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Dynamics of Cadmium Acclimation in *Daphnia pulex*: Linking Fitness Costs, Cross-Tolerance, and Hyper-Induction of Metallothionein

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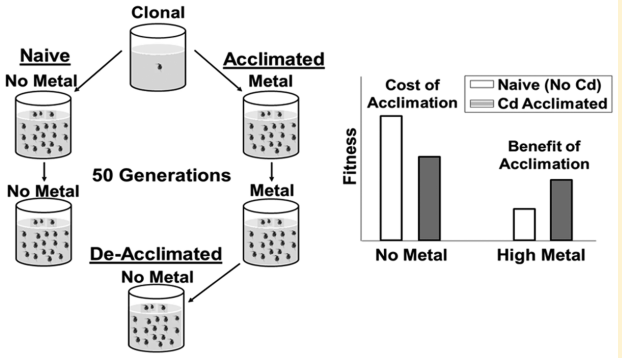
ABSTRACT: Acclimation increases tolerance to stress in individuals but is assumed to contribute fitness costs when the stressor is absent, though data supporting this widely held claim are sparse. Therefore, using clonal (i.e., genetically identical) cultures of *Daphnia pulex*, we isolated the contributions of acclimation to the regulation of the metal response gene, metallothionein 1 (MT1), and defined the reproductive benefits and costs of cadmium (Cd)-acclimation. *Daphnia pulex* were exposed for 50 parthenogenetic generations to environmentally realistic levels (1 μg Cd/L), and tolerance to Cd and other metals assessed during this period via standard toxicity tests. These tests revealed (1) increased tolerance to Cd compared to genetically identical nonacclimated cultures, (2) fitness costs in Cd-acclimated *Daphnia* when Cd was removed, and (3) cross-tolerance of Cd-acclimated *Daphnia* to zinc and silver, but not arsenic, thereby defining a functional role for metallothionein. Indeed, Cd-acclimated clones had significantly higher expression of MT1 mRNA than nonacclimated clones, when Cd exposed. Both the enhanced induction of MT1 and tolerant phenotype were rapidly lost when Cd was removed (1−2 generations), which is further evidence of acclimation costs. These findings provide evidence for the widely held view that acclimation is costly and are important for investigating evolutionary principles of genetic assimilation and the survival mechanisms of natural populations that face changing environments.



INTRODUCTION

Many phenotypes that have consequences on an organism's fitness, including stress tolerance, are environmentally plastic.[1] These reversible changes occur in response to environments experienced not only by the individual but also those by previous generations through parental-effect or epigenetic mechanisms.[2−4] Therefore, while the ecological fitness of a given genetic isolate at any time depends on the expressed phenotype in the current selective environment, phenotypic expression itself is likely to have been influenced by both current and historical environmental conditions. Moreover, since the fitness costs and benefits of a given phenotype can differ among environments, sustained environmental change in a given direction and changes in patterns of environmental variability can influence both the evolutionary trajectory of a trait and its plasticity.[3,5] In some cases, environmentally induced phenotypes, such as stress induced acclimation (i.e., acclimation defines nongenetic, physiological changes in tolerance occurring at the level of the individual and differs from adaptation that occurs when selection operating at population levels retains tolerant genotypes, e.g., Huchmuth et al.[6]), may become constitutively expressed in a process termed genetic assimilation (i.e., adaptation). This can occur in response to natural selection if the constitutively expressed phenotype is selectively advantageous in the presence of the environmental trigger or if constitutive expression is less costly than environmentally responsive phenotypic plasticity. These selective pressures are not mutually exclusive, and both may contribute simultaneously to adaptation.

Our study begins to tease apart these contributions by focusing on physiological acclimation of individuals to the model chemical stressor cadmium (Cd), considered by the United States Environmental Protection Agency (US EPA) to be a pollutant of greatest concern in the United States.[7] Cadmium can induce a strong acclimation response (i.e., steep norms of reaction) at the level of an individual. Furthermore, acquired tolerance to a stressor can be induced via multi-

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generation acclimation, leading to a transiently heritable physiological tolerance.[8,9] Tolerance in Cd-acclimated individuals has been associated with higher production of the metal-binding protein metallothionein (MT) upon exposure to Cd and other metals from groups IB and IIB of the Periodic Table of Elements.[10−12] While greater MT production and higher tolerance acquired during acclimation do not involve heritable genetic changes, they can nevertheless be passed to the next generation due to maternal or paternal effects on the phenotypes of progeny.[13,14] The fitness benefits associated with increased acclimation tolerance in the presence of stressors are generally thought to be balanced against costs in its absence, but evidence for this assumption is sparse. Potential costs associated with acclimated phenotypes can arise from altered energy allocation, modifications in gene function, or decreased plasticity,[15] resulting in lower reproduction and survival in the absence of the stressor. Thus, maintaining an acclimated phenotype in an environment where, spatially and temporally, the exposure to the stressor varies is thought to be particularly costly. Therefore, understanding and predicting the full impact of stressors on natural populations requires an assessment of the benefits and costs associated with tolerance, especially the potential for fitness costs in the absence of the stressor. These needs are critical for estimating the risks and assessing the long-term fate of populations, and to help establish protective guidelines for regulators, especially in times of rapid environmental change.

Daphnia species are targets of many studies of stressor induced tolerance acquisition.[8,9,12] *Daphnia* are keystone species that play a sentinel role in a broad array of aquatic ecosystems.[16,17] Long recognized as an environmental indicator species, *Daphnia* also provide an ideal experimental platform for uncoupling epigenetic and genetic contributions to tolerant phenotypes. They are cyclical parthenogens and are thus capable of either clonal or sexual reproduction. With clonal reproduction, their genetic background (i.e., genotype) can be held constant,[18,19] providing an effective means for comparisons of various treatments across multiple generations. They also have a maturing set of genome resources that facilitate molecular studies.[20,21] The connection of molecular and multigeneration population responses in these important indicator species not only provides mechanistic information about tolerance to common pollutants such as metals but also lays the groundwork for understanding individual variation in stress tolerance and response within populations. Such variation defines population susceptibility to stress and provides the information necessary for predicting the effects of pollution in freshwater ecosystems worldwide.

Our immediate objectives were to compare short and long-term Cd-induced fitness differences and any associated changes in the regulation of the metallothionein 1 (MT1) gene in two laboratory cultures founded from genetically identical sisters (hereafter referred to as isoclonal cultures), differing only in exposure to the metal stressor Cd. We focus on Cd because it is widespread and extremely toxic to aquatic life. Also, *Daphnia* species exhibit a remarkable range of sensitivity to it.[22] Here, we investigate whether multigeneration Cd acclimation induces tolerance to Cd and cross-tolerance to three other metals. *Daphnia* also provide an experimental advantage for these studies in that genetic variation can be eliminated at the start of the experiment by clonal reproduction. We developed isoclonal cultures of *D. pulex* and for 50 generations, compared the tolerance of cultures continuously exposed to environmentally realistic, low concentrations of Cd, i.e., Cd-acclimated, to genetically identical cultures not exposed to Cd, i.e., naïve. In other words, changes in tolerance at the individual level are studied in a replicable design in the absence of genetic variation. Isolates are assumed to be genetically identical because they were derived clonally from a single mother. Also, given the spontaneous mutation rate measured in *D. pulex* (10^{-9} mutation/site/generation)[23] we expect ∼10 single nucleotide mutations to arise per individual over 50 generations, i.e., contributing only 0.000005% variation among individuals. For cross-tolerance experiments, we exposed Cd-acclimated *D. pulex* to zinc (Zn) and silver (Ag) that like Cd reside in groups IIA and IIB of the Periodic Table of Elements for which MT is known to be protective. We also exposed them to the metalloid arsenic (As), which does not reside in group IIA or IIB for which MT protection is greatly reduced.[24] We also compared MT1 gene-expression following Cd exposure in both Cd-acclimated and naïve cultures. Additionally, to understand the fitness effects of acquired tolerance (i.e., benefits and costs,) we measured clonal reproduction and survivorship of acclimated and naïve cultures at two different Cd concentrations and in the absence of Cd.

By characterizing the benefits and costs of acclimation as well as the regulation of the key Cd defense gene, MT1, underlying the epigenetic contributions to metal tolerant phenotypes over multiple generations, we were able to test the following hypotheses: (i) previous exposure to Cd increases tolerance to Cd and other metals that are regulated by similar mechanisms; (ii) in the absence of metals, Cd-acclimated phenotype is rapidly lost; (iii) regulation of metallothionein gene expression is altered in Cd-acclimated *Daphnia*; (iv) induced metal tolerance is accompanied by fitness costs in the absence of the acclimated stressor.

MATERIALS AND METHODS

Animals and Cd Exposure. *Daphnia pulex* (subclade arenata) used in this study were obtained from isoclonal laboratory cultures of a daphniid collected from an ephemeral pond near the Pacific coast in Oregon (as described in Colbourne et al.[20]). *Daphnia pulex arenata* is a member of the *Daphnia pulex* complex,[25] and this isolate is from the same population as the strain whose genome was sequenced and assembled into a reference.[20] *Daphnia* were housed in 4 L borosilicate glass beakers (40 per beaker) held inside an environmental chamber at a constant temperature (19 ± 1 °C) and photoperiod (16:8 light-dark).[26] Organisms were maintained in nanopure water reconstituted to moderate hardness (89 ± 16 mg CaCO3/L),[27] renewed weekly, and fed *Ankistrodesmus falcatus* three times per week at a rate of 75000 cells/mL. Our pre-experimental procedure described in Folt et al.[28] controlled for maternal influence on variation in offspring traits that is independent of inherited genes[29] (e.g., lipid stores, feeding rates, dispersal[30]) by regulating density and food availability, while allowing only clonal reproduction for all demographic experiments and batch exposures. The experimental populations were started by isolating <24 h old neonates from the maintenance cultures, thus synchronizing the population with respect to age. These organisms referred to as "brood females" were used to seed both the acclimated and naïve cultures.

Cadmium acclimated and naïve cultures were established by isolating a single mother from the previously described brood females. For naïve cultures, offspring (<24 h) from the selected

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brood mother were collected at 21 days, transferred to a 4 L beaker, and cultured as previously described. Cadmium acclimated cultures were initiated using offspring (<24 h) from the same brood mother and clutch that founded the naïve cultures. These *Daphnia* were exposed to environmentally relevant Cd concentrations,[31,32] known to inhibit *D. pulex* reproduction as shown under laboratory conditions by the US EPA (i.e., EC20 for *Daphnia* reproduction range from 0.1 to 6 μg Cd/L)[33] and impact natural populations in the field (i.e., cladoceran biomass has been shown to decrease by as much as 70% in 1 μg Cd/L).[34−36] The Cd acclimated cultures were developed using the same methods as naïve cultures with the following exceptions: Twenty neonates (<24 h) were removed from the same maintenance cultures that created the naïve line and each neonate was individually placed into a 100 mL culture vessel that contained high levels (5 μg Cd/L) of Cd. Following 21 days of daily renewal, neonates from these cultures were isolated by transfer to a new 100 mL culture vessel with exposure concentration reduced to 1 μg Cd/L. After 21 days, neonates from these cultures were then selected based on reproductive success, using the top five producers to start the Cd-acclimated population, which was cultured in 4 L beakers as described for naïve cultures, but in concentrations of 1 μg Cd/L. Both the Cd acclimated and naive cultures were passaged every 21 days, which defines a generation. Tests reported in this manuscript were completed at 50 generations (i.e., 1050 days), though these cultures were maintained for much longer. Metal concentrations were analyzed in the Dartmouth Superfund Trace Metal Core Facility (Hanover, NH, U.S.A.) using a magnetic sector inductively coupled plasma/mass spectrometer (ELEMENT; ThermoElectron, Boston, MA, U.S.A.) fitted with a standard liquid sample introduction system (microconcentric nebulizer mCN-2; CETAC, Omaha, NE, U.S.A., and cooled Scott-type spray chamber). Primary stock solutions were made from analytical grade zinc chloride, silver chloride, cadmium chloride, and sodium (meta) arsenite, Sigma Chemical (St. Louis, MO, U.S.A.) dissolved in deionized water. Stock solutions were measured before and after dilution of test solution concentrations to ensure they were within 10% of nominal targets.

Acute Toxicity Tests. *Daphnia pulex* acute (48 h) toxicity tests for all metals were conducted according to recommendations given by the US EPA 2002 methods,[37] with slight modifications.[38] Test solutions were prepared immediately prior to testing from metal stock solutions dissolved in the synthetic water, as previously described. We have measured arsenic species and determined that arsenite is stable under these test conditions.[39] Toxicity tests employed a completely random design[37] consisting of five or six metal treatments and a control group arrayed in 2-fold serial dilutions. Ten neonates (<24 h old) were randomly placed into a 40 mL glass exposure chamber containing 30 mL of test solution. A minimum of three replicate exposure chambers were employed per treatment or control group. As required, daphniids were not fed during tests. Mortality was assessed for individuals in each container after a 48 h exposure. An individual was recorded as dead if it was unresponsive to gentle prodding. These tests were repeated, and results werecombined to determine lethal concentrations (LC*x* values, where *x* equals a given percent mortality) estimated from the probit transformed concentration−response curves.

Demographic Experiments. Chronic (21 d) toxicity tests followed a completely randomized design as given in ASTM methods.[40] A single <24 h old neonate was randomly placed into a 120 mL exposure chamber. Ten replicate chambers were used per test group. Treatment groups consisted of 1 and 2.5 μg Cd/L and control (0 μg Cd/L). Test waters were renewed every other day, at which point metal concentrations and general water quality parameters of temperature (19 °C ± 1 °C), dissolved oxygen (10 mg/L ± 1.5 mg/L), and pH (7.8 ± 0.8) were monitored. Mortality and reproduction were observed over the duration of the test, and end points included age to first reproduction, per capita birth rate, total neonates per adult, cumulative reproduction (R_o) and intrinsic rate of increase (r) as per Chen and Folt.[41] End points were analyzed using a two-way full factorial ANOVA followed by a posthoc t test using JMP.[42]

Metal Removal Experiments. To better understand acclimation, we conducted a series of experiments in which we removed acclimated *Daphnia* from Cd to determine when their tolerance to metal was lost. These tests involved measuring Cd tolerance in three laboratory cultures, Cd-acclimated, naïve, and Cd-acclimated cultures removed from Cd (Deacclimated), over multiple generations. For each population, neonates from the third brood clutch were used to populate the next generation and to define Cd tolerance using acute toxicity tests (previously described).

Metallothionein Gene Expression. Adult *D. pulex* from Cd-acclimated and naïve cultures were held in the absence of Cd for 48 h and then exposed in quintuplicate with 10 organisms per 100 mL glass exposure vessel to 0, 1, and 20 μg Cd/L for 24h. Total RNA was isolated from adult *Daphnia* using Qiashredder columns and RNeasy kits (Qiagen) according to manufacturer recommendations as detailed by Shaw et al.[43] DNA contamination was contained by DNase treatment (Ambion), and RNA was quantified by spectrophotometry (Nanodrop Technologies); quality was determined with a Bioanalyzer 2100 (Agilent). Quantitative real-time RT-PCR was used to quantify *D. pulex* metallothionein 1 gene expression. Previous studies demonstrated that this MT gene is Cd responsive.[43,44] The primers and *TaqMan* probe were designed using PRIMER EXPRESS, V (Applied Biosciences):

sense primer, AAACTACCCAACGGAATCAACAT;

antisense primer, CAGTTGGGTCCGCATTTG;

Taqman probe, CCACACGAGCATTTACCTTGGCAAC.

Reverse transcription was performed with the Omniscript reverse transcription kit from Qiagen. RNA was reverse transcribed for each sample (2 μg) using random primers (final concentration of 5 uM) and the standard protocol included with the kit. Real-time PCR was performed on the Applied Biosystems 7700 machine and included a standard curve in each run. The standard curve consisted of serial dilutions of cDNAs containing the sequence being amplified. Applied Biosystems Master Mix was used in each amplification which contains all PCR components necessary except the cDNA, primers (900 nM each, final concentration), and Taqman probe (250 nM, final concentration). The Taqman probe was FAM labeled and contained an MGB quencher. Controls to test for DNA contamination were always included even though DNase digestion was performed on the RNA before the reverse transcription. Amplification was the standard 40 cycles preceded by 2 min at 50 °C and 10 min at 95 °C to activate the enzyme. Each cycle included 15 s at 95 °C and 1 min at 60 °C. For each sample, the cycle at which amplification reached the exponential phase was recorded by the machine as the Ct value. Final transcript quantities were normalized to

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Table 1. Acute (48 h) Metal Challenges in Naïve and Cadmium Acclimated *Daphnia pulex*[ab]

Metal	Group	G_0	G_5	G_{10}	G_{20}	G_{50}
Cadmium	Naive	69.3 (61.7−76.7)	71.8 (62.6−81.2)	70.3 (60.9−78.8)	74.0 (61.8−84.9)	71.5 (61.1−81.0)
	Acclimated		136.6 (115.8−157.1)	170.2 (151.8−188.0)	151.1 (131.5−167.7)	99.7 (86.1−111.9)
Zinc	Naive	194.2 (152.8−237.6)	211.0 (174.8−254.4)	216.6 (178.8−261.9)	241.5 (202.3−288.7)	nd
	Acclimated		337.1 (270.7−439.0)	506.8 (427.7−602.4)	525.7 (454.6−610.8)	nd
Silver	Naive	0.39 (0.33−0.47)	0.51 (0.44−0.59)	0.44 (0.38−0.51)	0.47 (0.40−0.55)	nd
	Acclimated		0.86 (0.74−0.99)	2.7 (2.0−3.4)	2.7 (2.2−3.3)	nd
Arsenic	Naive	3340 (3015−3663)	3401 (2795−4072)	3210 (2775−3776)	3170 (2792−3551)	nd
	Acclimated		3868 (3305−4440)	3226 (2840−3721)	3528 (3045−3959)	nd

[a]Expressed as median lethal values (μg/L). [b]The 95% confidence intervals are given in parentheses.

direct measures of the cDNA level in each sample. In addition, serine-threonine kinase, which is known to exhibit stable expression under these conditions,[43] was used as a house-keeping gene employing the primers and methods given in Shaw et al.[43] Its expression did not differ across any treatment (data not shown).

RESULTS AND DISCUSSION

Acclimated Tolerance Acquisition. We tested the hypothesis that pre-exposure to Cd increased Cd-tolerance using standard metal toxicity tests with acclimated and naïve cultures of *D. pulex*. Prolonged exposure to sublethal Cd concentrations increased the tolerance, measured as an increase in the acute (48 h) median lethal concentrations (LC50), of *D. pulex* (Table 1). Acclimation was rapid, producing an almost 2-fold increase in LC_{50} value (i.e., from 72 to 137 μg Cd/L) within five generations, which was sustained for 50 generations in the presence of Cd. Differences between the Cd-acclimated and naïve cultures peaked after 10 generations (170 μg Cd/L), and the tolerance of acclimated cultures declined (i.e., from 151 to 100 μg Cd/L) between 20 and 50 generations but remained elevated compared to naïve cultures (Table 1). Cadmium acclimation seems to follow a rapid rise in tolerance, peaking after 10 generations and then decreasing in tolerance, but remains significantly higher than the naïve culture even after 50 generations. We offer no explanation for this phenomenon and highlight this finding for future studies.

Organisms are known to acclimate to a wide variety of environmental stressors, but persistence over multiple generations in tolerant but nonadapted (i.e., acclimated) organisms has rarely been studied. For example, thermal imprinting and optimum performance temperature[45,46] and caloric restriction and increased lifespan[47] have been demonstrated in arthropod models, but these studies are limited to single generations. Physiological acclimation to metals has been shown in a related daphniid, *D. magna*;[12,14,48−52] however, these studies followed acclimation over fewer generations (i.e., 2−8), compared to 50 in this study. In addition, the acquired tolerance was less pronounced in *D. magna* than in *D. pulex*.[8,48,49] This difference may also result from *D. magna* being more tolerant and responding differently to metal exposures in comparison to other daphniids.[53]

Tolerance Acquisition Strategies. Acclimation induced metal tolerance is hypothesized to result from (i) transport modifications that either reduce uptake or facilitate export of metals in target tissues[54] or (ii) detoxication pathways.[55,56] To evaluate these possibilities, we examined the cross-tolerance of Cd-acclimated *Daphnia* to a suite of metals that use several transport mechanisms and detoxication pathways.

Daphnia acclimated to Cd exhibited cross-tolerance to Zn and Ag, but not to As (Table 1). The dynamics of tolerance acquisition to Zn and Ag were similar across 50 generations to those observed for Cd. Levels of metal tolerance to Cd and Zn were comparable (i.e., LC_{50} ∼ 2.5 times greater than naïve populations), while much greater tolerance was observed for Ag (i.e., LC_{50} ∼ 7 times greater than naïve populations). Others have noted cross-tolerance between Cd and Zn, which are chemically and biochemically similar.[6,49,50] There are no reports of cross-tolerant phenotypes for Cd and Ag, as reported here, or the lack of cross-tolerance for Cd and As.

Taken together, these results suggest that detoxification pathways rather than transport mechanisms are producing the Cd tolerance in *Daphnia*. Cadmium and Zn share common transporters (e.g., calcium),[57] but Ag and As each have unique transport mechanisms. The main mechanism by which silver crosses biological membranes is via sodium channels thereby interfering with sodium homeostasis.[58−60] Arsenic uses specific aquaporin channels (arsenite) or phosphate/sulfate transport mechanisms (arsenate) to enter cells.[57,61] While Cd-acclimated *Daphnia* were tolerant to Cd and Zn, they also acquired tolerance to Ag, which has different known transport mechanisms and there are no reports of Cd influencing Ag (or sodium) transport. These observations suggest that transport mechanisms controlling decreased uptake or enhanced secretion are not responsible for the observed differences in tolerant phenotypes. This argument is strengthened by several reports that indicate that prolonged exposure to Cd results in increased Cd accumulation in *Daphnia magna*.[48−52]

In contrast to transport pathways, Cd, Zn, and Ag share a common detoxification pathway. They are all potent inducers and strongly bound by the metal binding protein, metallothionein (MT). Of these, Ag is the strongest inducer and is more tightly bound to MT,[10] which could explain the greater level of tolerance observed for Ag (Table 1). Though As induction of MT1 via metal response has been observed in cell culture,[62] As is not a potent inducer of MT via metal specific pathways and is predicted to bind poorly to the protein compared to Cd, Ag, and Zn.[10,63] This is likely why Cd acclimation did not confer protection to As exposure. Hence, it appears likely that *Daphnia* copes with some of these metals by detoxication via sequestration of the toxic metals by MT1.

Our inferences are supported by studies at the biochemical-level with *D. magna* demonstrating that both acute and chronic exposure to Cd increases the abundance of MT-like proteins (MT-LP) and that elevated MT-LP levels are associated with the Cd-acclimated phenotype.[12,48−52] However, in multi-

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generational studies, the correlation between MT-LP levels, Cd accumulation, and changes in Cd tolerance are not always clear.[51,52] These inconsistencies are likely due to the long (i.e., several weeks to months) biological half-life of the metal bound protein,[64] making it difficult to link levels of MT-LP to changes in accumulation and tolerance for an organism with a generation time of 9 to 14 days.[51,65] The critical determinant for establishing the link between MT and metal tolerance is its rate of synthesis,[12] which for this transcriptionally regulated protein can be inferred from mRNA levels.[66−68] The identification and characterization of the Cd inducible MT1 gene in *D. pulex*[43] allow an empirical test of our predictions about MT and Cd acclimation at the molecular-level.

Expression of the Metallothionein Gene. We tested the hypothesis that Cd acclimation alters the regulation of the MT1 gene using quantitative real-time PCR (qPCR) to assess differences in MT1 transcript levels between naïve and Cd-acclimated *Daphnia* in the presence (1 μg Cd/L, 20 μg Cd/L) and absence of Cd (Figure 1). Metallothionein 1 mRNA levels

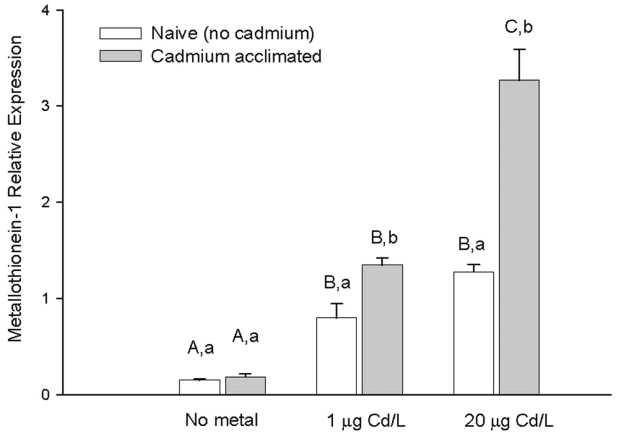

Figure 1. Increased MT1 mRNA expression levels in Cd-acclimated (filled-bar) *Daphnia pulex* after 50 generations compared to naïve (open bar) when exposed to Cd. MT1 relative expression levels were determined by qPCR for adult *D. pulex* from each population exposed to control water (No metal), 1 μg Cd/L, or 20 μg Cd/L for 24 h. Data were normalized to cDNA concentrations that were measured for each sample (n = 5). Capital letters signify statistically difference ($p < 0.05$) within population across treatments, and lowercase letters indicate statistical differences ($p < 0.05$) between populations within a treatment. The significant interactive term of the two-way full factorial ANOVA was further analyzed using a posthoc student's t test. All statistical analysis were performed in JMP.[62]

were similarly low for both naïve and Cd-acclimated cultures in control conditions (i.e., no Cd) and, as expected, transcription of the MT gene was induced in both populations following exposure to Cd for 24 h. However, the magnitude of this induction was significantly greater in the Cd-acclimated population at both exposure concentrations, i.e., 1 and 20 μg Cd/L.

This is the first whole animal study to report that multigeneration chronic exposure to environmentally relevant Cd concentrations influences regulation of the MT1 gene and the hyper-induction of MT in the presence of toxic concentrations of metals. Other studies have examined response in single generations. Li et al.,[12] showed similar results using pulse Cd exposures on *D. magna* in a single generation. Stennard et al.[69] also suggested hyper-induction following repeated Cd exposures in single generation studies with primary cultures of rat hepatocytes but were unable to make statistical determinations. Likewise, Hart et al.[70] studied repeated Cd exposures of sheep lungs but could not assess effects on MT mRNA because transcript levels remained elevated in animals receiving Cd pretreatment without any subsequent exposure. Nevertheless, MT is overexpressed in many Cd resistant cell-lines[71−73] and the MT gene has been functionally linked to the Cd sensitivity in transgenic and knockout mice.[68,74]

Benefits and Costs of Acclimation Tolerance Acquisition. We tested the hypothesis that fitness trade-offs (i.e., benefits and costs) increase in acclimated populations, using 21 d chronic toxicity tests with naïve and Cd-acclimated populations in the presence (1 and 2.5 μg Cd/L) and absence of Cd. The stressor Cd inhibited the birth rates of both populations relative to their respective controls (i.e., no Cd). However, Cd-acclimated *Daphnia* maintained higher rates of reproduction compared to the naïve group when metals were present, but this pattern was reversed when Cd was removed (Figure 2a). Cadmium pre-exposure also produced a delay in the age of first reproduction in Cd-acclimated *D. pulex* compared to their naïve counterparts in the absence of metal (13 vs 11 d, Figure 2b). Thus, the benefits associated with maintaining an increased tolerance to metal toxicity including hyper-induction of the MT gene are accompanied by costs in the absence of metal stressor that manifest at the population-level, reducing the ability to develop, reproduce, and ultimately sustain population growth.

Theory suggests that fitness trade-offs are necessary to maintaining a tolerant phenotype.[15,75−78] There are no better examples of such trade-offs than insecticide resistance mechanisms from among at least 590 species of insects that have genetically evolved resistance to around 300 compounds.[79] However, empirical evidence demonstrating the costs associated with physiological acclimation is sparse. Temple and Johnston[45] found that thermal acclimation in the short-horn sculpin improves their escape response in warm waters at the cost of slower escape times in cooler waters. Likewise, warm-acclimated lobsters can tolerate higher temperatures, but the range of temperatures they can tolerat is smaller than lobsters acclimated to cooler temperatures.[46] However, these trade-offs have not been directly linked to population-level outcomes across multiple generations as demonstrated in the present study and which some have argued is necessary for assessing fitness.[75] Our findings are supported by multigeneration studies with *D. magna*, discussed previously, that although not rigorously tested, observed apparent population-level costs for some Cd-acclimated populations when metal concentrations were reduced.[8,51] It has been suggested that due to these costs, the Cd-tolerant phenotypes would be rapidly lost in the absence of metal stressor,[75] a theory we were able to test in this study.

Loss of Metal Tolerance. We tested the hypothesis that in the absence of metal, the Cd-acclimated phenotype is rapidly lost, using standard metal toxicity tests to determine differences between three isoclonal cultures of *D. pulex*-Naïve, Cd-acclimated, and Cd-acclimated but removed from metal over multiple generations. Both the naïve and Cd-acclimated group produced a common (logistical/sigmoid) concentration−response curve, but the Cd-acclimated groups was shifted to the right (Figure 3), indicating increased tolerance. Tolerance was lost within two generations of being

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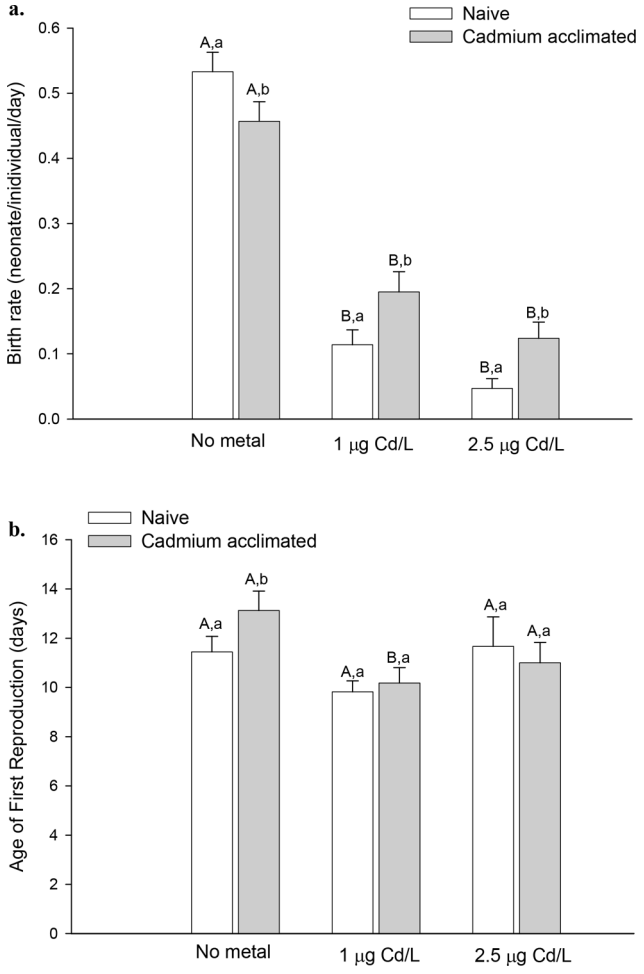

Figure 2. Per capita birth rate (a) and age to first reproduction (b) showed higher fitness for Cd-acclimated (filled-bar) *Daphnia pulex* compared to naïve (open bar) population. Per capita birth rate was calculated from chronic toxicity tests that exposed *Daphnia* (<24 h old) from each population to control water (no metal), 1 μg Cd/L, or 2.5 μg Cd/L for 21 days. Bars with different capital letters are significantly different ($p < 0.05$) for comparisons within populations, while bars with different lower-case letters are significantly different ($p < 0.05$) for comparisons between populations within treatments. Analysis were performed using a two-way full factorial ANOVA followed by a posthoc student's *t* test using JMP.[62] The significant interactive term of the two-way full factorial ANOVA was further analyzed using a posthoc student's *t* test. All statistical analyses were performed in JMP.[62] Per capita birth rate $n = 106$ and age to first reproduction $n = 100$.

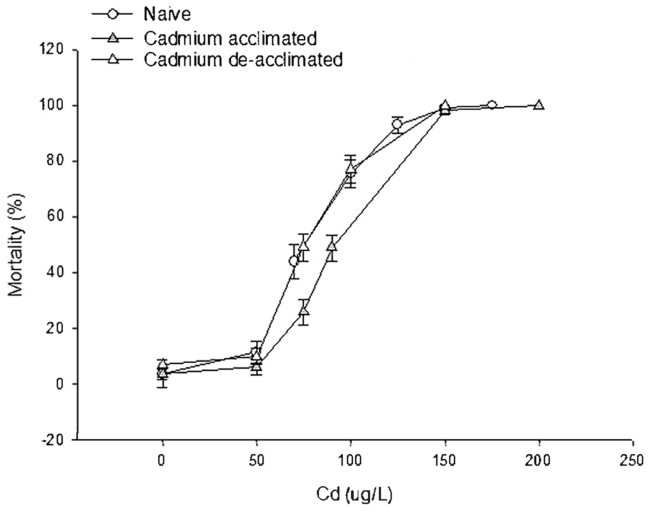

Figure 3. Acute toxicity (48 h) as a function of Cd concentration in three isoclonal *D. pulex* cultures, naïve, Cd-acclimated, and Cd deacclimated, showed acclimated populations removed from metal lost tolerance in within 2 generations. Error bars represent standard error.

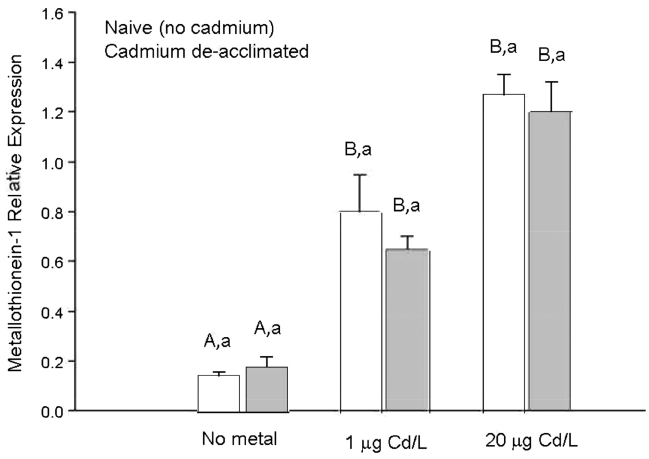

Figure 4. Metallothionein 1 mRNA relative expression levels do not statistically differ between naïve (open bar) and Cd deacclimated (closed bar) *Daphnia pulex* populations. For these experiments, Cd-acclimated *D. pulex* were removed from Cd over a single a generation (i.e., <24 h old to 14 days) and rechallenged with Cd on day 14. Metallothionein 1 relative expression levels were determined by qPCR for adult *Daphnia* from each population exposed to control water (no metal), 1 μg Cd/L, or 20 μg Cd/L for 24 h. Data were normalized to cDNA concentrations that were measured for each sample, $n = 5$. Capital letters signify statistically difference ($p < 0.05$) within population across treatments and lowercase letters indicate statistical differences ($p < 0.05$) between populations within a treatment. The significant interactive term of the two-way full factorial ANOVA was further analyzed using a posthoc student's *t* test. All statistical analysis were performed in JMP.[62]

removed from metals, demonstrated by a return (left shift) of the concentration−response curve to that of the naïve population (Figure 3). To determine if decreased expression levels of the MT1 gene accompanied the observed loss of tolerance, we also evaluated MT1 gene expression following the removal of Cd. Expression level differences between naïve and Cd-acclimated cultures removed from Cd were determined in the presence (1 or 20 μg Cd/L for 24 h) and absence of Cd. While changes in MT1 induction were expected to occur over several generations once Cd exposure was terminated, the hyper-induction was lost within a single generation (Figure 4). As before (Figure 1), there were no differences in transcript-levels for either population maintained in control conditions and both populations increased expression of the MT1gene following a subsequent Cd challenge. However, when Cd-acclimated *Daphnia* were removed from Cd there were no differences in the magnitude of this induction when compared to naïve populations. In agreement with our demographic studies, the speed at which tolerant phenotypes and their associated mechanisms were lost (within 2 generations) suggests that there are significant costs associated with maintaining acclimation phenotypes in the absence of the stressor.

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We conclude that multigeneration exposure to environmentally relevant levels of Cd can expand the physiological limits of Cd tolerance and cross-tolerance to toxic concentrations of Zn and Ag, but not As, in *Daphnia*. Gene expression studies suggested that the metal binding protein MT1 is important for the acquired Cd-tolerant phenotype, in that acclimated animals were able to produce more MT1 transcript than naïve animals in the presence of metals, but there were no differences in transcript levels in the absence of metals. Chronic toxicity studies indicated that Cd tolerance increased reproductive fitness in the presence of Cd. However, Cd-acclimated *Daphnia* exhibited a reduction in reproductive fitness compared to naïve *Daphnia* when Cd was removed, demonstrating that there are costs associated with the tolerant phenotype. Furthermore, the tolerant phenotype was lost within two generations (G_n to G_{n+1}), while the enhanced ability to induce MT1 transcription was lost within a single generation following metal removal. These rapid losses further suggest there are costs associated with the tolerant acclimation phenotype. The environment dependent plasticity of this heritable, stress-tolerance phenotype was rapid and stable in stressor conditions (i.e., Cd exposure), and costly to maintain. An understanding of acclimation processes is necessary to assess the long-term fate of populations, especially in changing environments, since they contribute to evolved tolerance (i.e., adaptation) in complex ways. By providing a deeper understanding of acclimation, these studies will also assist regulators' ability to assess the risk of toxic stressors in the environment and help establish appropriate metrics for remediation of contaminated sites, which may carry negative fitness consequences if organisms have acclimated to polluted conditions.

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The authors declare no competing financial interest.

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