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Strong Delayed Interactive Effects of Metal Exposure and Warming: Latitude-Dependent Synergisms Persist Across Metamorphosis

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Supporting Information

ABSTRACT: As contaminants are often more toxic at higher temperatures, predicting their impact under global warming remains a key challenge for ecological risk assessment. Ignoring delayed effects, synergistic interactions between contaminants and warming, and differences in sensitivity across species' ranges could lead to an important underestimation of the risks. We addressed all three mechanisms by studying effects of larval exposure to zinc and warming before, during, and after metamorphosis in *Ischnura elegans* damselflies from high- and low-latitude populations. By integrating these mechanisms into a single study, we could identify two novel patterns. First, during exposure zinc did not affect survival, whereas it induced mild to moderate postexposure mortality in the larval stage and at metamorphosis, and very strongly reduced adult lifespan. This severe delayed effect across metamorphosis was especially remarkable in high-latitude animals, as they appeared almost insensitive to zinc during the larval stage. Second, the well-known synergism between metals and warming was manifested not only during the larval stage but also after metamorphosis, yet notably only in low-latitude damselflies. These results highlight that a more complete life-cycle approach that incorporates the possibility of delayed interactions between contaminants and warming in a geographical context is crucial for a more realistic risk assessment in a warming world.

■ INTRODUCTION

Current approaches to assess the risk of contaminants to aquatic ecosystems fail to adequately protect biodiversity.[1−3] This may not be surprising as these approaches are developed for single stressors while stressors may interact,[4] often do not include delayed postexposure effects,[5,6] and ignore geographic variation in the sensitivity to pollutants.[7] However, studies integrating these three key aspects are lacking, yet crucial to arrive at a more realistic ecological risk assessment. This is furthermore important in the context of global warming, which represents a serious threat that may strongly magnify the impact of contaminants.[8−11] Integrating warming into risk assessment of contaminants has therefore been identified as a major challenge for ecotoxicology.[12,13]

Delayed postexposure effects of contaminants can be equally strong or even stronger than the initial effects during exposure (e.g., refs 5, 6, 14−17). In animals with a so-called complex life cycle, such delayed effects may be revealed or magnified at metamorphosis, which has been identified as a sensitive, stressful event ("stressful metamorphosis hypothesis").[18] This is because the process of metamorphosis involves substantial tissue construction and reorganization, and requires a considerable amount of energy.[19,20] Recent studies demonstrated severe lethal effects of contaminants in aquatic insects during metamorphosis despite the absence of any effects during the preceding larval exposure period.[21−23] Yet, also after successful metamorphosis, there is still potential for delayed effects.[14,23,24] While studies are more frequently considering such delayed effects of single stressors across metamorphosis, and despite the increasing concern that interactions between stressors also occur when they are encountered sequentially,[25,26] delayed postmetamorphic effects of combined larval exposure to contaminants and other stressors, particularly warming, are very limited (but see refs 27, 28). Understanding how aquatic stressors affect aquatic insect emergence and their postmetamorphic survival is important for managing both aquatic and riparian ecosystems as aquatic insects subsidize the diets of terrestrial insectivores such as spiders and birds.[29,30]

Populations from the same species may differ widely in their sensitivity to contaminants[31−33] and to warming[34,35] because of geographic differences in the evolution of adaptation to these

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stressors. While adaptation to one stressor may ameliorate the negative consequences of exposure to another stressor,[36−38] this has been poorly studied in the context of warming and contaminants, yet crucial to arrive at a better risk assessment in a warming world.[8] Studies focusing on how differential thermal adaptation in populations from different latitudes creates a geographic signal in the interactive effects of warming and contaminants may be especially revealing. The comparison of contaminant effects in high-latitude populations with low-latitude populations currently experiencing the higher temperatures that are predicted to occur at the high latitude under global warming, may inform about the impact of gradual thermal evolution on the toxicity of contaminants in a warming world ("space-for-time substitution").[39]

To address these issues in an integrated way we investigated the effects of larval exposure to a metal and warming on larval and adult fitness-related traits in replicated high- and low-latitude populations of an aquatic insect. More specifically, we documented the combined effects of a metal and warming during and after the metal-exposure period in the larval stage (survival, growth rate, development time), during emergence (emergence ratio), as well as in the terrestrial adult stage (size at emergence, lifespan) in a common garden experiment with the damselfly *Ischnura elegans*. This allowed testing for delayed mortality caused by larval metal exposure and interactive effects with warming before, during and after metamorphosis. High-latitude populations were collected in southern Sweden, where the species is semivoltine (one generation per two years), whereas low-latitude populations were collected in southern France, where the species is multivoltine (three to four generations per year).[40] This difference in life-history strategy is expected to translate into a different susceptibility to contaminant exposure, with the more fast-lived southern populations being more susceptible, because of a trade-off between growth and investment in detoxification and repair mechanisms.[41] We further tested for a signal of thermal adaptation in how damselflies from both latitudes differed in their responses to the metal and temperature treatment (which matched the IPCC 4 °C warming scenario RCP8.5[42] at the higher latitude). By comparing the performance of the high-latitude populations at the local cooler and predicted warmer temperatures, predictions can be made about how high-latitude would perform under global warming in the absence of thermal evolution. Comparing them with the low-latitude populations (which have been locally adapted to warmer temperatures), however, simulates a global-warming scenario that also takes into account the performance change due to gradual thermal evolution.

■ MATERIALS AND METHODS

Sampling and Rearing. Using a common garden rearing experiment, we quantified key life-history traits before, during and after metamorphosis in the damselfly *Ischnura elegans* from two latitudes spanning more than 1500 km. Eggs were collected at three high-latitude locations in southern Sweden (56−57°N) and three low-latitude locations in southern France (43°N) (see Supporting Information, Appendix S1 for details).[43] At each location between 9 and 22 mated females were collected and placed individually in small jars with wet filter paper for oviposition. The eggs were transferred to the laboratory in Belgium where they hatched. Larvae were placed individually in 200 mL vials with dechlorinated tap water and assigned to a rearing temperature treatment of 20 or 24 °C. These temperatures reflect the mean water temperatures in shallow water bodies during summer in southern Scandinavia and southern France, respectively.[44] The 4 °C temperature difference between these latitudes matches the predicted temperature increase in southern Scandinavia by 2100 under IPCC scenario RCP8.5.[42] Therefore, the experimental setup allows a space-for-time substitution whereby the low-latitude populations reared at 24 °C provide a proxy for the evolutionary response of the high-latitude populations to global warming. Temperature was manipulated using water baths, across which rearing vials were regularly reshuffled. Throughout the experiment, the larvae were kept at the assigned rearing temperature and a constant photoperiod of 14:10 h light:dark. During the pre-exposure period, the larvae were fed *Artemia nauplii* ad libitum 5 days per week.

Experimental Setup. When a larva moulted into the final instar, it was randomly assigned to a six-day zinc exposure treatment (control or 100 mg L^{-1} zinc). This resulted in a nested full factorial design with three sampling locations per latitude ×2 latitudes (high/Sweden and low/France) × 2 temperatures (20 and 24 °C) × 2 zinc treatments (zinc present and absent). The chosen zinc concentration was previously shown to reduce growth rate in *I. elegans*.[32] Because of the low metal sensitivity of damselflies[45] and the strictly aqueous exposure route in our experiment (the dominant exposure route for metals is generally through the diet),[46,47] this concentration strongly exceeds commonly occurring environmentally concentrations (but see ref 48). Note, however, that our aim was not to mimic a realistic field situation, but to test for a proof-of-principle of the idea that susceptibility to a contaminant across metamorphosis can be shaped by temperature and latitude. Zinc solutions were prepared from a stock solution of $ZnCl_2$ (5 g zinc L^{-1} dissolved in milli-Q water) that was stored in the dark at 4 °C and that was renewed monthly. To prepare exposure media, the stock solution was further diluted with synthetic pond water, which was also used as control medium (for the composition see ref 49). The larvae were exposed to 100 mL of the medium in the same vials as they had been reared in. To minimize concentration changes due to evaporation, the medium was renewed every 2 days. The measured zinc concentrations in the experimental vials when the medium was freshly renewed was 93.10 ± 1.65 mg L^{-1} (mean ± SD, $n = 5$ pooled start samples, taken throughout the experiment); after 48 h (just before renewal of the medium) the concentration was 95.31 ± 2.84 mg L^{-1}, as verified with ICP-OES. The associated water quality parameters were (mean ±1 SD, $n = 10$ samples) the following: conductivity: 387 ± 17 μS/cm, pH: 6.7 ± 0.1, dissolved oxygen: 8.0 ± 0.2 mg L^{-1}, hardness: 132 ± 4 mg L^{-1} $CaCO_3$ and dissolved organic carbon: 4.9 ± 1.3 mg L^{-1}. After the six-day zinc exposure, all larvae were transferred to synthetic pond water and kept at their rearing temperature until emergence. This assured that all larvae were exposed to zinc for the same duration. Throughout the final instar, larvae were daily fed *Artemia nauplii* ad libitum, supplemented with 3 larvae of *Chironomus riparius* per week.

After emergence, the damselflies were kept overnight in their rearing vials to ensure complete hardening of the exoskeleton and wings before handling. Adults that emerged successfully were individually marked by writing a number on one of the wings with a permanent marker (Staedtler Lumocolor permanent F), after which they were transferred to insectaries (36 × 30 × 30 cm^3) to record lifespan; for logistic reasons this was only done for a subset of the adults. Males and females were kept in separate insectaries with maximum 10 adults of similar ages per insectary and were provided with ad libitum fruit flies. The insectaries were kept in a room at 22 °C ± 1 °C with an air humidity between 70% and 80% and were sprayed twice per day with dechlorinated tap water. By keeping all adults at this intermediate temperature (between the two larval rearing temperatures) any effects of temperature on adult lifespan could be attributed to differences in larval rearing temperature (hence, carry-over effects) without confounding with the temperature experienced in the adult stage.

Initial sample sizes varied between 112 and 133 animals per combination of latitude, temperature and zinc treatment (total =969). Between 44 and 102 animals emerged successfully per treatment combination (total =583). Sample sizes for the subset on which lifespan was recorded varied between 18 and 52 per combination of latitude, temperature and zinc treatment (total =291). The imbalance in sample size was due to differential mortality and emergence success in the different treatment combinations (see Results). Sample sizes per treatment combination for each end point are presented in Appendix S2.

Response Variables. We quantified larval growth rates both during the six-day exposure period and during the subsequent first 4 days of the postexposure period. To quantify growth rates, each larva was weighed to the nearest 0.01 mg at the start and at the end of the zinc exposure and again 4 days later using an electronic balance (AB135-S, Mettler Toledo, Zaventem, Belgium). Individual growth rates were calculated as ($\ln \text{mass}_{\text{final}} - \ln \text{mass}_{\text{initial}}$)/number of days (see ref 50). Rearing vials were checked twice per day for survival and adult emergence. To obtain a detailed picture on when larval mortality occurred we separately considered larval survival during the six-day exposure period and during the remaining postexposure period (until emergence). In addition, we calculated the emergence ratio as the percentage of larvae surviving until the end of the final instar that emerged successfully (i.e., complete emergence without wing malformations). To obtain an integrated picture of larval survival and emergence ratio we also quantified the total emergence success as the percentage of individuals initially in the experiment that emerged successfully. Development time was calculated as the number of days between molting to the final instar and adult emergence. Adults that emerged successfully were weighed to the nearest 0.01 mg and sexed. Insectaries were checked twice per day to record adult lifespan.

Statistical Analyses. Effects of latitude, rearing temperature and zinc exposure on the response variables were analyzed with (generalized) linear mixed-models (GLMMs/LMMs) using the lme4 package[51] in R.[52] In each model all interactions between latitude, rearing temperature and zinc exposure (fixed factors) were included, as well as sampling location nested in latitude as random effect (but see below for survival). Effects on larval survival were tested with a GLMM with binomial error distribution and a logit link function that also included the fixed factor period (before/after zinc exposure) and its interaction with the other fixed factors. For the analysis of emergence ratio and total emergence success, we ran GLMMs with binomial error distribution and a logit link function. The two successive larval growth rates were modeled with a repeated measures LMM that also included the fixed factor period and its interaction with the other fixed factors, and individual as random effect. The models for development time, mass at emergence, and lifespan also included sex as fixed factor, together with its interactions with the other fixed factors. However, as this is not the focus of this manuscript, results on sex differences are not reported. For lifespan a GLMM with a Poisson error distribution and a log link function was used. Because of nonconvergence of the model for larval survival when including sampling location as a random factor, we reduced the complexity of this model by including this factor here as a nested fixed (instead of a random) effect (following refs53, 54). Whenever an interaction between stressors was detected, we calculated the interaction effect size (estimated as Hedges'd) with its 95% confidence interval following Jackson et al.[55] An interaction effect size larger than zero indicates a synergistic interaction among stressors.

RESULTS AND DISCUSSION

Latitude-Wide Delayed Effects of Zinc. While zinc exposure barely affected the survival of the larvae during the six-day exposure period (−1.5%), it considerably reduced survival after the exposure period (−12.4%; period × Zinc; Table 1; Figure 1a,b). Zinc furthermore decreased larval growth rate (main effect Zinc, Table 1). Again, this zinc effect was stronger after than during the exposure period (period × Zinc; Table 1; Figure 1c,d). Zinc neither induced a latitude-wide effect on emergence ratio, nor on total emergence success (Table 2; Figure 2a,b), although there were pronounced latitude-specific effects of zinc on these end points (see below). Previous zinc exposure did not lead to a longer development time (Table 2; Figure 3a), but did result in a lower mass at emergence (main effect Zinc; Table 2; Figure 3b). Finally, adult lifespan was strongly reduced (i.e., by ca. 7 days) by larval zinc exposure (main effect Zinc; Table 2; Figure 3c).

Table 1. Results of the (G)LMMs Testing for the Effect of Latitude, Rearing Temperature, and Zinc Exposure on Larval Survival and Growth Rate of *Ischnura elegans* in the Final Instar[a]

	larval survival		larval growth rate	
effect	χ^2_1	P	χ^2_1	P
latitude (lat)	0.52	0.47	202.34	**<0.001**
temperature (temp)	5.35	**0.021**	13.47	**<0.001**
zinc (Zn)	26.13	**<0.001**	46.01	**<0.001**
lat × temp	0.59	0.44	70.75	**<0.001**
lat × Zn	12.12	**<0.001**	12.38	**<0.001**
temp × Zn	3.37	0.066	0.35	0.55
lat × temp × Zn	4.39	**0.036**	6.35	**0.012**
period (per)	104.38	**<0.001**	102.46	**<0.001**
per × lat	3.77	0.052	8.83	**0.0030**
per × temp	14.31	**<0.001**	6.02	**0.014**
per × Zn	16.43	**<0.001**	5.48	**0.019**
per × lat × temp	0.72	0.40	13.26	**<0.001**
per × lat × Zn	4.33	**0.037**	3.79	0.052
per × temp × zinc	5.21	**0.022**	2.38	0.12
per × lat × temp × Zn	0.49	0.48	0.21	0.65

[a]Both life-history traits were quantified during and after a six-day exposure to zinc, indicated by the (repeated) factor Period. Significant P values ($P < 0.05$) are printed in bold.

These results show the presence of strong delayed carry-over effects of larval metal exposure, not only in the larval stage after the exposure period (survival, growth rate) but also after emergence in the adult stage (adult mass and lifespan), and this despite zinc imposing no biologically significant effects on

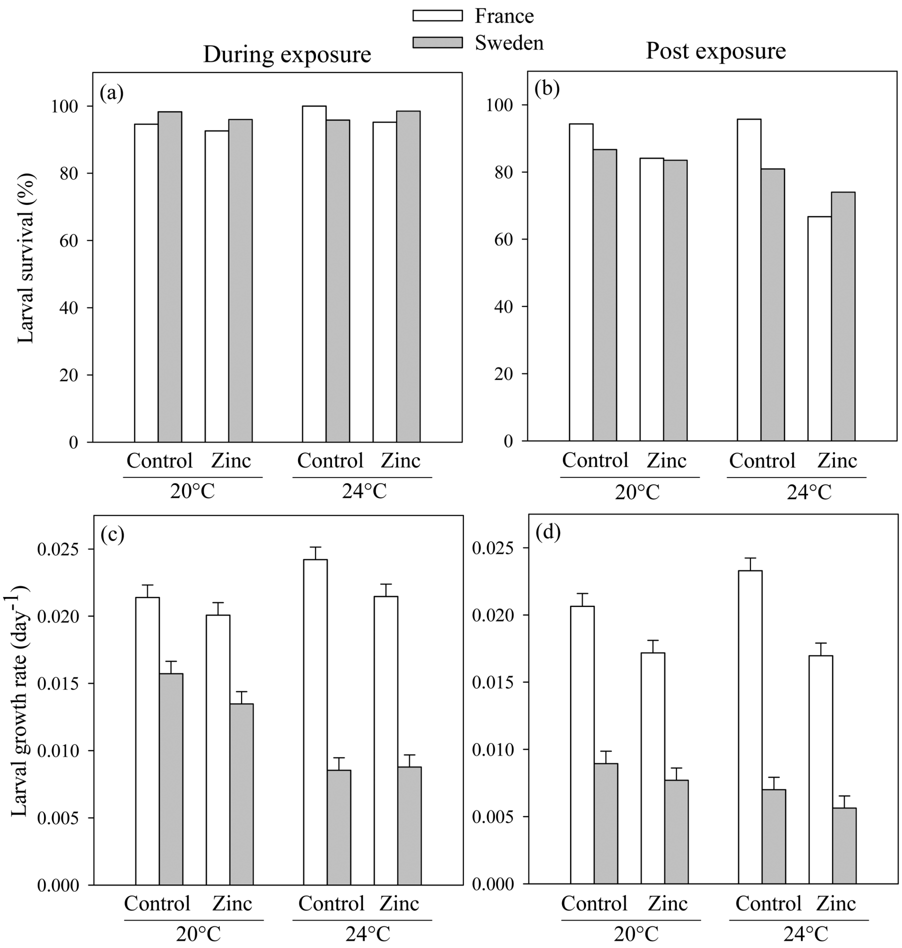

Figure 1. Percentage survival (a,b) and mean (+1 SE) growth rate (c,d) of *Ischnura elegans* larvae in the final instar during and after a six-day exposure to zinc as a function of latitude, rearing temperature and zinc exposure.

Table 2. Results of the (G)LMM Testing for Effects of Latitude, Temperature and Zinc on the Emergence Ratio (% Larvae Surviving until the End of the Final Instar That Emerged Successfully), Total Emergence Success (% Individuals Initially in the Experiment That Emerged Successfully), Development Time (Final Instar Duration), Mass at Emergence, and Adult Lifespan of *Ischnura elegans*[a]

	emergence ratio		total emergence success		development time		mass at emergence		adult lifespan	
effect	χ^2_1	P	χ^2_1	P	χ^2_1	P	χ^2_1	P	χ^2_1	P
latitude (lat)	35.59	**<0.001**	28.74	**<0.001**	59.03	**<0.001**	189.53	**<0.001**	15.59	**<0.001**
temperature (temp)	35.88	**<0.001**	36.08	**<0.001**	65.55	**<0.001**	41.76	**<0.001**	5.60	**0.018**
zinc (Zn)	2.98	0.084	18.44	**<0.001**	0.026	0.87	14.46	**<0.001**	445.86	**<0.001**
lat × temp	1.31	0.25	4.37	**0.037**	372.09	**<0.001**	4.68	**0.030**	0.72	0.40
lat × Zn	20.88	**<0.001**	32.57	**<0.001**	0.43	0.51	2.11	0.15	2.25	0.13
temp × Zn	0.10	0.75	1.93	0.16	0.13	0.72	1.24	0.26	0.23	0.63
lat × temp × Zn	0.60	0.44	6.09	**0.014**	0.030	0.86	0.042	0.84	4.26	**0.039**

[a] Significant P values ($P < 0.05$) are printed in bold.

survival during the six-day exposure period. These delayed effects and particularly the lethal effects during metamorphosis of larval exposure to metals (see below) confirm the important role of metamorphosis as a survival bottleneck in metal-contaminated environments.[21,29,56] Besides the important effects on survival, also the sublethal effect on mass at emergence may result in a lower fitness, as body size is positively related with fecundity in damselflies.[57] Yet more importantly, we showed that larval zinc exposure also caused a remarkably strong reduction in adult lifespan, a crucial fitness measure in damselflies.[58] This reduction in lifespan would have particularly strong fitness implications as it reduced adult lifespan to ca. 2 days. Given that sexual maturation takes ca. 6 days in the study species,[59] no reproduction would occur in the damselflies that emerged successfully from the zinc treatment. This strong postmetamorphic lethal effect of zinc complements the very few documented adverse metal effects across metamorphosis: lowered survival in bryozoans,[14] lowered fecundity in mosquitoes,[60] lowered immune function and delayed reproduction in flies,[27] and reduced body condition in juvenile frogs.[28] Although some elimination of metals can happen during metamorphosis (e.g., via the shed larval skins),

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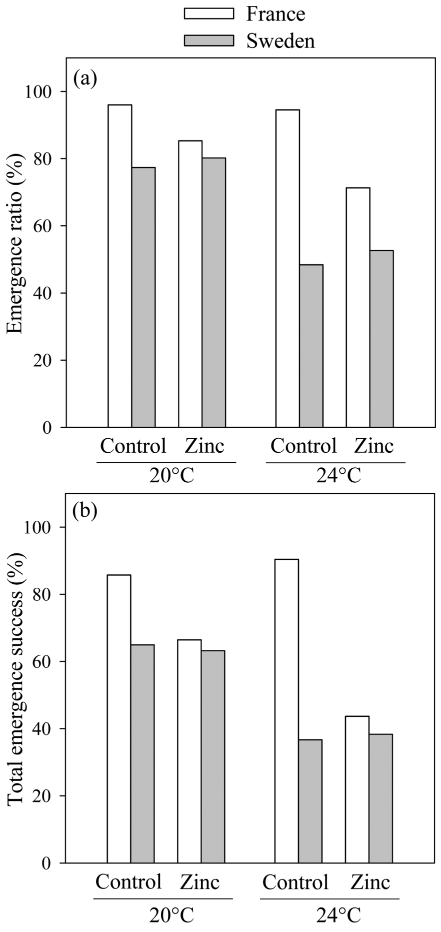

Figure 2. Emergence ratio (% larvae surviving until the end of the final instar that emerged successfully) (a) and total emergence success (% individuals initially in the experiment that emerged successfully) (b) of *Ischnura elegans* larvae as a function of latitude and previous rearing temperature and zinc exposure in the larval stage.

metal body burdens after metamorphosis can still remain very high.[61,62] Moreover, in addition to the stress that metamorphosis itself creates, the remobilization of stored metals during tissue reorganization at metamorphosis has been suggested to magnify the effects of metals.[56,63]

Latitude-Specific Delayed Effects of Zinc. The survival of French larvae was more strongly reduced by zinc than that of Swedish larvae, especially after the exposure, where zinc reduced the survival of the French larvae by ca. 20% and that of Swedish larvae by ca. 6% (period × latitude × Zinc, Table 1, Figure 1a,b). Also the zinc-induced reduction in larval growth rate was stronger in French damselflies (latitude × Zinc, Table 1, Figure 1c,d). While the emergence ratio of Swedish damselflies was not affected by previous zinc exposure, that of French damselflies was reduced by ca. 18% (latitude × Zinc; Table 2, Figure 2a). This, in combination with the different larval survival rates between the latitudes, resulted in a strong zinc-induced decrease in total emergence success in French, but not in Swedish damselflies (latitude × Zinc; Table 2; Figure 2b). Development time and mass at emergence were similarly affected by previous zinc exposure across latitudes (Table 2). [Results on life-history differences in the control treatment between damselflies from both latitudes are presented and discussed in Appendix S3.]

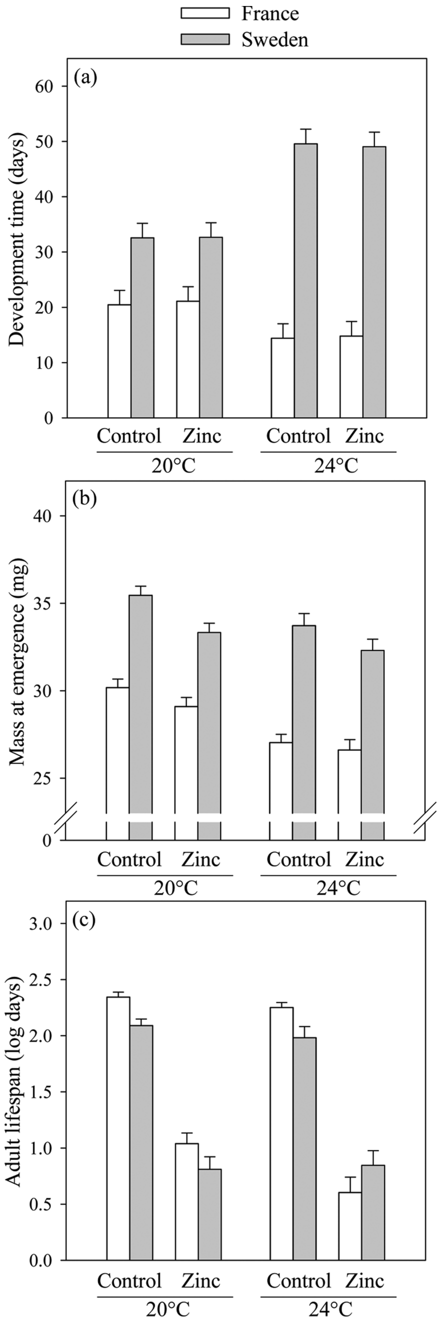

Figure 3. Mean (+1 SE) development time (number of days in the final instar) (a), mass at emergence (b), and adult lifespan (log transformed) (c) of *Ischnura elegans* as a function of latitude, previous rearing temperature and zinc exposure in the larval stage. All adults were kept under identical conditions.

The negative impact of zinc thus strongly differed between latitudes with all effects being more pronounced (and sometimes only present) in French larvae. As French showed adaptation to higher temperatures (see below), this pattern of an increased vulnerability to contaminants has been predicted as a population-level trade-off between tolerance to warming and tolerance to toxicants by Moe et al.[8] This might furthermore indicate a role of energy-based life-history trade-offs shaping sensitivity to contaminants.[41] In such case, fast growth is traded off against a lower investment in detoxification and repair mechanisms, which would make fast-lived individuals more susceptible for contaminants. Furthermore, fast growth often covaries with a higher food intake, a smaller size (hence a higher surface-to-volume ratio), and a faster metabolic rate,[64−67] traits that can lead to a higher uptake or accumulation of contaminants.[9,68,69]

Although zinc-induced effects were consistently stronger in French damselflies, even in Swedish adults, which showed only a low zinc-induced reduction in larval survival (6.2% postexposure) and no zinc-induced mortality during metamorphosis, zinc reduced adult lifespan by ca. 71%. This strong reduction happened despite a potentially long recovery period between the end of the exposure and emergence (i.e., on average 27 days at 20 °C and 43 days at 24 °C). This finding particularly underscores the importance of covering the full life cycle in ecological risk assessment of pollutants.[70,71] If our study would have been limited to premetamorphic life stages (e.g., ref 32) or had ended just after metamorphosis (e.g., ref 21), we would have erroneously concluded that Swedish animals would only suffer negligible zinc-induced effects, whereas the strong delayed effect on lifespan implicates a complete failure to reproduce.

Latitude-Specific Delayed Synergistic Effects between Zinc and Warming. While in Swedish larvae the effect of zinc on survival was similar across temperatures, the survival of French larvae was more strongly reduced by zinc at 24 °C than at 20 °C (latitude × temperature × Zinc; Table 1; Figure 1a,b). This indicated a synergistic interaction between the temperature and zinc treatments in French larvae, which was supported by the positive interaction effect size both during (Hedges' d = 0.049, 95% CI: [0.030,0.068]) and especially after the exposure period (Hedges' d = 0.36, 95% CI: [0.34,0.38]). Similarly, only in French larvae growth rate was more strongly affected by zinc at 24 °C than at 20 °C (period × latitude × Zinc; Table 1; Figure 1c,d; interaction effect size during the exposure period: Hedges' d = 0.023, 95% CI: [0.0016,0.044]; after the exposure period: Hedges' d = 0.037, 95% CI: [0.014,0.060]). Likewise, only in French damselflies, total emergence success was more strongly reduced by zinc at 24 °C (ca. 52%) than at 20 °C (ca. 23%) (interaction effect size: Hedges' d = 0.47, 95% CI: [0.45,0.48]) (latitude × temperature × Zinc; Table 2; Figure 2b). Finally, the strong effect of zinc on adult lifespan was more pronounced at 24 °C, but again only in French damselflies (latitude × temperature × Zinc). This indicated a synergistic interaction between the temperature and zinc treatments in French adults (interaction effect size: Hedges' d = 0.17, 95% CI: [0.09,0.24]).

We thus observed the expected synergism of metals being more toxic at higher temperatures for larval survival and growth, emergence success and adult lifespan.[9,10] Importantly, the synergistic interactions were mainly delayed (occurred after the exposure period ended) and had a strong geographic signal as these were only present in French damselflies. Despite the concern that trace metals become more toxic at higher temperatures[9,10] and the potential of strong delayed effects of metals during and after metamorphosis (see above), delayed interactive effects between metals and warming have been largely ignored. The only exception are two studies showing delayed sublethal effects of previous exposure to metals being magnified under warming (for adult body mas: ref 27, for juvenile body condition: ref 28). Here, we showed that also across metamorphosis delayed effects of a metal can be magnified under warming, and even translated in a synergistic lethal effect by shortening adult lifespan. The strong geographic signal in the delayed synergistic interaction also indicated that thermal adaptation of French damselflies to 24 °C (see below) did not alleviate the adverse effects of zinc. Possibly, at 24 °C the French damselflies allocated a higher percentage of resources to growth than at 20 °C, and this at the expense of investment in detoxification and repair, whereas the Swedish damselflies, which grew even slower at the higher temperature than at the lower temperature, might have been better able to maintain investment in detoxification and repair at an optimal level.

Signals of Thermal Adaptation. We found widespread evidence of latitude-associated thermal adaptation (e.g., refs 72−74), with the French damselflies being adapted to higher environmental temperatures than the Swedish damselflies. While French larvae had a higher growth rate at 24 °C (the current mean water temperature in southern France[44]) than at 20 °C, Swedish instead had a reduced growth rate at 24 °C than at 20 °C (the current mean water temperature in Sweden.[44]) (latitude × temperature; Table 1). French damselflies had a high total emergence success in the control treatment at both temperatures, but that of Swedish damselflies was strongly reduced (by ca. 43%) at the higher temperature (latitude × temperature × Zinc; Table 2; Figure 2b). This was partly due to a lower larval survival at the higher temperature, but mainly to a lower emergence ratio. Furthermore, while French damselflies had a ca. six-day shorter development time at 24 °C than at 20 °C, the Swedish increased their development time at the higher temperature by ca. 18 days (latitude × temperature, Table 2, Figure 3a). The differential thermal responsiveness of larval growth and development resulted in a lower mass at emergence in damselflies reared at 24 °C compared to 20 °C (main effect temperature), and this mass reduction at the higher temperature was stronger in French than in Swedish damselflies (latitude × temperature; Table 2, Figure 3b).

Implications for Risk Assessment in a Warming World. In conclusion, our integrated study not only identified three key mechanisms challenging current risk assessment (i.e., delayed postexposure effects,[5] interactions between stressors,[4] and geographic variation in the sensitivity to pollutants[7]), but more importantly, also documented interactions between these mechanisms. A key observation was that the effects of zinc were very mild during the exposure period, yet were strongly magnified after the exposure period and carried over to the adult stage. We further observed a strong geographic signal in the sensitivity to zinc, with the French damselflies consistently more strongly affected than the Swedish, especially when the larvae had experienced a higher rearing temperature. Moreover, the synergistic interaction between the metal and the warming treatment in the French damselflies bridged metamorphosis and strongly affected (post-) metamorphic traits. However, even though Swedish damselflies experienced only mild effects of zinc on larval and metamorphic end points, they did show a very strong reduction in adult lifespan when exposed to zinc in the larval stage.

Predicting and understanding the impact of contaminants under warming remains a key challenge at the interface of ecotoxicology and global change biology.[8,10] Even though the zinc concentration used in our study is much higher than those that would be encountered in realistic scenarios, our study provided a proof of principle, adding two key related insights that may contribute to the much needed development of a more realistic risk assessment,[1−3] especially with regard to the impact of contaminants in a warming world.[12,13] First, our

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results refined the well-known pattern that trace metals are more toxic at higher temperatures by showing this synergistic interaction may persist across metamorphosis and may show a strong geographic signal (being only present in French damselflies). This geographic signal was consistent with a trade-off scenario between thermal adaptation and tolerance to toxicants,[8] illustrating the complexity of predicting the impact of metals in a warming world. Using a space-for-time substitution our results thereby tentatively suggest that warming at the high latitude would not make zinc more toxic to the study species, unless high-latitude populations show gradual thermal evolution and convert to the current French phenotype.

Second, our study refined the hypothesis of aquatic insect emergence as a sensitive indicator of aquatic metal toxicity[21] in two important ways that are directly relevant for ecological risk assessment. (i) Metamorphosis success may strongly depend on latitude as we only observed a zinc-induced mortality during metamorphosis in French animals and not in Swedish animals. (ii) Postmetamorphic survival may even be more important than larval survival and emergence success; this result again showed the importance of the geographical context, as this pattern was especially striking for the Swedish animals. Taken together our results thereby challenge current water quality standards (such as EQS, EU Directive 2008/105/EC) which assume that protection of aquatic stages will translate into the protection of adults (but see ref 71), and ignore geographical patterns in sensitivity to contaminants. Aquatic insects are important vectors of transfer of nutrients from aquatic to terrestrial ecosystems,[30,75] and aquatic ecosystems are especially vulnerable to warming[76,77] and pollutants.[78] Combined with current findings this highlights that risk assessment for aquatic systems can highly benefit from a more complete life-cycle approach addressing delayed synergisms under warming in a geographical context in order to obtain more ecologically relevant water quality standards.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b04989.

> Information on the sampled locations (S1), sample sizes (S2), and presentation of the results and discussion on baseline life-history differences between the latitudes (S3) (PDF)

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The authors declare no competing financial interest.

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