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Combined Effects of Cadmium Exposure and Temperature on the Annual Killifish (*Nothobranchius furzeri*)

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Abstract: Freshwater organisms are increasingly exposed to combinations of stressors. However, because it is time-consuming and costly, research on the interaction of stressors, such as compound toxicity and global warming on vertebrates, is scarce. Studies on multigenerational effects of these combined stressors are almost nonexistent. In the present study, we tested the combined effects of 4 °C warming and cadmium (Cd) exposure on life-history traits, biomarkers, bioaccumulation, and multigenerational tolerance in the turquoise killifish, *Nothobranchius furzeri*. The extremely short life cycle of this vertebrate model allows for assessment of sublethal and multigenerational effects within 4 mo. The applied Cd concentrations had only limited effects on the measured endpoints, which suggests that *N. furzeri* is more resistant to Cd than fathead minnow and rainbow trout. In contrast, the temperature increase of 4 °C was stressful: it delayed female maturation and lowered adult mass and fecundity. Finally, indications of synergistic effects were found on peak fecundity and embryonic survival. Overall, these results indicate the importance of studying chronic and multigenerational effects of combined stressors. *Environ Toxicol Chem* 2018;37:2361–2371. © 2018 SETAC

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INTRODUCTION

Organisms in polluted environments are typically exposed to a cocktail of stressors. Trace metals are, for instance, omnipresent in freshwater ecosystems; and combined with the effects of global warming they can be harmful at low concentrations to aquatic organisms including fish (Noyes and Lema 2015), amphibians (Hallman and Brooks 2016), and insects such as damselflies (Debecker et al. 2017). This is attributable to the fact that environmental warming increases the body temperature of ectothermic animals and will alter physiological and biochemical reactions but also will affect the stability of biological molecules (Sokolova and Lannig 2008). Still, ecological risk-assessment methods mainly focus on single–stressor exposure regimes (Kimberly and Salice 2013), and interaction effects among combined stressors are often neglected (Holmstrup et al. 2010).

Temperature–toxicity relationships generally show that higher temperatures alter the toxicokinetics of metals and increase their toxicity (Sokolova and Lannig 2008; Noyes et al. 2009). Underlying reasons can be increased uptake, a higher metabolic rate, or loss of respiration efficiency, which results in up-regulated ventilation and feeding rates (Noyes et al. 2009). Even when temperature acclimation is possible, key physiological mechanisms could be altered, resulting in a lower resistance to additional stressors, like toxic compounds (Noyes et al. 2009). Because contaminants are typically tested at the optimal culture temperature of the studied model organism and only during short time exposure, chronic interactive effects could be overlooked and the toxicity of compounds underestimated (Stoks et al. 2015). Fathead minnow (Lapointe et al. 2011) and Japanese eel (Yang and Chen 1996) were, for instance, shown to be more sensitive to copper and cadmium (Cd) at higher temperatures, respectively. Likewise, exposure to metals decreases the ability of an organism to resist additional stress (Folt et al. 1999). For example, metal exposure has been found to alter the organism-specific temperature tolerance range in, among others, rainbow trout and silver perch (Patra et al. 2007).

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Apart from having negative effects within a generation, parental exposure to metal and an elevated temperature can also negatively affect subsequent generations. Alternatively, repeated exposure to combined stressors across generations may result in an increased tolerance, by means of genetic adaptation or epigenetic effects (Franks and Hoffmann 2012). We can differentiate between 2 types of experiments to study these effects on following generations. Multigenerational studies expose several generations of an organism to the stressors (Skinner 2008). This can be achieved by exposing the pregnant females, by exposing the offspring to the same stressor, or combining the 2 methods. In transgenerational studies, the stressor is transmitted over generations in an indirect way, making sure at least one germ line is not exposed directly to the stressor (Skinner 2008). Both positive and negative multigenerational effects have been found for metals, with parental metal exposure making offspring more vulnerable to metal pollution in nematodes (Yu et al. 2013) and zooplankton (Tsui and Wang 2004) and more tolerant in snails (Plautz and Salice 2013). In fish, research on trans- and multigenerational effects is scarce because their life cycle is generally much longer than that of invertebrate model organisms. The few studies describing multigenerational effects of metals such as reduced spermatozoa motility, disturbed embryonic development, and death in the next generation (Jezierska et al. 2009) only exposed adult pairs during breeding. Yet, for persistent contaminants, the multigenerational impact of lifetime parental exposure is more relevant.

Cadmium was chosen as a toxic compound because it is a nonessential metal and has harmful effects after acute and chronic exposure at low concentration levels (Cuypers et al. 2010). Weathering of rock is the major natural source of Cd in the environment (Walker et al. 2012). Furthermore, industrial and agricultural activities such as mining, smelting, ore refining, and electroplating processes contribute to Cd pollution in the environment (Zhang et al. 2014). In surface waters in the United States, the concentration generally does not exceed 0.1 μg/L (Colt 2006). However, in regions with effluents from heavy industry or attributable to spills or sudden incidence, concentrations may peak. During recent decades, Cd pollution has also increased globally because of the increasing use of phosphate fertilizers containing Cd (Roberts 2014). In 2007, about 240 tons of Cd entered the soil in the European Union (European Union 2007). It is the seventh toxicant on the "Top 20 Hazardous Substance" list, compiled by the US Environmental Protection Agency; and it is one of 6 pollutants banned by the European Union in the "Restriction on Hazardous Substances" directive (Nogawa et al. 2004). Cadmium is persistent and, combined with a high bioconcentration factor, accumulates within organisms, which drives biomagnification through the food chain (Croteau et al. 2005). Fish predominantly take up Cd through gill respiration because of the direct uptake of the dissolved particles in the water by calcium transport pathways (Amundsen et al. 1997). Metals such as Cd affect gills by changing their morphology, which causes imbalanced respiration and osmoregulation (Evans 1987). When fish are exposed to sublethal Cd levels, this can negatively affect their life history (Cazan and Klerks 2015), physiology (Pereira et al. 2016), and behavior (Qi et al. 2017). However, fish can partly protect themselves against the damaging effects of metal exposure by metallothionein (MT) induction in the liver and kidneys (Carpene et al. 1994).

Instead of performing long-term exposure experiments to identify effects of chronic exposure to toxins, early warning signals may provide information on future effects. This reduces the length of experiments and minimizes suffering of laboratory animals, without losing key information on the effects of the applied stressors. For trace metals, MTs act as nonspecific biomarkers (Sarkar et al. 2006), whereas for temperature stress critical thermal maximum (CTmax) values may act as nonlethal predictors of general resistance of fish to temperature stress. The CTmax value is defined as the temperature at which the critical thermal endpoint is reached and an organism loses its equilibrium by failing to maintain a dorsoventrally upright position (Patra et al. 2007). Finally, the energy reserves (e.g., fat, glycogen, and protein content) of an organism can provide important and early signs of stressor effects on the metabolism (Sibly et al. 2013).

In the present study, we studied the individual and combined long-term effects of Cd exposure and a temperature increase of 4 °C in the turquoise killifish, *Nothobranchius furzeri* Jubb, 1971 (Cyprinodontiformes). We chose this model organism because of its short life cycle, which enables us to study the interactive and multigenerational effects of exposure to Cd in just over 4 mo. We investigated whether Cd exposure in combination with a temperature increase affected life-history traits (maturation time, fecundity, mass, and growth), biomarkers (CTmax, MTs, energy reserves), total bioaccumulation, and multigenerational tolerance to both stressors. We used the definition of Folt et al. (1999) to define a synergism based on the additive effects model, whereby the combined effect is greater than the sum of effects elicited by the individual stressors. The temperature in a typical *N. furzeri* habitat ranges between 14 and 37 °C (Cellerino et al. 2016). Killifish have adapted their physiology to cope with large changes in abiotic conditions in their highly variable habitat during short periods (i.e., diurnal fluctuations; Schulte 2015). However, when extended, constant temperature variation could cause chronic stress when populations are preadapted to life under a different fixed temperature. In our laboratory cultures, naturally derived populations of this species have been reared for 3 generations at 24 °C prior to experimenting. As temperature stress, we opted for an exposure regime of +4 °C because a temperature rise of 4 °C is predicted by the Intergovernmental Panel on Climate Change (2014) at the end of the 21st century under scenario AR5. Although continuous exposure to 4 °C above their normal culturing temperature could compromise the stability of physiological functions in fish (Cairns et al. 1975), they are known to adapt their thermal tolerance in response to increased temperatures. We hypothesize that higher temperatures will up-regulate metabolism and increase the speed of development, possibly trading off with energetically costly traits such as fecundity and general performance. Furthermore, we expect delayed maturation, impaired growth, and lower fecundity as a result of Cd exposure. Combined with temperature stress, we expect that these effects will be exacerbated. Finally, when *N. furzeri* is continuously exposed

to Cd for 2 generations, we expect that the F_1 offspring will be more sensitive to Cd than the parental generation.

MATERIAL AND METHODS

Maintenance of the test animals

All fish were second-generation (F_3) individuals from wild-caught (F_0) fish of the natural population NF414 located in the Limpopo River basin in the south of Mozambique, sampled by the Czech Institute of Vertebrate Biology in 2012 (Bartáková et al. 2013). *Nothobranchius furzeri* eggs (stored in moist peat) were hatched synchronically by inundating them in 1 cm of 12 °C dechlorinated tap water in 2-L tanks. Afterward, water temperature gradually converged to room temperature (22 °C). Healthy, buoyant larvae were housed individually in 0.5-L glass jars with rearing medium (see *Experimental setup*) from 48 h posthatching onward. The medium was renewed every 2 d, to ensure a constant water quality. All glass jars used for Cd exposure were complexed with Cd ($CdCl_2[H_2O]_{2.5}$) prior to exposure trials by filling them with the corresponding exposure medium and leaving them overnight. Experiments were performed in temperature-controlled water baths, to ensure a constant temperature of either 24 or 28 °C. Both temperatures are within the range of thermal variation recorded in the habitats of *N. furzeri* (Polačik et al. 2016). Fish were subjected to a 14:10-h light:dark cycle, and jars were randomized within their respective water bath every other day.

At the start of the experiment, mortality was checked daily, whereas abiotic parameters (pH, percentage of dissolved oxygen, conductivity, and temperature) were measured every second day in a randomly chosen control jar for each temperature treatment. Juveniles were fed ad libitum with *Artemia* nauplii (Ocean Nutrition) twice per day, 7 d per week. After day 24, fish were moved into 2-L jars, with the water refreshed every week. From then onward, the same set of abiotic parameters was measured once a week. Average abiotic water quality values are presented in Supplemental Data, Table S1. At the start of the adult phase (day 43), the ad libitum *Artemia* diet was complemented with chopped *Chironomus* larvae (Ocean Nutrition). Another 2 wk later, fish were fed ad libitum twice a day with frozen *Chironomus* larvae only.

Experimental setup

The parental generation was incubated under 2 Cd concentrations, C1 (15 μg/L Cd) and C2 (30 μg/L Cd), and a control treatment without cadmium (C0), fully crossed with 2 rearing temperatures, 24 and 28 °C, resulting in 6 treatments. Each treatment was replicated 26 times. Treatment concentrations were based on a range-finding experiment with 4 Cd concentrations (5, 10, 20, and 40 μg/L Cd) with 6 replicates each. Cadmium was added from a stock solution of 250 mg/L $CdCl_2(H_2O)_{2.5}$ that was kept in the dark at 4 °C. The experimental medium was prepared using dechlorinated tap water. Water samples were taken in week 7 of the experiment and analyzed using inductively coupled plasma—mass spectrometry (ICP-MS) to measure the actual Cd concentrations in the medium. Analysis of the exposure medium showed concentrations of 1.18 ($n = 8$, standard deviation [SD] = 0.52), 15.50 ($n = 8$, SD = 1.65), and 30.75 ($n = 8$, SD = 2.68) μg/L Cd for C0, C1, and C2, respectively. The parental exposure was stopped after 129 d to prevent mortality effects from the natural aging process.

Response variables (parental generation)

Mortality was checked daily. Maturation time for males was scored according to the protocol that was used in other killifish studies as the age at which the first signs of nuptial coloration are visible (Polačik et al. 2016; Grégoir et al. 2017; Philippe et al. 2017). For females, the age at which the first egg was produced was used as an exact measure of maturity. Fecundity was measured as the number of eggs laid per week. Because we started the trial with newborn fish, for which sex cannot yet be identified, we were unable to manipulate the sex ratio. This resulted in a skewed sex ratio in a number of treatments for the assessment of fecundity. Mature females were allowed to spawn with a male of the same treatment 3 times a week until death. For spawning, couples were put in separate 1-L aquaria provided with a sand substrate for 2 h. Afterward, fish were transferred back to their individual 2-L glass jars. The sand was sieved (mesh size 500 μm) to count the eggs. The eggs were placed on moist peat at 28 °C for rapid development and checked for fungal growth every 2 d. Body size was measured weekly by photographing every fish individually in a Petri dish that was placed on millimeter paper. The images were analyzed digitally using the open source AnalysingDigitalImages software (Pickle 2008). In addition, all fish that survived until the last experimental day were weighed (0.1 mg accuracy) after gently patting them dry. Fish were weighed 4 h after the last feeding.

The measurement of the CTmax (Patra et al. 2007) was performed at day 105 in a series of 5 randomly chosen individuals. Five 1-L aquaria, each holding one individual, were placed in a plastic 30-L rectangular water bath that was heated starting from the rearing temperature (24 or 28 °C) by a HETO therm heater (Anker Schmitt; Type Grant TC120) at a constant rate of 0.33 °C/min (standard error [SE] 0.04 °C), comparable with the heating rate used in other heating experiments on fish (LeBlanc et al. 2012). The water circulated, and its temperature was monitored using a digital thermometer (accuracy 0.1 °C). The CTmax was scored as the temperature at which fish lost their balance and failed to maintain a dorsoventrally upright position (Patra et al. 2007). At the end of each trial, fish returned to their rearing tank for recovery.

Deceased fish were rinsed with dechlorinated tap water and stored dry at –20 °C in a microcentrifuge tube (2 mL; BRAND) for further analysis. After 129 d, all surviving fish were killed ($n = 69$) by sedating them in ice water and transferring them to liquid nitrogen at –196 °C. Fish were stored at –80 °C to prevent protein degradation. Afterward, various biomarkers were measured, including energy reserves and MT content. In addition, we quantified bioaccumulation of Cd using ICP-MS. Young fish (age <60 d) were analyzed as a whole for Cd only. Because of the small size of the organisms, dry mass values could

not always be determined accurately using a Mettler AT261 DeltaRange balance (precision of 0.01 mg; Mettler-Toledo). Consequently, wet mass was used to calculate the relative concentration of Cd in the whole body (micrograms of Cd per gram wet mass). Fish that weighed <0.02 g were excluded from the analysis because of large calculation errors. Older (age >60 d) fish were homogenized on ice with 4 mL Milli-Q water. This homogenate was divided in subsamples for to determine the bioaccumulation of Cd and MT concentration. Both were measured on all fish that survived until the last day of the experiment, as described in Philippe et al. (2017).

Because energy in an organism is limited, the increasing maintenance costs attributable to stressors will result in less energy for growth and reproduction (Kimberly and Salice 2013). To study energy reserves of the fish, we chose to measure total glycogen, fat, and protein content because these provide insight into the metabolism of potentially stressed fish. Furthermore, this could be helpful to exclude food deprivation as an explanation for stress in fish reared at an elevated temperature. Energy reserves were measured on all fish that survived until the last day of the experiment. Protein was determined using a standard curve of bovine serum albumin (Bradford 1976). Total lipid was extracted using methanol chloroform and measured by comparing the results with a tripalmitin standard curve (Bligh and Dyer 1959). Glycogen was measured using Anthron reagent and a glycogen standard curve (Roe and Dailey 1966).

Multigenerational effects on offspring

We also tested for multigenerational effects of parental exposure to Cd and a temperature rise. The goal of this multigenerational exposure was to assess if the short-term sensitivity to Cd would change when the parental generation was sublethally exposed to Cd. For this, we acutely exposed all offspring of each parental treatment to Cd at the rearing temperature of their parents. The protocol of this acute assay is outlined in Philippe et al. (2017). We exposed the 48-h-old offspring of experimental fish to the 72-h median lethal concentration (LC50; 0.36 mg/L Cd in both temperature regimes [Philippe et al. 2018]) and assessed mortality over 14 d. All healthy eggs that had developed into the hatchable DIII phase were used; therefore, the number of replicate fish in every treatment differed and ranged from 3 to 37 per Cd × temperature combination (see Table 1).

TABLE 1: Sample size for each treatment in the multigenerational exposure experiment[a]

Treatment	*n*
0 µg/L, 24 °C	3
15 µg/L, 24 °C	7
30 µg/L, 24 °C	37
0 µg/L, 28 °C	11
15 µg/L, 28 °C	11
30 µg/L, 28 °C	0

[a]Replicates consist of the offspring of each parental treatment that developed to the hatchable DIII phase.

Data analyses

Escapees (3 out of 156 fish) were excluded from the survival analysis. Survival curves were constructed by plotting the survival ratio for every treatment against time (in days) and compared between treatments (temperature, concentration) and between sexes. A right-censor index was included to indicate if a fish died during the experiment (status 1) or was euthanized at the end of the experiment (day 129; status 0). Survival between treatments was compared using the log-rank test in the survreg package (R Development Core Team 2015). Maturation time was analyzed for both sexes separately because maturation was differently scored. For both sexes, maturation time was analyzed using a general linear model with concentration and temperature as fixed factors. Growth was assessed with Von Bertalanffy growth models (Von Bertalanffy 1950) for each fish, parameterized with the maximum body size of the fish (L_{max}) and the growth factor (K), using the nls function (stats package; R Development Core Team 2015) to estimate the parameters of the growth model. Von Bertalanffy parameters were then analyzed using full factorial general linear models with concentration, temperature, and sex as fixed factors. Body size was analyzed at the start of the experiment (week 1), at maturation (week 7), and at the end of the experiment (week 15), using linear models with concentration, temperature, and sex (in weeks 7 and 15) as fixed factors. Fecundity (number of eggs per week) was analyzed using a generalized linear mixed model with a Poisson distribution and concentration and temperature as fixed factors and time (in weeks) as well as individual fish as random factors. Cumulative fecundity and fecundity at peak production were analyzed using a generalized linear model with a Poisson distribution and concentration and temperature as fixed factors. Mass was analyzed as a linear model with categorical factors concentration, sex, and temperature, as well as their interaction. Also, CTmax was analyzed as a general linear mixed model with concentration, temperature, sex, and body size as fixed factors and series as a random factor. Bioaccumulation was analyzed as a linear model with concentration, temperature, and sex as fixed factors. Glycogen, total fat, protein, and MT levels were analyzed as linear models with concentration, temperature, and sex as fixed factors. Analyses of glycogen and MT were corrected for heterozygous variances using white-adjusted, heteroscedasticity-corrected standard errors. Offspring survival in the acute exposure experiment was analyzed using Cox proportional hazards regression models.

Statistical analyses were performed in R (R Development Core Team 2015). We used the packages survival (differences between survival plots), lme4 (likelihood ratio test), multcomp and lsmeans (post hoc tests), car (analysis of variance), stats (generalized linear models), and mass (StepAIC). Tukey's honestly significant difference test was performed to test multiple pairwise comparisons using the "multcomp" package (Hothorn et al. 2008).

RESULTS

Survival

There was no effect of Cd exposure ($\chi^2_{2,152} = 3.31$, $p = 0.191$), rearing temperature ($\chi^2_{1,153} = 1.85$, $p = 0.174$), or

their interaction ($\chi^2_{5,149} = 2.85$, $p = 0.240$) on survival of the fish (Figure 1). The mean survival of control fish (57%) after 18.5 wk is considered normal for this species because median survival in captivity has been reported to be between 17.5 and 29 wk (Cellerino et al. 2016).

Maturation time

There was no effect of Cd exposure on female maturation time ($F_{2,36} = 0.977$, $p = 0.386$; Figure 2A). Temperature had a significant effect ($F_{1.36} = 4.20$, $p = 0.048$), with females maturing earlier at 24 °C (77 ± 6.9 d) than at 28 °C (82 ± 10.3 d). Cadmium exposure had an effect on male maturation time ($F_{2,31} = 3.848$, $p = 0.032$; Figure 2B), with C1 males maturing later compared to control males ($p = 0.025$; 57.7 ± 4 and 49 ± 8 d, respectively). However, C2 males (52 ± 7 d) did not mature later compared to control ($p = 0.442$) and C1 males ($p = 0.277$). This could be a false-positive result, attributable to the small SE in the C1 treatment compared to the control and C2 treatment. Temperature did not affect male maturation time ($F_{1,39} = 3.284$, $p = 0.080$).

Body size and growth

Von Bertalanffy growth models were used to calculate the growth rate (K) and estimated maximal length (L_{max}) coefficients for each individual. Growth rate was not different between sexes ($F_{1,68} = 0.621$, $p = 0.433$), temperatures ($F_{1,68} = 0.125$, $p = 0.725$), or Cd concentrations ($F_{2,68} = 1.083$, $p = 0.344$). Maximal length, however, differed between males and females ($F_{1,76} = 27.52$, $p < 0.001$), with males growing to a larger average size (35.0 ± 5.2 mm) than females (32.6 ± 5.3 mm). Maximal body length was not affected by Cd concentration ($F_{2,76} = 2.365$, $p = 0.101$) or temperature ($F_{1,76} = 0.037$, $p = 0.848$).

One-week-old fish did not differ in body size between any of the treatments (concentration $F_{2,101} = 1.41$, $p = 0.249$; temperature $F_{1,101} = 0.260$, $p = 0.611$). Body size of maturing fish (week 7) was affected by the sex of the fish ($F_{1,75} = 5.077$, $p = 0.027$), with females being on average 1.2 mm (6%) smaller than males. Furthermore, the interaction of Cd concentration and temperature was significant ($F_{2,74} = 3.75$, $p = 0.029$). Fish reared at 28 °C had a tendency to be smaller with increasing Cd concentrations ($F_{2,33} = 3.25$, $p = 0.052$; C0 28 ± 2.6 mm, C1 21 ± 2.0 mm, C2 19 ± 1.4 mm), whereas Cd did not have an effect on body size at 24 °C ($F_{2,40} = 1.57$, $p = 0.22$; C0 21 ± 2.5 mm, C1 20 ± 2.4 mm, C2 22 ± 1.8 mm; Figure 3A). The same pattern was found at the end of the experiment (week 15), where sex ($F_{1,64} = 6.18$, $p = 0.016$) as well as the interaction between Cd concentration and temperature ($F_{2,63} = 3.41$, $p = 0.039$) affected body size (Figure 3B). Females were on average 1.6 mm (6%) smaller than males. In addition, body size at 28 °C decreased with increasing concentration (C0 29 ± 3.1 mm, C1 29 ± 2.2 cm, C2 27 ± 2.1 cm), whereas this was not the case at 24 °C (C0 30 ± 3.9 mm, C1 28 ± 2.6 cm, C2 30 ± 1.8 cm).

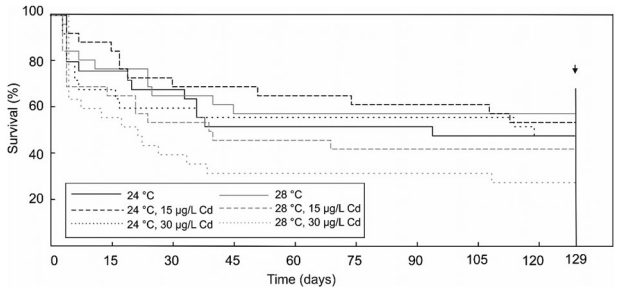

FIGURE 1: Survival curves showing the percentage of surviving individuals in the 6 treatments. The experiment was terminated at day 129 (indicated by a black arrow).

Fecundity

Fecundity (measured as number of eggs per week per living female) was not affected by exposure to Cd (likelihood ratio test = 0.625, $p = 0.732$; Figure 4A). Temperature affected fecundity significantly (likelihood ratio test = 21.6, $p < 0.001$), with a water temperature of 28 °C resulting in a clearly reduced egg production compared to a water temperature of 24 °C.

When focusing on peak fecundity (week 11), the generalized linear model showed a significant interaction effect between Cd treatment and temperature ($\chi^2_{2,39} = 23.76$, $p < 0.001$), resulting in a reduction from a mean of 20 eggs in the control treatment compared to 1.75 eggs in the combined stressor treatment. Also, both Cd treatment ($\chi^2_{2,39} = 8.227$, $p = 0.016$) and temperature treatment ($\chi^2_{1,39} = 93.16$, $p < 0.001$) were significant as main factors.

The analysis of total (cumulative) fecundity showed a significant interaction effect between Cd treatment and temperature ($\chi^2_{2,29} = 14.69$, $p < 0.001$). In addition, both Cd treatment ($\chi^2_{2,29} = 23.71$, $p < 0.001$) and temperature treatment ($\chi^2_{2,29} = 403.1$, $p < 0.001$) were significant as main factors (Supplemental Data, Figure S1).

CTmax

Values of CTmax were all between 38.9 and 41.7 °C. All fish recovered from the CTmax assay without loss of buoyancy. The behavior of *N. furzeri* near the thermal maximum was similar to the behavior described in other studies that evaluated this endpoint (Beitinger et al. 2000; Patra et al. 2007): erratic swimming, increased opercular movement, and loss of ability to remain in a dorsoventrally upright position. Values of CTmax were not affected by exposure to Cd ($\chi^2_{2,37} = 0.341$, $p = 0.843$), sex ($\chi^2_{1,37} = 1.888$, $p = 0.169$), or body size ($\chi^2_{1,37} = 0.975$, $p = 0.324$). However, temperature had a strong effect on the thermal maximum ($\chi^2_{1,37} = 85{,}56$, $p < 0.001$), and fish reared at 28 °C had an approximately 1 °C higher CTmax value than fish reared at 24 °C (Figure 4B).

Adult mass

Mass of the fish was affected by temperature ($F_{1,62} = 24.26$, $p < 0.001$), sex ($F_{1,62} = 15.73$, $p < 0.001$), and body size of the

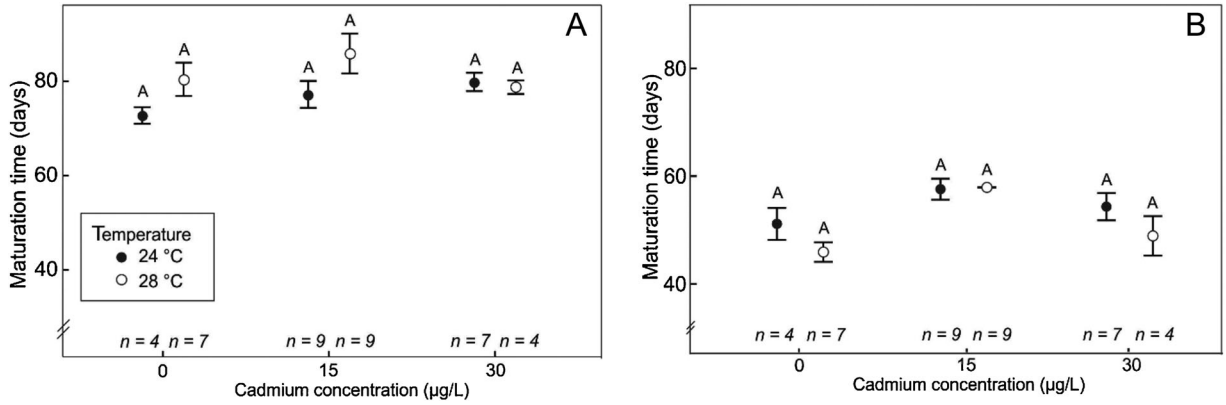

FIGURE 2: Mean maturation time of *Nothobranchius furzeri* exposed to different cadmium concentrations. (**A**) Age (in days) at which females produced their first eggs. (**B**) Age (in days) at which the first signs of coloration appeared in males. Nominal concentrations are shown. Values are presented as mean ± standard error. Different letters indicate statistically significant differences between groups.

fish ($F_{1,62} = 8.74$, $p = 0.004$; Figure 5A). Cadmium exposure did not affect mass ($F_{2,62} = 0.479$, $p = 0.622$). Males weighed on average 62.5 mg (18%) more than females (429.7 and 364.3 mg, respectively), and fish in the 24 °C treatment weighed on average 72.9 mg (20%) more than those from the 28 °C treatment (430.8 and 357.9 mg, respectively).

Bioaccumulation of Cd

Bioaccumulation was not affected by sex ($F_{1,43} = 2.525$, $p = 0.119$) or temperature ($F_{1,43} = 2.010$, $p = 0.164$). Fish accumulated more Cd when exposed to increasing Cd concentrations ($F_{2,43} = 124.3$, $p < 0.001$; Figure 5B). On average, fish accumulated 0.0005 ± 0.001, 0.1131 ± 0.030, and 0.1887 ± 0.030 µg Cd/g wet mass when exposed to C0, C1, and C2, respectively. Out of 28 control samples, 20 contained a Cd concentration below the measurable threshold. Because 0.001 µg Cd/L is the minimum resolution of the ICP-MS analyses, we adopted this value for fish homogenates in which the concentration was below the detection limit. This probably resulted in an artificially high mean Cd concentration in control fish.

Energy parameters and MT concentration

Glycogen concentrations were not affected by exposure to Cd ($F_{2,69} = 1.039$, $p = 0.359$), sex ($F_{1,69} = 0.521$, $p = 0.473$), or temperature ($F_{1,69} = 2.162$, $p = 0.146$). Females had a higher fat content compared to males ($F_{1,69} = 11.72$, $p = 0.001$; female 36 768 ± 6854 µg/g tissue, male 30 875 ± 5919 µg/g tissue; Figure 6A). Exposure to Cd ($F_{2,69} = 1.112$, $p = 0.335$) or warming ($F_{1,69} = 0.667$, $p = 0.417$) did not affect fat content (Figure 6B). Protein content was affected by temperature ($F_{1,69} = 12.441$, $p < 0.001$), with fish reared at 28 °C having a 31% higher protein content. There was also an effect of sex ($F_{1,69} = 4.375$, $p = 0.040$), with females having a 14% lower protein content than males (Figure 6C). However, an effect of Cd exposure was not found ($F_{2,69} = 0.26$, $p = 0.771$). Finally, MT concentration was affected by temperature ($F_{1,69} = 4.54$, $p = 0.037$) and sex ($F_{1,69} = 26.4$, $p < 0.001$) but not by exposure to Cd ($F_{2,69} = 0.16$, $p = 0.848$). Males had a 75% higher MT concentration compared to females. Fish reared at 28 °C had a 24% lower MT concentration compared to fish reared at 24 °C (Figure 6D). There was no correlation between accumulated cadmium and MT concentration ($r = 0.04$, $t_{68} = 0.334$, $p = 0.740$).

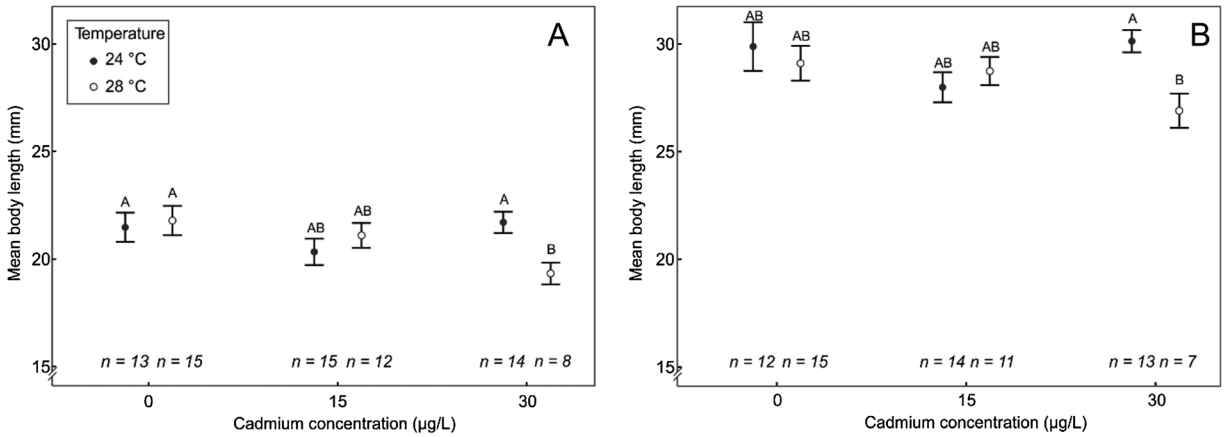

FIGURE 3: Mean body size of *Nothobranchius furzeri* exposed to different concentrations of cadmium and 2 temperatures at (**A**) week 7 and (**B**) week 15. Nominal concentrations are shown. Values are presented as mean ± standard error. Different letters indicate statistically significant differences between groups.

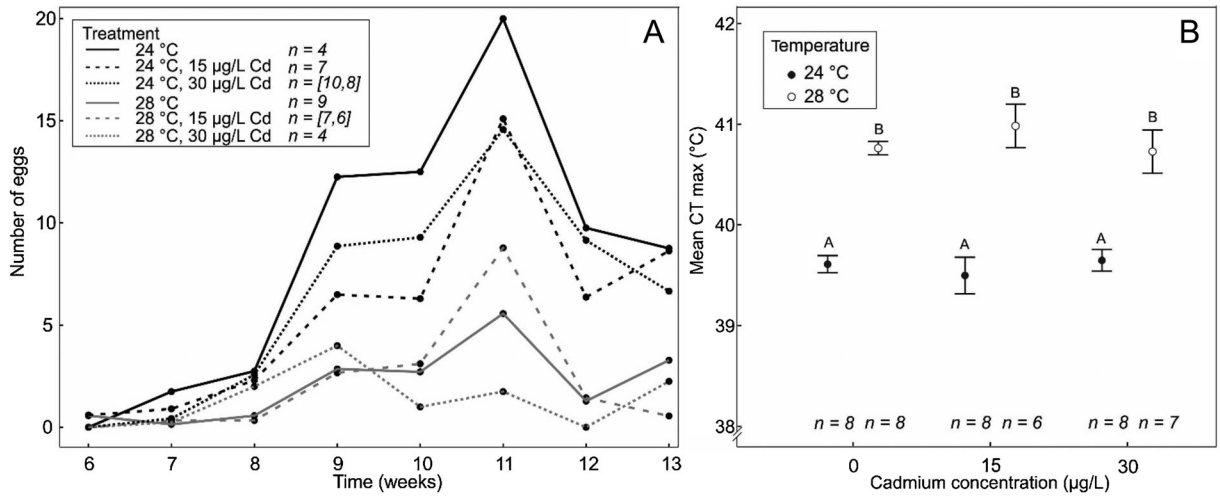

FIGURE 4: (A) Fecundity through time, measured as number of eggs per week for each temperature treatment. To improve the readability and interpretability of the figure, error bars are not shown on the graphs. The number of females in each treatment at the beginning and end of the egg-laying period is indicated. (B) Mean critical thermal maximum of fish exposed to different concentrations of cadmium and 2 temperatures. Nominal concentrations shown. Values are presented as mean ± standard error. CTmax = critical thermal maximum. Different letters indicate statistically significant differences between groups.

Multigenerational sensitivity to Cd

The treatment with exposure to 30 µg/L Cd at 28 °C could not be included because all eggs produced by these fish died before hatching. Figure 7 shows the cumulative mortality of the offspring generation for the remaining 5 treatments at every measured time point. Because the parental generation had a 72-h LC50 of 0.36 mg/L Cd in both temperature regimes, we expected that offspring of both temperature treatments would have about 50% survival after 72 h when exposed to this concentration in the absence of a multigenerational effect (black dot in Figure 7). Survival in the C2T0 condition was excluded from the analysis because there were no surviving C2T1 larvae to analyze the effects of both Cd and temperature stress in a full factorial way. We found a trend of temperature having an effect on offspring survival ($\chi^2_{1,30} = 3.724$, $p = 0.054$), with offspring reared at a parental temperature of 28 °C having a lower survival compared with offspring reared at a parental temperature of 24 °C. The parental Cd treatment had no effect on offspring survival ($\chi^2_{1,30} = 0.146$, $p = 0.702$).

DISCUSSION

The temperature-dependent toxicity of chronic exposure to metals has been studied for a number of organisms (reviewed in Noyes et al. 2009; Holmstrup et al. 2010) but rarely for vertebrates because of cost and time constraints. Yet, because vertebrates have a distinct sensitivity and stressor interaction effects may be different from those on invertebrates (Jackson et al. 2016), such studies are needed. We used the annual killifish, *N. furzeri*, which is one of the most short-lived vertebrate models, to assess the impact of chronic exposure to Cd and increased temperatures within and across generations. Increased temperature affected maturation time of females, fecundity, CTmax, body mass, and protein content. In addition, a trend of reduced offspring survival emerged. Cadmium exposure affected maturation time of males and was shown to accumulate. Both stressors combined decreased adult body size, peak fecundity, and embryonic survival because all eggs that were produced at 30 µg/L Cd and 28 °C died before hatching.

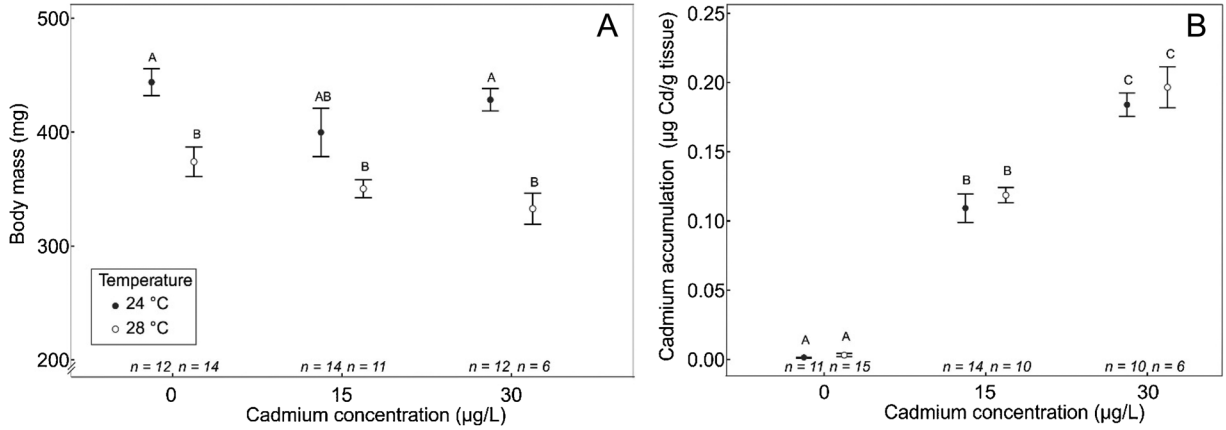

FIGURE 5: (A) Mean mass and (B) mean cadmium accumulation of fish exposed to different concentrations of cadmium and 2 temperatures. Nominal concentrations are shown. Values are presented as mean ± standard error. Different letters indicate statistically significant differences between groups.

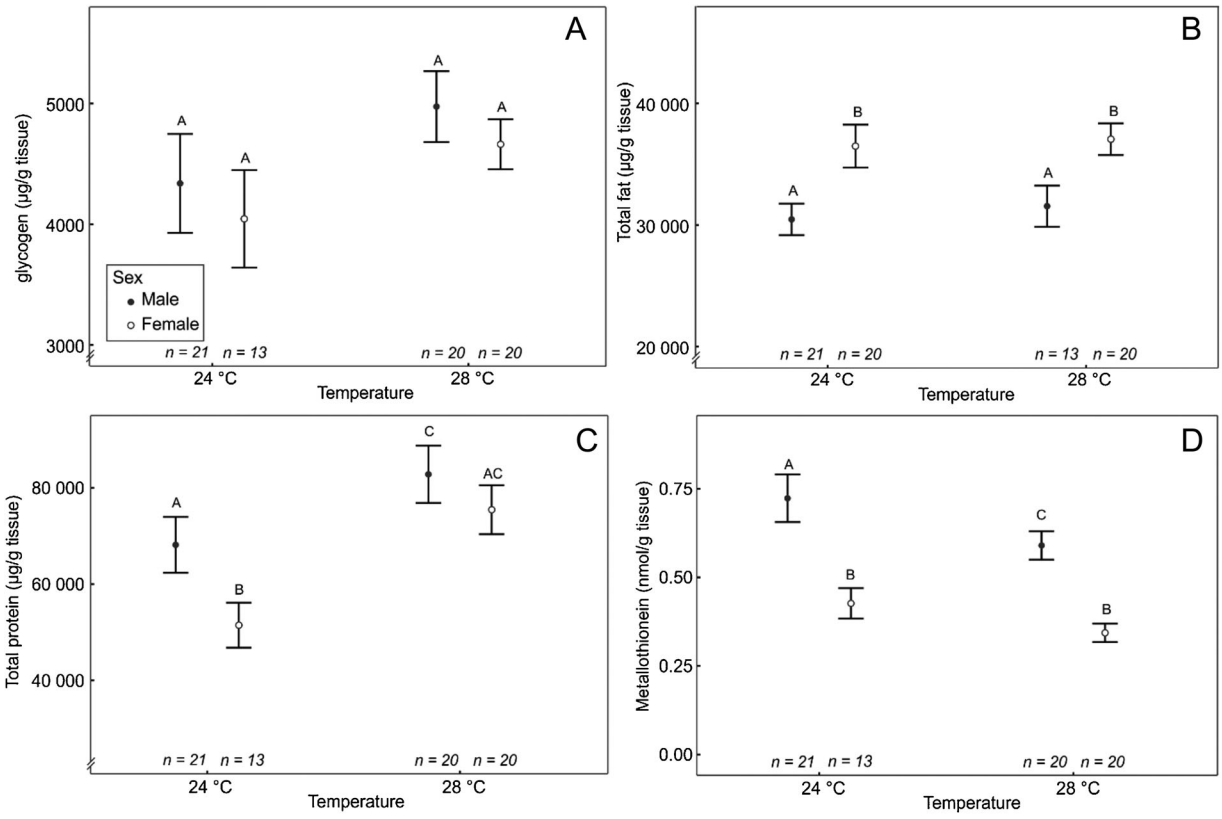

FIGURE 6: Energy reserves and metallothionein (MT) induction in *Nothobranchius furzeri* exposed to different concentrations of cadmium and 2 temperatures. (**A**) Glycogen content, (**B**) total fat content, (**C**) total protein content, and (**D**) MT induction (all as wet mass) of males and females at different rearing temperatures. Values are presented as mean ± standard error. Different letters indicate statistically significant differences between groups.

Effects of chronic Cd exposure

The absence of any Cd effect on survival indicates that the chosen concentration range was sufficiently low to measure more sensitive, sublethal endpoints, as was the goal of the present study. *Nothobranchius furzeri* fish appear to be more resistant to Cd exposure than other tested fish species. Although exposure to Cd delayed the maturation of males in the 15 µg/L (C1) treatment by about 9 d compared to fish in the control condition, this effect was not maintained in the 30 µg/L Cd (C2) treatment. For rainbow trout, Brown et al. (1994) showed that 5.48 µg/L Cd was the lowest-observed-effect concentration (LOEC) for delayed maturation and that the LOEC for survival was as low as 29.1 µg/L, which corresponds to C2 (30 µg/L) in the present study. In addition, for fathead minnow, effects of chronic exposure to Cd were already observed at a concentration of 10 µg/L Cd (Spehar and Fiandt 1986). The relative resistance of *N. furzeri* to Cd is also supported by the fact that the total MT concentration did not differ between control and Cd-exposed fish, even though the bioconcentration of Cd linearly increased with increasing exposure concentrations. The absence of this signal might be an indication that existing defense mechanisms at the cellular level were sufficient to protect the fish from excessive damage and that investment in costly extra defense mechanisms was unnecessary.

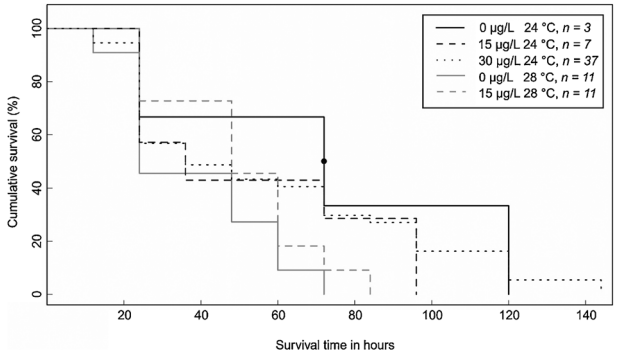

FIGURE 7: Cumulative survival of the offspring of each parental treatment exposed to 0.36 mg/L cadmium at the exposure temperature of their parents. Black dot represents the expected survival after 72 h in the absence of multigenerational effects.

Temperature stress and acclimation

Even though mortality in the parental generation was not influenced by temperature, a temperature increase of 4 °C was stressful. Three previous generations have been reared at a constant temperature of 24 °C, which might have caused acclimation and suboptimal performance at 28 °C. This might be realized in several ways. First of all, the increased rearing temperature delayed maturation of females by about 5 d. Acclimation to a higher temperature may have been costly, partly causing a delay in female maturation, which is likely

related to delayed gonad development and egg production. Although no similar effect emerged for males, this could be an artifact of the method that was used to determine male maturity. Male maturation time was only scored indirectly using the first signs of their nuptial coloration as a proxy, and this may not be an exact enough measure. Possibly, males showed full nuptial coloration before they produced active gametes. Second, adult body mass was also reduced in the high temperature regime. When fish are reared at a temperature that exceeds the optimal rearing temperature, mass increases less because of the higher cost spent on maintaining body homeostasis (Van Ham et al. 2003). Costs of maintaining body regulation at +4 °C may have overruled the benefits of a potentially accelerated digestive process. Although the body size of fish at different temperatures did not differ, fish at 28 °C weighed 20% less than fish at 24 °C, indicating a lower body condition. In addition, fish that were reared at 28 °C might have experienced food stress attributable to the higher food demand, resulting in a lower mass compared to fish reared at 24 °C. The latter mechanism appears less likely because energy reserves were comparable in both temperature treatments, except for protein content which was even higher in fish reared at 28 °C. In zebrafish, an opposite effect was found, with liver protein content being lower with higher temperatures and muscle protein content being equal among fish from different temperature treatments (Vergauwen et al. 2010). Third, fecundity of fish reared at 28 °C was reduced by >50%. This could also be explained by the higher metabolic costs associated with life at higher than optimal temperatures. Such impaired fecundity has been previously described in, for instance, the copepod *Acartia tonsa* (Holste and Peck 2006) and the coral reef damselfish *Acanthochromis polyacanthus* (Donelson et al. 2010). Finally, the offspring survival of fish reared at 28 °C showed a trend of being lower than that of fish reared at 24 °C. Although this finding implies that 28 °C caused stress, this setup was not designed to determine if this was attributable to the sensitivity of the larvae or to multigenerational sensitivity to this temperature.

In contrast to the concept of "toxicant-induced climate change sensitivity" (Noyes and Lema 2015; Op de Beeck et al. 2017), whereby organisms lose their resistance to heat stress (reduced CTmax) after exposure to a toxicant, fish exposed to Cd did not have a reduced CTmax. This may reflect strong selection to maintain high heat tolerance in natural *N. furzeri* habitats, which are shallow water bodies that are characterized by large temperature fluctuations (Cellerino et al. 2016), or be an indication of the limited toxic effects that these sublethal Cd concentrations had on *N. furzeri*. In line with thermal acclimation, the higher rearing temperature did increase the upper thermal limit. This is a common phenomenon in freshwater fish (Healy and Schulte 2012) and other taxa (e.g., damselflies [Op de Beeck et al. 2017]) that may plastically adapt their thermal tolerance depending on the environmental temperature. These results thus indicate that while populations of *N. furzeri* are naturally exposed to broad temperature fluctuations, they plastically adjust their maximal thermal tolerance after being exposed to an average temperature rise of 4 °C.

Interactive effects of Cd exposure and temperature stress

When fish were exposed to Cd in combination with a 4 °C temperature increase, adult body size was reduced by 7.6% compared to fish that were only exposed to Cd at the standard rearing temperature of 24 °C. Because warming in itself did not have this effect, the pattern suggests a more-than-additive or a synergistic interaction between both stressors. Although this reduction in size is limited, it could impact the competitive abilities of males and could be associated with other fitness-related traits including pathogen resistance and fecundity. Moreover, size reduction may have been limited because all factors except the imposed stressors were kept optimal in (and previous to) our experiment. In an ecological context this effect may well be much stronger given that the fish are exposed to less than optimal physical and physiological conditions (Liess et al. 2016).

The most stressful combination of Cd and temperature resulted in a strongly decreased peak fecundity. This is an important result because fecundity is the most fitness-affecting endpoint. On top of that, it resulted in low embryonic survival, which further supports the synergism between the 2 stressors. This result should, however, be interpreted with care because egg survival was only monitored. Also, we cannot disentangle the effect of embryonic exposure and multigenerational effects because eggs were produced in water from the different treatments and, as such, were directly subjected to the stressors. Moreover, it is known that metals have the largest effect on embryos during the swelling phase immediately after fertilization. At this stage, the egg shell is still highly permeable and Cd can easily penetrate (Jezierska et al. 2009).

Synergistic effects of Cd and temperature stress were also demonstrated in zebrafish larvae, which showed increased levels of malformation (Hallare et al. 2005). Increased Cd toxicity at higher temperatures may be explained in several ways. First of all, uptake of Cd at 28 °C may simply have been higher because of an increased Cd diffusion rate at higher temperatures (Patra et al. 2007). However, because we did not find a higher Cd accumulation at higher temperatures, this appears unlikely. Second, while oxygen demand increases in a body that is exposed to chemicals, oxygen uptake is less efficient at higher temperatures (Eddy and Handy 2012), resulting in a detrimental mismatch between oxygen uptake and oxygen need.

Overall, the present findings support the need to assess the combined effects of multiple stressors. Although we found individual effects of temperature stress, almost no direct effects of Cd exposure emerged. Still, when combined with temperature stress, fish were severely affected at the same Cd levels and, besides additive, also synergistic effects of temperature stress and Cd emerged. We conclude that the extent to which multiple stressors exerted adverse effects individually or combined was dependent on the endpoint evaluated as well as the intensity of stress. The present results illustrate the possibility to measure a battery of endpoints on this fish species in a short time span, adding to the potential of *N. furzeri* as a model for chronic ecotoxicity testing.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.4182.

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Data Availability—Data are available on request (charlotte.philippe@kuleuven.be).

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