Log (wt) family and feeding	7.63×10^{-9}	0.1183	0.0035	0.8107	3.83117	16.3377
Star	Log (wt) order and feeding		0.2086	0.0712	0.0263	-9.75819	31.5164
PGLS	Log (wt) order and feeding		0.0741	0.6520	0.0507	-11.2680	34.5360
OU	Log (wt) order and feeding	0.6989	0.0818	0.4583	0.0416	-8.62619	31.2524

Best-fit model indicated in bold.