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Metamorphosis Enhances the Effects of Metal Exposure on the Mayfly, *Centroptilum triangulifer*

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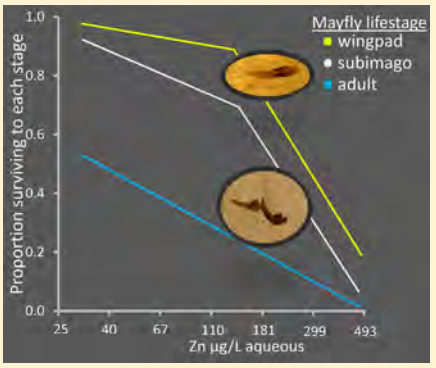
Supporting Information

ABSTRACT: The response of larval aquatic insects to stressors such as metals is used to assess the ecological condition of streams worldwide. However, nearly all larval insects metamorphose from aquatic larvae to winged adults, and recent surveys indicate that adults may be a more sensitive indicator of stream metal toxicity than larvae. One hypothesis to explain this pattern is that insects exposed to elevated metal in their larval stages have a reduced ability to successfully complete metamorphosis. To test this hypothesis we exposed late-instar larvae of the mayfly, *Centroptilum triangulifer*, to an aqueous Zn gradient (32−476 μg/L) in the laboratory. After 6 days of exposure, when metamorphosis began, larval survival was unaffected by zinc. However, Zn reduced wingpad development at concentrations above 139 μg/L. In contrast, emergence of subimagos and imagos tended to decline with any increase in Zn. At Zn concentrations below 105 μg/L (hardness-adjusted aquatic life criterion), survival between the wingpad and subimago stages declined 5-fold across the Zn gradient. These results support the hypothesis that metamorphosis may be a survival bottleneck, particularly in contaminated streams. Thus, death during metamorphosis may be a key mechanism explaining how stream metal contamination can impact terrestrial communities by reducing aquatic insect emergence.



INTRODUCTION

Water quality criteria are typically derived by measuring the response of noninsect model organisms to pollutants in the aquatic ecosystem.[1−3] However, aquatic habitats are dominated by insects, nearly all of which undergo metamorphosis from aquatic larvae to winged terrestrial adults.[4,5] This water-land transition is ecologically important for two reasons: (1) it allows reproduction and dispersal by winged aquatic insects, and (2) it represents a pathway for nutrients, energy, and contaminants to move from aquatic to terrestrial food webs.[6,7] For example, adult aquatic insects subsidize the diets of terrestrial insectivores like birds, spiders, bats, and lizards.[8−12] They also serve as potential vectors of contaminant transport from aquatic to terrestrial food webs.[7,13] Understanding how aquatic stressors affect aquatic insect emergence is therefore important for managing both aquatic and riparian ecosystems.[13−15]

It is often assumed that aquatic insect emergence is directly related to larval insect density.[16,17] In other words, if we know the factors affecting survival of larvae, we can then predict the density of adults. However, recent field work contradicts this assumption. In alpine streams, insect emergence was decoupled from benthic insect density across a gradient of stream metal contamination in the central Rocky Mountains.[15] As a result, insect emergence density was a more sensitive indicator of stream metal contamination than the more commonly studied end point of benthic insect density. Importantly, the data from Schmidt et al.[15] suggested that while current aquatic life criteria are reasonably protective of the larval aquatic stages of insects, they may not be protective of adults. This finding means that aquatic life criteria may not be sufficient to protect the ecosystem services that emerging aquatic insects provide to terrestrial ecosystems.[13,15]

One hypothesis to explain the decoupling between larvae and adults is that stream metal concentrations that are sublethal to larvae become lethal during the physiologically demanding process of metamorphosis. Metamorphosis between larval and adult stages is a common feature of insects.[18] It has evolved, in part, because it enhances dispersal.[18] This benefit is balanced by the physiological stress caused by metamorphosis.[19−22] For example, oxidative stress increased during metamorphosis in amphibians and damselflies.[20,21] In damselfies, this increase in stress led to morphological asymmetry in adults, even though the larvae of those adults were unstressed.[21] This leads to the reasonable hypothesis that metamorphosis in aquatic insects

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functions as a stressor (i.e., the "stressful metamorphosis hypothesis"[21]). Thus, aquatic insects in stressful larval environments may be able to cope with sublethal environmentally induced stress, but unable to survive the additional stress of metamorphosis. For aquatic insects exposed to metals, emergence may represent a bottleneck in which larvae survive metal exposure and leave the benthos to emerge, only to die during emergence.

We tested the hypothesis that mortality during metamorphosis is affected by metal contamination by exposing a laboratory culture of the mayfly, *Centroptilum triangulifer*, to an aqueous gradient of Zn. We measured survival of different end points during metamorphosis (late instar larva, dark wingpad development, subimago, and imago). Based on observations from field data[15] and evidence in the literature that metamorphosis is physiologically stressful[20,21] and can be altered by contaminant exposure,[23] we predicted that survival would decline during metamorphosis, and that the magnitude of this decline would change across the Zn gradient.

MATERIALS AND METHODS

Microcosms. The experiment took place at the Aquatic Experimental Laboratory (AXL) at the USGS Fort Collins Science Center (Fort Collins, CO). Microcosms consisted of sterile plastic mouse cages (model no. PC7115HT, Allentown Caging Equipment Co. Inc., Allentown, NJ) filled with 2.5 L of moderately hard water reconstituted from reverse-osmosis filtered water (Table 1). This filled the cage approximately $^1/_4$ full, leaving ~7 cm of the cage wall as a substrate for subimagos to cling to during imago emergence. Cages were placed in an environmental chamber with a 16:8 light:dark cycle and constant air temperature (27 °C), producing an average water temperature of 24.8 °C. Other studies have shown that water temperatures of 25 °C maximize growth rates in this species with high survival.[24,25] Water was continuously oxygenated with air stones and cages were covered with plastic wrap to prevent emerged adult mayflies from escaping. Conductivity, dissolved oxygen, pH and water temperature were measured weekly (HQ40, Hach Company, Loveland, CO; Table 1). Weekly 80% water replacement was made to maintain conductivity below ~350 uS, following Hammer.[24]

Table 1. Summary Statistics of Average Water Temperature, Conductivity, pH, Dissolved Oxygen, and Hardness in Cages. Data Are the Means of Means Estimated Weekly in Each Cage

	mean	SD	min	max
temperature (°C)	24.8	0.3	24.1	25.2
conductivity (μS)	259	38	229	370
pH	8.6	0.1	8.5	8.7
dissolved oxygen (mg/L)	7.1	0.2	6.7	7.4
hardness	67.4	9.6	59.2	96.6

Design. We obtained *C. triangulifer* eggs from a permanent mayfly culture at AXL. This culture is ultimately derived from a laboratory colony (WCC-2 clone) developed by the Stroud Water Resource Center (Avondale, PA) from a population in White Clay Creek, PA.[25] Eggs from three females (~1000 eggs from each female) had been stored in separate 15 mL glass vials for ~3 weeks at 10 °C air, a temperature which keeps eggs alive but prevents hatching for several months. To induce hatching, we slowly increased the temperature to 25 °C by raising the temperature by 2 °C per day in an environmental chamber. Eggs began hatching on 6 June 2013, after 2 days at 25 °C. On this date, we transferred the larvae from each vial to 1000 mL glass beaker and added a 1 mL slurry of diatoms from laboratory stocks (*Nitzschia* sp. and *Synedra* sp.) for food (Table S-1, Supporting Information (SI)). Eggs continued to hatch for 4 days. After 4 days, 10 June 2013, we combined hatched larvae in a watch glass to ensure that larvae came from multiple females. Under a dissecting microscope, we transferred 30 early instar *C. triangulifer* larvae to each of 24 experimental cages using steril plastic 5 mL pipets. On the day of transfer, larvae were 1−4 days old as estimated from the initial date when larvae began hatching from eggs. Larvae were acclimated in the cages for 16 days and fed diatoms. We added diatom slurries twice weekly in 25 mL aliquots. Visible diatom mats were apparent several days after the initial diatom slurry was added, confirming that diatoms were growing in the cages and that larvae were not food limited.

When larvae were approximately 20 days old (late-instar, 26 June, SI Table S-1), we added dissolved $ZnSO_4$ to randomly assigned cages during an 80% water replacement to achieve six nominal Zn concentrations (0, 30, 75, 150, 300, or 600 μg/L Zn), marking the start of the experiment (Day 0). Zn was also added during subsequent weekly water exchanges to maintain nominal concentrations (see below). While we did not measure metal uptake by diatoms, a previous experiment in our laboratory demonstrated a bioconcentration factor in diatoms of >1500, based on an aqueous concentration of 300 μg/L Zn, though this was measured only once, and we do not know if it represented a steady state condition. Further, Kim et al.[26] found that Zn bioacummulation in C. triangulifer was driven primarily through dietary uptake, and De Schamphelaere et al. Twenty-seven found that exposure to dietary Zn reduced reproduction by 40% in *Daphnia magna*. Metals such as Zn might also affect organisms indirectly by reducing the nutritional quality of their food.[28] While we did not measure the specific route of exposure, it is likely that effects of Zn exposure in this experiment were from a mix of aqueous and dietary uptake, along with potential nutritional effects in high Zn environments.

Prior to Zn addition, we noted that eight of the 24 cages did not appear to have visible larvae. Some mortality of early instar larvae is normal in these cultures, particularly if nymphs do not feed within 12 h of hatching,[29] and the replicated regression design was chosen to account for possible mortality in the acclimation phase. However, *C. triangulifer* larvae are often too small to accurately see until they are >20 days old, so we added Zn to these cages according to their preassigned nominal concentrations. None of these eight cages produced visible larvae, even after ~40 days. Because this occurred at least once in each treatment (one cage each in 0, 30, 75, and 300 μg/L treatments and two cages in the 150 and 600 treatements), and was consistent with observations before the addition of Zn, we assumed that mortality was not a result of Zn exposure and therefore eliminated these cages.

In the remaining 16 cages, we qualitatively confirmed live larvae on Day 0, but could not make a full count of larvae until Day 6 (SI Table S-1). Full counts prior to this date would have required a dissecting microscope, necessitating a disturbance by draining water and removing larvae from their cages. The range of starting densities estimated by visual counts on Day 6 was 16−29 individuals or 53−97% of the initial 30 larvae (mean 74% ± 14%). There was no effect of Zn on larvae at Day 6 (see

Results), indicating that the variation in starting densities was unrelated to zinc exposure. At the end of the experiment (Day 16) four cages still had live larvae (n = 10 total larvae, <3% of starting density (SI Table S-1)). These larvae were excluded from survival calculations because we could not assess their survival to these stages (SI Table S-2). All larvae in the other cages had either died or emerged at the end of the experiment. On rare occasions (n = 3 cages) the number of larvae counted on Day 6 was lower than the subsequent number of emerging mayflies. In those cases, we used the number of emerging mayflies to correct our Day 6 counts. Thus, we used these corrected starting densities (range: 16−29 larvae per cage) as the basis for estimates of larval survival and the rate of survival to wingpad, subimago, and imago, rather than using the initial 30 larvae (SI Table S-2). We chose this as a conservative measure to account for any pre-experiment mortality. However, the shape of the response curves are similar whether the starting densities are based on all 30 larvae or based on corrected densities (SI Figure S-1). In seven cages, >80% of the original 30 larvae survived both the acclimation period and the six day exposure period. To determine whether our results changed if only high-survival cages were used (i.e., >80%), we reran the analyses (see Analyses) using only these seven cages.

We measured aqueous Zn concentrations once before Zn additions (Day 0) and six times after (SI Table S-1; Figure 1).

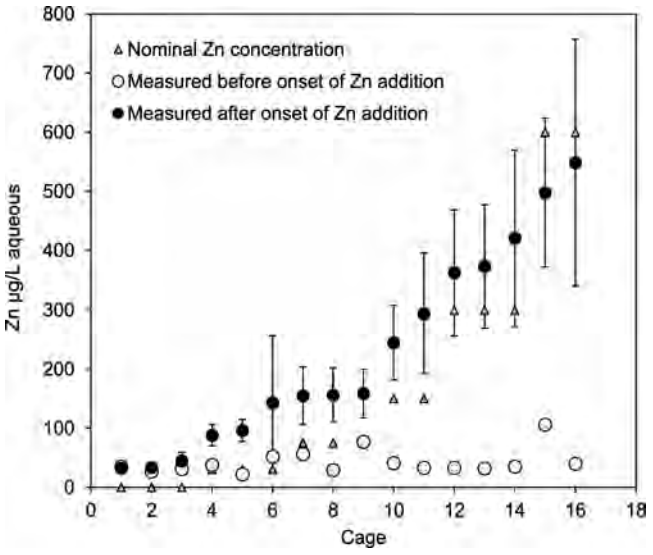

Figure 1. Nominal and measured Zn concentration in experimental cages before (June 26, Day 0) and after (average of six measurements) Zn addition. Error bars represent standard deviations.

For each sample, we filtered 15 mL of water through a 0.45 μm filter (Acrodisc syringe filter with Supor membrane) into an analytically clean 15 mL Falcon tube. We then acidified the sample with two drops of ultrapure HNO_3. Trace metal concentration was analyzed at the Crustal Geophysics and Geochemical Science Center (U.S. Geological Survey, Denver, CO) using inductively coupled plasma mass spectrometry (ICP-MS). Sample recovery of three replicate standards was high, averaging 101.9% (range: 100.2−102.9%), and three replicate blank samples (DI water) were below detection (<0.5 μg/L).

Emergence began on Day 6. Beginning on this date, we made daily counts of larvae, subimagos, and imagos. We also counted the number of larvae with dark wingpads, and noted whether subimagos or imagos had morphological defects (i.e., shriveled wings). These stages represent the dominant transition stages in mayflies, which are hemimetabolous insects that do not have a pupal stage. Dark wingpad development marks the end of the larval stage in mayflies. At this point they have stopped feeding and growing in preparation for emergence. The transition between the wingpad stage and emergence takes less than 24 h for most species,[30] including *C. triangulifer*. Mayflies first emerge as immature, winged subimagos (subadults), usually in the evening. Sexually mature adults (imagos) emerge from the subimago several hours later through early morning. For this reason, we made counts in the morning to allow enough time for subimagos to metamorphose to imagos. If a subimago was still alive in the morning, but had not metamorphosed to imago, we left it in the cage until it either died or metamorphosed. All emerging mayflies (live and dead imagos and dead subimagos) were removed daily to avoid double counts. A subset of dead subimagos and imagos were collected in individually labeled vials, dried for >24 h at 60 °C, and weighed to the nearest 0.01 mg.

Analysis. Measured Zn concentrations were different from nominal targets in most cages (average difference of measured minus nominal, 32 ± 72 μg/L Zn, Figure 1). We used the resulting Zn gradient to analyze the data as a simple linear regression (or piecewise, see Analysis) rather than a replicated regression across fixed nominal groups. The four end points were larval density on Day six, the total proportion of larvae that developed wingpads, the total proportion that successfully emerged to at least subimago, and the total proportion that successfully emerged to adulthood. We defined successful emergence to either subimago or imago as a mayfly that fully shed its larval or subimago exuvia and did not show obvious morphological defects (i.e., shriveled wings (Figure 2)).

Figure 2. *Centroptilum triangulifer* subimago with shriveled wings (left) and emerged imago with normal wings (right). Photo credit, J. Wesner.

Because metamorphosis can be a source of mortality even in low stress environments,[22] we assumed that there would be a drop in survival as metamorphosis progressed through time regardless of Zn exposure (i.e., more insects would successfully develop wingpads than would emerge to subimago, and more would emerge to subimago than would emerge to imago). However, the key question is whether this drop is constant across the Zn gradient. To test this, we subtracted survival at a given stage from survival at the next stage for each tank. For example, if wingpad development in a cage was 90% and subimago emergence was 70%, that would be a difference of −20 percentage points in the transition between wingpad and subimago. We tested whether this difference varied across the Zn gradient using separate linear regressions on treatments that

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were either below (n = 5 cages) or above (n = 11 cages) the hardness-adjusted aquatic life criterion (105 μg/L). We did separate analyses to account for the fact that survival to a given stage was not a constant function of Zn concentrations, but was instead defined by thresholds (see *Results*). As a result, the drop in survival between stages was expected to be small at low Zn levels, increase at moderate Zn levels, and become small again at high Zn levels as survival to all stages converged toward zero. Because all thresholds identified in the piecewise regressions were above 105 μg/L, analyzing the data around this cutoff is both environmentally relevant and conservative.

Zn concentrations were natural log transformed prior to analysis to improve linearity and normality, as confirmed by residual plots. We used Akaike's Information Criterion corrected for small sample size (AIC_C) to determine which model (linear or piecewise) best explained the relationship between an end point and Zn concentration. To compare survival of each end point to an environmentally relevant Zn concentration, we used the regression results to estimate survival at the hardness-adjusted aquatic life criterion for Zn.[31]

To determine whether Zn exposure affected the body size (dry mass) of individual emerging mayflies (as a proxy for fecundity), we used linear regression to test the relationship between subimago or imago body size and natural log-transformed Zn concentrations. To determine if metal exposure delayed development,[32−34] we estimated the date at which mayflies first developed wingpads, first emerged as subimagos, and first emerged as imagos. We then used linear regression to test the relationship between these stages and natural log-transformed Zn concentrations. We also estimated the date at which at least 50% of mayflies developed wingpads or emerged as subimagos. Imagos were excluded from this analysis because survival to imago was below 50% for 14/16 cages, likely due to imagos being trapped by water droplets (see Results).

All analyses were performed in R.[35] We used the package siZer[36] to model the piecewise regressions.[37] We chose siZer because it minimizes spurious detection in small data sets (<50 observations[38]).

RESULTS

Effects of Zn Exposure on Mayfly Life Stages. Larval density after 6 days of exposure was not affected by Zn concentrations (r^2 = 0.06, p = 0.36, Figure 3a). Larvae developed dark wingpads in all but one treatment (366 μg/L). On the first day of larval counts (Day 6), 14/367 larvae (4%) had developed wingpads or already emerged. Over the entire experiment, the piecewise model best described the relationship between the proportion of larvae that developed wingpads and Zn concentration (Table 2, Figure 3b). The model indicated a threshold at 138 μg/L Zn. Below this level, wingpad development averaged 95 ± 2% (mean ± standard error; range: 86−100%). Above this level, wingpad development averaged 47 ± 18% (range: 0−92%), and declined by 61 percentage points with every natural log unit increase in Zn (Figure 3b). The piecewise linear equation predicted that 89% of larvae developed wingpads at the hardness-adjusted U.S. EPA aquatic life criterion.[31]

Subimagos emerged from 14/16 cages. 86% of emergence occurred between days 8 and 13. On these days, 36−56 mayflies emerged daily. On all other days, less than 12 mayflies emerged daily. Five subimagos had shriveled wings following eclosure from their larval exuvia (Figure 2). All of these

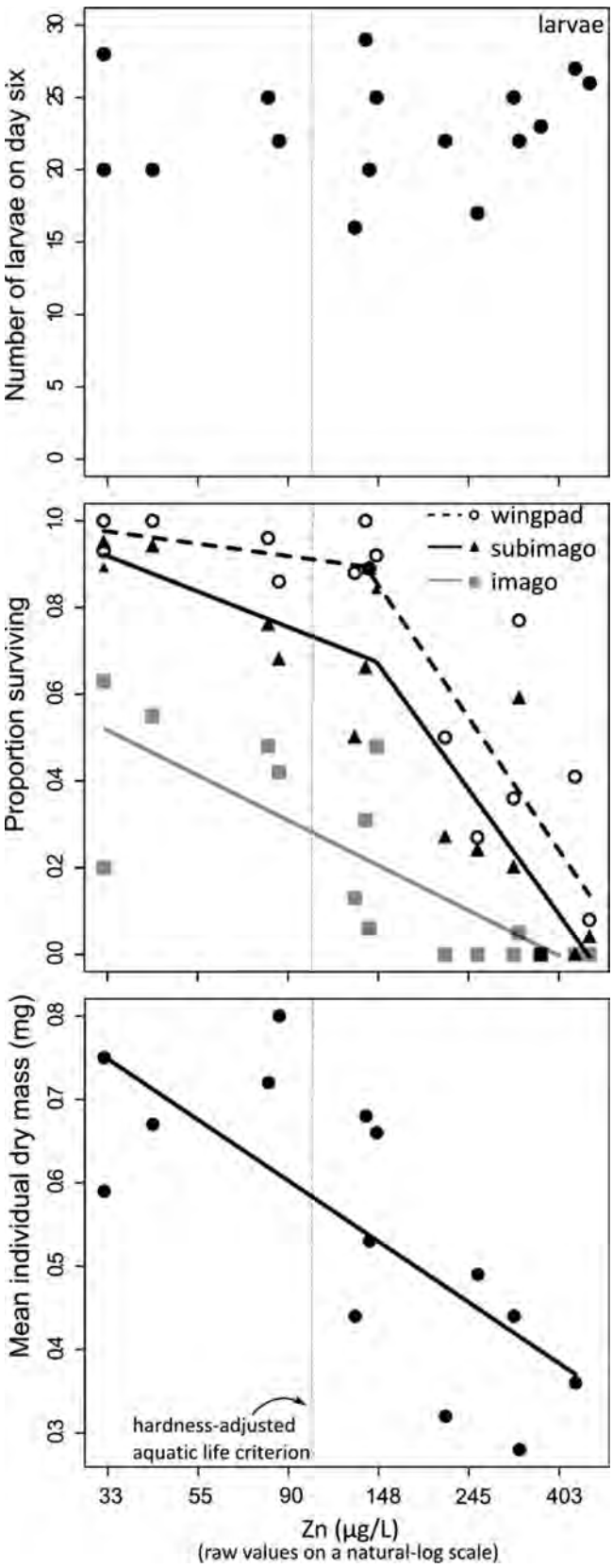

Figure 3. Simple linear and piecewise regressions for the effects of aqueous Zn exposure on (a) number of larvae on Day 6, (b) proportion of initial larvae surviving to a given developmental milestone (dark wingpad, subimago, imago), and (c) average of individual subimago body size. Arrows indicate the hardness-adjusted aquatic life criterion for Zn (105 μg/L).

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Table 2. Model Selection among Piecewise and Linear Models to Explain the Relationship between Survival to Each Stage (Wingpad, Subimago, Imago) And the Natural Log of Zn Aqueous Exposure

survival stage	model	K	AIC_C	ΔAIC_C	ω_i	threshold	95% CI	model slope[a]	95% CI
wingpad	piecewise	3	−1.5	0.0	0.84	4.9	4.1−5.8	−0.06	−0.13, 0.47
	linear	2	2.1	3.6	0.16			−0.31	−0.45, −0.17
subimago	piecewise	3	−4.5	0.0	0.62	4.9	3.8−5.7	−0.16	−0.27, 0.19
	linear	2	−3.5	1.0	0.38			−0.34	−0.46, −0.22
imago	piecewise	3	−8.8	2.4	0.23	3.7	3.5−5.6	0.5	−0.42, 2.78
	linear	2	−11.3	0.0	0.77			−0.21	−0.30, −0.12

[a]Only initial slopes are given for piecewise models.

individuals came from three cages with Zn concentrations between 216 and 325 μg/L.

The linear and piecewise models were nearly equivalent (<2 ΔAIC_C) in their explanation of the negative relationship between subimago emergence and Zn concentration (Table 2, Figure 3b). In Figure 3b we plot the piecewise model because it was the top-ranked model. It indicated a threshold of 147 μg/L. Below this level, survival to subimago averaged 79 ± 5% (mean ± standard error, range: 50−95%), and there was a negative, but nonsignificant, relationship between subimago emergence and natural log-transformed Zn (initial slope of −0.16 compared to −0.06 in the prethreshold wingpad regression, though 95% CI's overlap; Table 2). Above this level, survival to subimago averaged 19 ± 18% (range: 0−59%), and the proportion of larvae surviving to subimago declined by 58 percentage points with every natural log unit increase in Zn (Figure 3b). The piecewise equation predicted that 75% of larvae survived metamorphosis to subimago at the hardness-adjusted aquatic life criterion. Subimago body size declined linearly with increasing Zn concentration (r^2 = 0.54, p < 0.01; range, 0.28−0.80 mg; slope ± standard error, −0.15 ± 0.04; Figure 3c), decreasing by about 3-fold over the exposure gradient.

On average, survival to subimago was 15 ± 13 (mean ± SD) percentage points lower than wingpad survival, but this decline changed across the Zn gradient. At Zn concentrations below the hardness-adjusted aquatic life criterion, the drop in survival between the wingpad and subimago stage increased 5-fold from −4 to −20 percentage points across the Zn gradient (r^2 = 0.96, p = 0.004). Above this threshold, the drop in survival ranged from 0 to −41 percentage points, but was not related to Zn concentration (r^2 = 0.02, p = 0.64).

Imagos emerged from 10/16 cages. The proportion of larvae surviving to imago ranged from 0 to 63%. The linear model provided the best explanation of the negative relationship between imago emergence and Zn concentration (Table 2, Figure 3b). On average, only 26% of larvae survived to imago at the hardness-adjusted aquatic life criterion. At concentrations above 147 μg/L, survival to imago approached zero (complete failure in six cages and 5% success in one cage; Figure 3). Many of the failed imagos (i.e., dead subimagos) in all cages were found stuck to the water droplets on the cage walls. This would not have affected larva-subimago emergence, but likely artificially reduced our estimates of imago survival.

On average, survival to imago declined by 32 ± 23 (mean ± SD) percentage points relative to subimago survival. At Zn concentrations below the hardness-adjusted aquatic life criterion, the drop in survival between the subimago and imago stage ranged from −29 to −75 percentage points, but was not related to Zn concentrations (r^2 = 0.31, p = 0.33). Above the criterion, the drop in survival ranged from 0 to −83 percentage points, and was positively related to Zn concentration (r^2 = 0.46, p = 0.02). This reflects the fact that the drop in survival declined with increasing Zn as both subimago and imago survival approached zero.

The relationship between Zn concentration and imago body size trended negative but was not significant (r^2 = 0.11, p = 0.36; range, 0.29−0.80 mg, n = 10). However, because six of the seven cages with the highest Zn concentrations never produced imagos, this test has limited power. For the cages in which both subimagos and imagos emerged (n = 10/16), there was a positive, but nonsignificant relationship between subimago body size and imago body size (r^2 = 0.34, p = 0.08). This supports the idea that the weak relationship between imago body size and Zn concentration was indeed driven by data limitation, rather than a shift in the relationship between body size and Zn during the transition between subimago and imago.

Emergence Delay. We found no evidence that Zn exposure delayed metamorphosis to either the wingpad stage or emergence. This was true whether metamorphosis was measured as time to the first development of wingpads (r^2 = 0.004, p = 0.82), time to the first emergence as subimagos (r^2 = 0.05, p = 0.44), time until 50% of larvae developed wingpads (r^2 = 0.009, p = 0.82), or time until 50% of larvae emerged as subimagos (r^2 = 0.15, p = 0.27).

Reanalysis with Only High Survival Cages. All patterns above were retained, and the thresholds were nearly identical, when the analysis was limited to only those cages in which >80% of the original 30 larvae survived the acclimation phase and the six day Zn exposures (SI, Figure S-2; Table S-3).

DISCUSSION

Aquatic insects are the dominant taxa in most freshwater ecosystems[4], but are underrepresented in laboratory toxicity tests used to determine streamwater quality criteria.[1,39] In addition, the relatively few toxicity tests performed on aquatic insects through typically short-term exposures suggest that they are considerably more tolerant of metals than standard test species (e.g., cladocerans and fathead minnows;[3,24,40]). Our results support this concensus, showing no evidence of Zn effects on survival of late-instar *C. triangulifer*. It is important to emphasize that our exposure of late-instar larvae is less severe than natural exposures which include all life stages of aquatic organisms. A longer exposure through the entire larval development from egg to emergence may have increased larval mortality rates.

In contrast to laboratory studies, field studies of aquatic insects suggest that they are sensitive indicators of stream contamination.[3] One explanation for the disconnect between laboratory and field studies is that most laboratory studies do not account for major life-history events, such as egg-larval hatch, early instar survival, or metamorphosis (but see refs

41−43). Field studies implicitly account for these events by measuring the response of insects that have been exposed across multiple generations. In our experiment, the failure of many *C. triangulifer* to successfully emerge even at moderate Zn concentrations suggests that metamorphosis, a major life-history event, is likely a population bottleneck. Had we limited our study to premetamorphic larval exposure only, as is common in toxicity tests, we would have concluded no effect or potentially moderate effects, even though there was a clear effect on survival and potential reproduction of *C. triangulifer* as measured through metamorphosis. Our results complement recent calls for more realistic toxicity testing that includes, among other things, better taxonomic representation,[44] better understanding of the dynamics and physiology of metal uptake,[44] and better incorporation of critical life-history events.[38]

Survival to any stage of metamorphosis approached zero at Zn concentrations above ∼360 μg/L. This is notable given that laboratory studies of other mayfly species have found only minimal effects at concentrations up to 2 orders of magnitude higher. Brinkman and Johnston[40] estimated that 50% of *Rhithrogena hageni* (Heptageniidae) survived a 96 h exposure at 50 500 μg/L Zn. Moreover, after 10-days they found a lowest observed effect concentration (first concentration to significantly reduce survival) of 10 800 μg/L Zn, more than 20-fold higher than the highest concentration in this study. Clements[45] exposed a field collected larval insect assemblage to an aqueous Zn concentration ranging from 0 to 792 μg/L and found no significant relationship between mayfly survival and Zn. However, these experiments did not explicitly test survival during the critical stages of metamorphosis.

In our experiment, it is possible that the mayflies experienced additional stress during acclimation. For example, during the acclimation phase (i.e., before Zn additions), we observed 100% mortality of early instar larvae in 8 of 24 cages, indicating that our culture conditions during this phase were suboptimal. Weaver et al.[29]found high early instar mortality (67−72%) in some lab generations of *C. triangulifer*, but this disappeared when larvae were fed diatom slurries within ∼12 h after hatching. In our experiment, early instar larvae had access to food, but we could not confirm whether they actually fed. Further, it is unclear why early instar mortality prior to dosing was high in some cages, but not others, since all cages were treated identically. Either the factors causing mortality in the eight cages were not present in the remaining 16 cages, or they were present and the mayflies in those cages were simply robust. Though we cannot test this, if the latter case is true, then our results should be interpreted as reflecting Zn exposure only on larvae that had survived a period of stress (dietary or otherwise) in early life. Dietary limitation seems unlikely, however, given that the size of adults across all treatments averaged 0.6 ± 0.2 mg (mean ± SD). Sweeney and Vanotte[25] found nearly identical adult sizes (0.6 ± 0.1 mg) for *C. triangulifer* raised with unlimited food at 25 °C. Finally, our results remained the same when reanalyzed using only cages in which total larval survival up to 6 days of exposure was >80% (SI). This indicates that in cages in which initial larval habitat quality was presumably high, Zn still caused substantial mortality during metamorphosis.

Six days of exposure at the hardness-adjusted aquatic life criterion (105 μg/L) was protective of larval survival and dark wingpad development for *C. triangulifer*. However, the transition between dark wingpad development and emergence was negatively affected by Zn concentrations at or below the aquatic life criterion. For example, in two treatments with moderate Zn concentrations of 81 and 85 μg/L, 96 and 86% of larvae developed dark wingpads, but only 76 and 68% of larvae emerged to the subimago stage, respectively. This means that ∼20% died in the transition between water and land at these exposure levels. In contrast, at low Zn concentrations (∼30 μg/L), the drop in survival between wingpad development and subimago emergence was less than 5%, demonstrating that the "cost" of metamorphosis increases with increasing Zn. Other researchers have demonstrated similar costs of metamorphosis. For example, Sibley et al.[43] found enhanced mortality at the pupal stage of midges (*Chironomus tentans*) exposed to Zn relative to larvae (see also ref 46). Additionally, McCauley et al.[22] found that metamorphosis failure of dragonflies (*Leucorrhinia intacta*) increased from 2 to 11% when larvae were exposed to nonlethal predator stress of caged fish. Finally, Scheifler et al.[40] found that carabid beetles (*Chrysocarabus splendens*) died only during pupation when larvae were exposed to dietary cadmium. These results further support the stressful metamorphosis hypothesis[21] by showing that larval stress can magnify innate costs associated with metamorphosis, with potential consequences for our interpretations of water quality criteria.

While our results are limited to one species in one experiment, the different response between larval and emergent insects is consistent with a recent broad field survey. Schmidt et al.[15] analyzed data from benthic insects at 125 sites in the central Rocky Mountains and emerging insects at 14 of those sites[13] to estimate the relationship between stream metals and larval or emerging densities of aquatic insects. They found that larval insect density declined rapidly between moderate to high metal concentrations, but was relatively constant between low to moderate concentraions. In contrast, adult densities from the same streams declined even at low metal concentrations.[13] Our results are consistent with these patterns, and clearly point to metamorphosis as a critical mechanism that may explain the different density responses of larval and adult insects to stream contamination in the field.

Adult aquatic insects provide an ecosystem service when they are eaten by terrestrial predators, transferring aquatic-derived energy and nutrients to riparian food webs.[6,8,10,11] Likewise, the larval stages of aquatic insects are integral to nearly all freshwater food webs on earth.[4,47] If these stages respond differently to aquatic stressors like metals, as demonstrated here and in the field,[13,15] then current aquatic life critera that protect only the larval stage may not be sufficient to protect the adult stage. As a result, new models focusing on the response of emerging aquatic insects are needed to minimize the ecological impact of stream pollution and other stressors on aquatic-terrestrial linkages.[13,14,3] Even emergence does not guarantee that insects can leave the water surface, as evidenced by the *C. triangulifer* in our experiment that had shriveled wings. They were unable to fly and unable to metamorphose to imagos. Though technically "emerged", in a natural stream, these insects would likely drown or be eaten by surface-feeding fish, further reducing energy and nutrient transfer between aquatic and terrestrial food webs.

There was a major drop in survival during the transition from subimago to imago. Part of this drop was likely an experimental artifact, in which adults tried to emerge, but failed because the subimago was stuck to water droplets on the cage walls. This artifact was presumably consistent across treatments. We urge

caution in interpreting our imago survival estimates, but suggest that the linear relationship between survival to adult and Zn concentrations would be similar even in the absence of an artifact.

Our focus in this study was on survival in response to Zn, but we also found a nearly 3-fold linear decline in adult body size across the Zn gradient. Reduced body size is a common response of insects exposed to contamination,[43] and can be caused either directly by metal toxicity or indirectly through a change in food quality.[28] In *C. triangulifer* (as well as many other insects), reductions in body size are linearly related to reductions in fecundity.[44] Thus, the large decline in adult body size we observed indicates a similarly large reduction in reproductive potential. As a result, our results based on survival likely represent a conservative estimate of the true population consequences of stream metal exposure.[43]

This study supports the hypothesis that aquatic insect emergence can be a more sensitive indicator of stream metal toxicity than larval survival. It also supports the hypothesis that metamorphosis is a critical event that can exacerbate the negative effects of larval stress.[21,22] Given the importance of aquatic insects as both indicators of stream health and vectors of aquatic-terrestrial energy transfer,[6,11,48,49,6] our results have implications for the derivation of water quality standards. Current standards do not account for the terrestrial stages of aquatic organisms,[15] but assume that protection of aquatic stages will translate to protection of adults. Yet both our results and those of recent field studies indicate that emerging aquatic insects respond differently to metal contamination than larval aquatic insects. To account for this different response, new models for determining the effects of stream contamination on adult aquatic insects are needed.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Timeline of events (Table S-1), raw data (Table S-2), model selection of high survival cages (Table S-3), comparison of survival using the full 30 larvae versus corrected larval densities (Figure S-1), comparison of survival using only high survival cages (Figure S-2). This material is available free of charge via the Internet at http://pubs.acs.org/.

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Notes

The authors declare no competing financial interest.

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