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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, since 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 non-existent. Here, we tested the combined effects of 4 °C warming and cadmium exposure on life history traits, biomarkers, bio-accumulation 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 four months. The applied cadmium concentrations had only limited effects on the measured endpoints, which suggests that *N. furzeri* is more resistant to cadmium 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. This article is protected by copyright. All rights reserved

Keywords: Freshwater toxicology, Bioaccumulation, Cadmium, Ecotoxicology, Killifish, Nothobranchius, Fish model

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 due to the fact that environmental warming increases the body temperature of ectothermic animals and will alter physiological and biochemical reactions but also affects 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 an increased uptake, a higher metabolic rate or loss of respiration efficiency, which results in upregulated 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). Since 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 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).

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 two 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 combine both. In transgenerational studies, the stressor is transmitted over generations in an indirect way, making sure at least on 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 as their life cycle is generally much longer than that of invertebrate model organisms. The few studies describing multigenerational effects of metals such as a 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 since it is a non-essential 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 US, the concentration generally does not exceed 0.1 μg/L (Colt 2006). However, in regions with effluents from heavy industry, or due to spills or sudden incidence, concentrations may peak. During the last decades, cadmium pollution has also increased globally due to the increasing use of phosphate fertilizers containing cadmium (Roberts 2014). In 2007, about 240 tons of cadmium entered the soil in the European Union (EU RAR 2007). It is the 7th toxicant on the "Top 20 Hazardous Substance" list, compiled by U.S. Environmental Protection Agency and it is one of six pollutants banned by the European Union in the "Restriction on Hazardous Substances (RoHS)" directive (Nogawa et al. 2004). Cadmium is persistent and combined with a high bio-concentration factor, accumulates within organisms which drives bio-magnification through the food chain (Croteau et al. 2005). Fish predominantly take up cadmium 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 cadmium affect gills by changing their morphology which causes imbalanced respiration and osmoregulation (Evans 1987). When fish are exposed to sublethal cadmium levels, this can negatively affect their life history (Cazan and Klerks 2015), physiology (Pereira et al. 2016) and behaviour (Qi et al. 2017). However, fish can partly protect themselves against the damaging effects of metal exposure by metallothionein 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, metallothioneins act as

nonspecific biomarkers (Sarkar et al. 2006) while for temperature stress critical thermal maximum (CTmax) values may act as non-lethal predictors of general resistance of fish to temperature stress. CTmax is defined as the temperature at which the critical thermal end point is reached and an organism loses its equilibrium by failing to maintain a dorso-ventrally 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).

Here, we studied the individual and combined long-term effects of cadmium 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 cadmium in just over four months. We investigated whether cadmium exposure in combination with a temperature increase affected life history traits (maturation time, fecundity, mass and growth), biomarkers (CTmax, metallothioneins, energy reserves), total bio-accumulation and multigenerational tolerance to both stressors. We will use 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 °C 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 pre-adapted to life under a different fixed temperature. In our lab cultures, natural-derived populations of this species have been reared for three generations at 24 °C prior to experimenting. As temperature stress, we

opted for an exposure regime of +4 °C since a temperature rise of 4 °C is predicted by the Intergovernmental Panel on Climate Change at the end of the 21th century under scenario AR5 (IPCC 2014). Although continuous exposure to 4 °C above their normal culturing temperature could compromise the stability of physiological functions in fish (Cairns Jr et al. 1975), they are known to adapt their thermal tolerance in response to increased temperatures. We hypothesize that higher temperatures will upregulate metabolism and increase the speed of development, possibly trading off with energetically costly traits such as fecundity and general performance. Furthermore, we expect a delayed maturation, impaired growth and lower fecundity due to cadmium exposure. Combined with temperature stress, we expect that these effects will be exacerbated. Finally, when *N. furzeri* is continuously exposed to cadmium for two generations, we expect that the F1 offspring will be more sensitive to cadmium 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. Afterwards, 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 below) from 48 h post hatching onwards. The medium was renewed every two days to ensure a constant water quality. All glass jars used for cadmium exposure were complexed with cadmium ($CdCl_2(H_2O)_{2.5}$) 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 °C 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 14h:10h 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, % 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, Essen, Belgium) twice per day, 7 days per week. After day 24, fish were moved into 2 L jars of which the water was refreshed every week. From then onwards, the same set of abiotic parameters was measured once a week. Average abiotic water quality values are presented in Table S1. At the start of the adult phase (day 43), the *ad libitum Artemia* diet was complemented with chopped *Chironomus* larvae (Ocean Nutrition, Essen, Belgium). Another two weeks later, fish were fed *ad libitum* twice a day with frozen *Chironomus* larvae only.

Experimental setup

The parental generation was incubated under two cadmium concentrations, C1 (15 µg/L Cd) and C2 (30 µg/L Cd) and a control treatment without cadmium (C0), fully crossed with two rearing temperatures, 24 °C and 28 °C, resulting in six treatments. Each treatment was replicated 26 times. The treatment concentrations were based on a range finding experiment with four cadmium concentrations (5, 10, 20 and 40 µg/L Cd) with six 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 seven of the experiment and were analysed using ICP-MS to measure the actual cadmium

concentrations in the medium. Analysis of the exposure medium showed concentrations of 1.18 (n = 8, 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 days in order to prevent mortality effects due to the natural ageing 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 colouration 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. Since we started the trial with newborn fish, of 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 three times a week until death. For spawning, couples were put in separate 1 L aquaria provided with a sand substrate for two hours. Afterwards, 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 two days. Body size was measured weekly by photographing every fish individually in a Petri dish that was placed on millimetre paper. The images were analysed digitally using the open source *Analysing Digital Images* software (Pickle 2008). Additionally, 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 critical thermal maximum, CTmax (Patra et al. 2007), was performed at day 105 in series of five 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 °C or 28 °C) by a HETO therm heater (Anker Schmitt) (Type Grant TC120) at a constant rate of 0.33 °C/min (SE 0.04 °C), comparable to 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). CTmax was scored as the temperature at which fish lost their balance and failed to maintain a dorso-ventrally 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 days, 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. Afterwards, various biomarkers were measured, including energy reserves and metallothionein (MT) content. In addition, we quantified bio-accumulation of cadmium using ICP-MS. Young fish (age < 60 days) were analysed as a whole for cadmium only. Due to 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 AG, Greifensee, Switzerland). Consequently, wet mass was used to calculate the relative concentration of cadmium in the whole body (µg Cd/g wet mass). Fish that weighed less than 0.02 g were excluded from the analysis due to large calculation errors. Older (age > 60 days) fish were homogenized on ice with 4 ml milli-Q water. This homogenate was divided in subsamples for the determination of bio-accumulation of cadmium 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.

As energy in an organism is limited, the increasing maintenance costs due 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 as 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 methanolchloroform 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 cadmium and a temperature rise. The goal of this multigenerational exposure was to assess if the short-term sensitivity to cadmium would change when the parental generation was sublethally exposed to cadmium. For this, we acutely exposed all offspring of each parental treatment to cadmium 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 72h-LC_{50} (0.36 mg/L Cd in both temperature regimes (Philippe et al. 2018)) and assessed mortality during 14 days. All healthy eggs that had developed into the hatchable DIII phase were used and therefore the number of replicate fish in every treatment differed and ranged from 3 to 37 per cadmium x temperature combination (see Table 1).

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 were 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 v3.2.3). Maturation time was analysed for both sexes separately, as maturation was differently scored. For both sexes, maturation time was analysed 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 v3.2.3) to estimate the parameters of the growth model. Von Bertalanffy parameters were then analysed using full factorial general linear models with concentration, temperature and sex as fixed factors. Body size was analysed 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 week 7 and 15) as fixed factors. Fecundity (number of eggs per week) was analysed using a generalised 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 analysed using a generalised linear model with a Poisson distribution and concentration and temperature as fixed factors. Mass was analysed as a linear model with categorical factors concentration, sex and temperature, as well as their interaction. CTmax was analysed as a general linear mixed model with concentration, temperature, sex and body size as

fixed factors and series as a random factor. Bio-accumulation was analysed as a linear model with concentration, temperature and sex as fixed factors. Glycogen, total fat, protein and MT levels were analysed as linear models with concentration, temperature and sex as fixed factors. The 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 analysed using Cox proportional hazards regression models.

Statistical analyses were performed in R v3.2.3 (R Development Core Team, 2016). We used the packages *survival* (differences between survival plots), *lme4* (likelihood ratio test), *multcomp* and *lsmeans* (post-hoc tests), *car* (Anova), *stats* (generalized linear models) and *mass* (StepAIC). Tukey's Honestly Significant Difference (Tukey's-HSD) test was performed to test multiple pair-wise comparisons using the "multcomp" package (Hothorn et al. 2008).

RESULTS

Survival

There was no effect of cadmium 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 weeks is considered normal for this species, as median survival in captivity has been reported to be between 17.5 and 29 weeks (Cellerino et al. 2015).

Maturation time

There was no effect of cadmium 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 days) than at 28 °C (82 ± 10.3 days). 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 days and 49 ± 8 days respectively). However, C2 males (52 ± 7 days) did not mature later compared to control (P = 0.442) and C1 males (P = 0.277). This could be a false positive result, due 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 cadmium 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 mm ±5.2) than females (32.6 mm ±5.3). Maximal body length was not affected by cadmium 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 cadmium 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 cadmium concentrations ($F_{2,33}$ = 3.25, P = 0.052) (C0: 28 mm ± 2.6, C1: 21 mm ± 2.0, C2: 19 mm ± 1.4), whereas cadmium did not have an effect on body size at 24 °C ($F_{2,40}$ = 1.57, P = 0.22) (C0: 21 mm ± 2.5, C1: 20 mm ± 2.4, C2: 22 mm ± 1.8) (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 cadmium 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. Also here, body size at 28 °C decreased with increasing concentration (C0: 29 mm ± 3.1, C1: 29 cm ± 2.2, C2: 27 cm ± 2.1), while this was not the case at 24 °C (C0: 30 mm ± 3.9, C1: 28 cm ± 2.6, C2: 30 cm ± 1.8).

Fecundity

Fecundity (measured as number of eggs per week per living female) was not affected by exposure to cadmium (LRT = 0.625, $P = 0.732$) (Figure 4A). Temperature affected fecundity significantly (LRT = 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 focussing on peak fecundity (week 11), the generalised linear model showed a significant interaction effect between cadmium 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 cadmium 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 cadmium treatment and temperature ($\chi^2_{2,29} = 14.69$, $P < 0.001$). Also, both cadmium 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 (Figure S1).

CTmax

Values of the critical thermal maximum (CTmax) were all between 38.9 °C and 41.7 °C. All fish recovered from the CTmax assay without loss of buoyancy. The behaviour of *Nothobranchius furzeri* near the thermal maximum was similar to the behaviour 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 dorso-ventrally upright position. CTmax was not affected by exposure to cadmium ($\chi^2_{2,37} = 0.341$, $P = 0.843$), 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 a ca. 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 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 mg 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 mg and 357.9 mg, respectively).

Bio-accumulation of cadmium

Bio-accumulation 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 cadmium when exposed to increasing cadmium concentrations ($F_{2,43} = 124.3$, $P < 0.001$) (Figure 5B). On average, fish accumulated 0.0005 ± 0.001 µg, 0.1131 ± 0.030 µg and 0.1887 ± 0.030 µg cadmium/g wet mass when exposed to C0, C1 and C2, respectively. Out of 28 control samples, 20 contained a cadmium concentration below the measurable threshold. Since 0.001 µg Cd/L is the minimum resolution of the ICM-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 cadmium concentration in control fish.

Energy parameters and MT concentration

Glycogen concentrations were not affected by exposure to cadmium ($F_{2,69}$ = 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) (F: 36768 ± 6854 µg/g tissue, M: 30875 ± 5919 µg/g tissue) (Figure 6A). Exposure to cadmium ($F_{2,69}$ = 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 cadmium 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 cadmium ($F_{2,69}$ = 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).

Multigenerational sensitivity to cadmium

The treatment with exposure to 30 µg/L Cd at 28 °C could not be included, as all eggs produced by these fish died before hatching. Figure 7 shows the cumulative mortality of the offspring generation for the remaining five treatments at every measured time point. As the parental generation had a 72h-LC_{50} of 0.36 mg/L Cd in both temperature regimes, we expect 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 on Figure

7). The survival of the C2T0 condition was excluded from the analysis since there were no surviving C2T1 larvae to analyse the effects of both cadmium 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 the parental temperature of 28 °C having a lower survival compared to offspring reared at the parental temperature of 24 °C. The parental cadmium treatment had no effect on the 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 and Holmstrup et al. 2010) but rarely for vertebrates due to cost and time constraints. Yet, since 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 *Nothobranchius furzeri*, which is one of the most short-lived vertebrate models, to assess the impact of chronic exposure to cadmium 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 since all eggs that were produced at 30 µg/L Cd and 28 °C died before hatching.

The effects of chronic cadmium exposure

The absence of any cadmium effect on survival indicates that the chosen concentration range was sufficiently low to measure more sensitive, sublethal endpoints, as was the goal of this study. *Nothobranchius furzeri* fish appear to be more resistant to cadmium exposure than other

tested fish species. Although exposure to cadmium delayed the maturation of males in the 15 µg/L (C1) treatment by about 9 days 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 LOEC for delayed maturation and the LOEC for survival was as low as 29.1 µg/L (Brown et al. 1994), which corresponds to C2 (30 µg/L) in our study. Also for fathead minnow, effects of chronic exposure to cadmium were already observed at a concentration of 10 µg/L Cd (Spehar and Fiandt 1986). The relative resistance of *N. furzeri* to cadmium is also supported by the fact that the total MT concentration did not differ between control and cadmium-exposed fish, even though the bio-concentration of cadmium 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.

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 realised in several ways. First of all, the increased rearing temperature delayed maturation of females by about 5 days. 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 artefact 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 colouration before they produced active gametes. Secondly, 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 due to 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 due to the higher food demand, resulting in a lower mass compared to fish reared at 24 °C. The latter mechanism appears less likely since 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). Thirdly, 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 tona* (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 set-up was not designed to determine if this was due to the sensitivity of the larvae or as a result of multigenerational sensitivity to this temperature.

In contrast with the concept of 'toxicant-induced climate change sensitivity' (Noyes and Lema 2015; Op de Beeck et al. 2016), whereby organisms lose their resistance to heat stress

(reduced CTmax) after exposure to a toxicant, fish exposed to cadmium 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 characterised 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. 2016)), 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 cadmium exposure and temperature stress

When fish were exposed to cadmium in combination with a 4 °C temperature increase, adult body size was reduced by 7.6% compared to fish that were only exposed to cadmium at the standard rearing temperature of 24 °C. As 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 also be associated with other fitness related traits including pathogen resistance and fecundity. Moreover, size reduction may have been limited as 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 optimal physical and physiological conditions (Liess et al. 2016).

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

Synergistic effects of cadmium and temperature stress were also demonstrated in zebrafish larvae, which showed increased levels of malformation (Hallare et al. 2005). Increased cadmium toxicity at higher temperatures may be explained in several ways. First of all, uptake of cadmium at 28 °C may simply have been higher because of an increased cadmium diffusion rate at higher temperatures (Patra et al. 2007). However, since we did not find a higher cadmium accumulation at higher temperatures this appears unlikely. Secondly, 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, our findings support the need to assess combined effects of multiple stressors. Although we found individual effects of temperature stress, almost no direct effects of cadmium exposure emerged. Still, when combined with temperature stress, fish were severely affected at the same cadmium levels and besides additive also synergistic effects of temperature stress and cadmium 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. Our 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.xxxx.

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

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

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 colouration appeared in males. Nominal concentrations are shown. Values are presented as mean ± SE.

Figure 3. Mean body size (in mm) of *Nothobranchius furzeri* exposed to different concentrations of cadmium and two temperatures at A) week 7 and B) week 15. Nominal concentrations are shown. Values are presented as mean ± SE.

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 begin and end of the egg laying period is indicated using the letter n. B) Mean critical thermal maximum (CTmax) of fish exposed to different concentrations of cadmium and two temperatures. Nominal concentrations shown. Values are presented as mean ± SE.

Figure 5. A) Mean mass and B) mean cadmium accumulation of fish that were exposed to different concentrations of cadmium and two temperatures. Nominal concentrations are shown. Values are presented as mean ± SE.

Figure 6. Energy reserves and MT induction in *Nothobranchius furzeri* exposed to different concentrations of cadmium and two temperatures A) Glycogen content (µg/g wet mass), B) Total fat content (µg/g wet mass), C) Total protein content (µg/g wet mass) and D) MT induction (mmol/g wet mass) of males and females at different rearing temperatures. Values are presented as mean ± SE.

Figure 7. Cumulative survival of the offspring of each parental treatment, exposed to 0.36 mg/L Cd at the exposure temperature of their parents. The black dot represents the expected survival after 72 h in the absence of multigenerational effects.

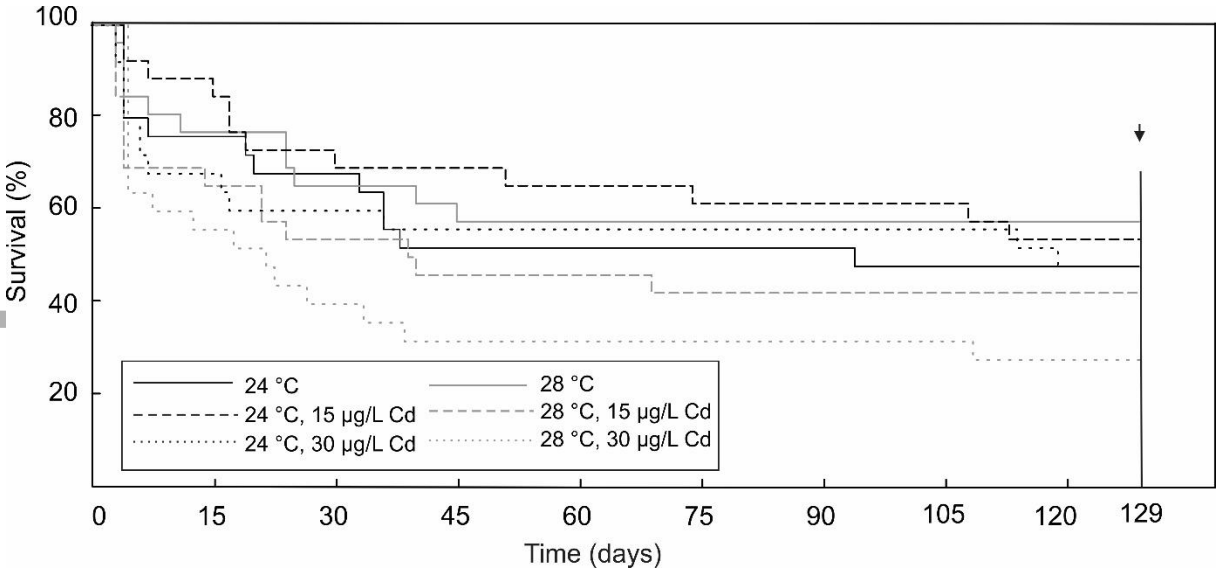

Figure 1

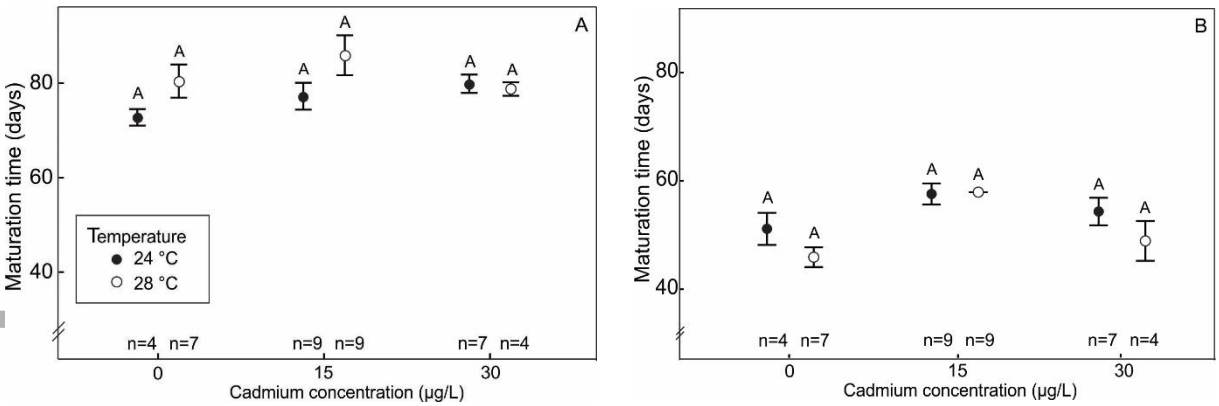

Figure 2

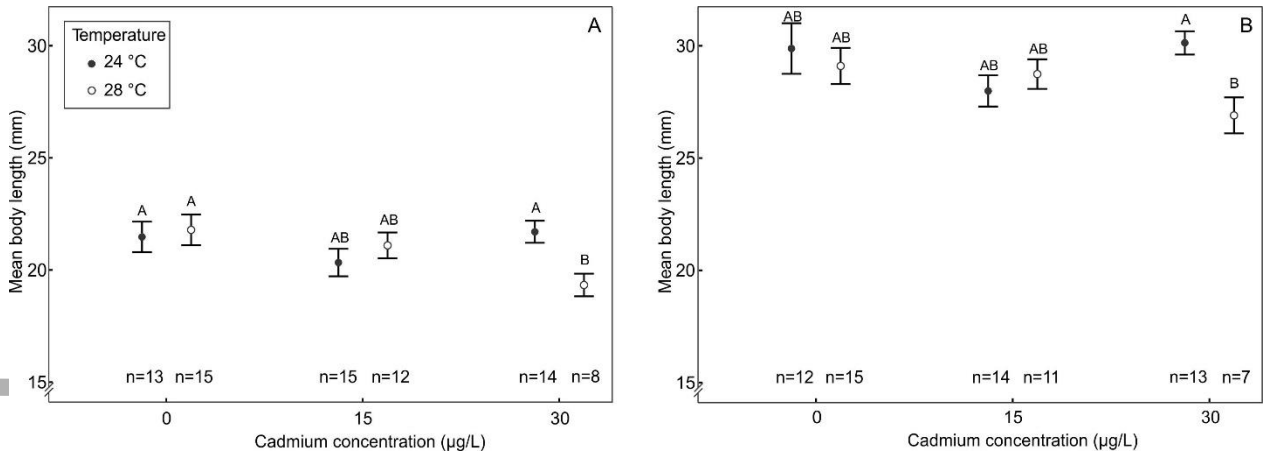

Figure 3

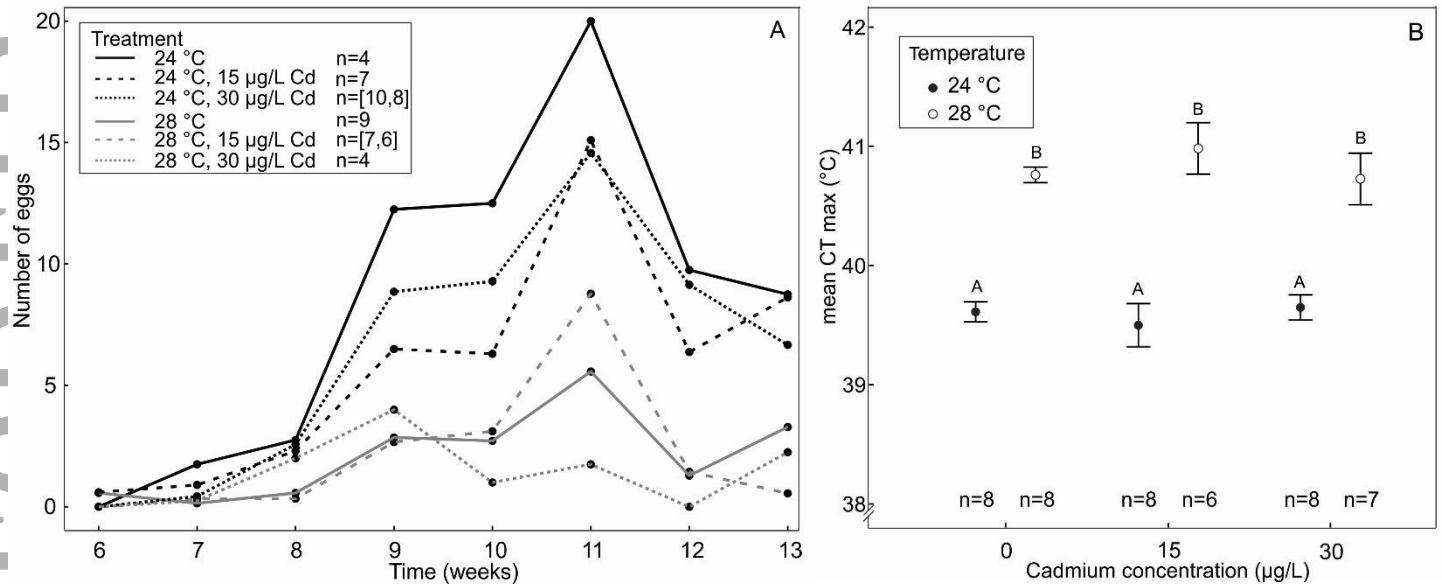

Figure 4

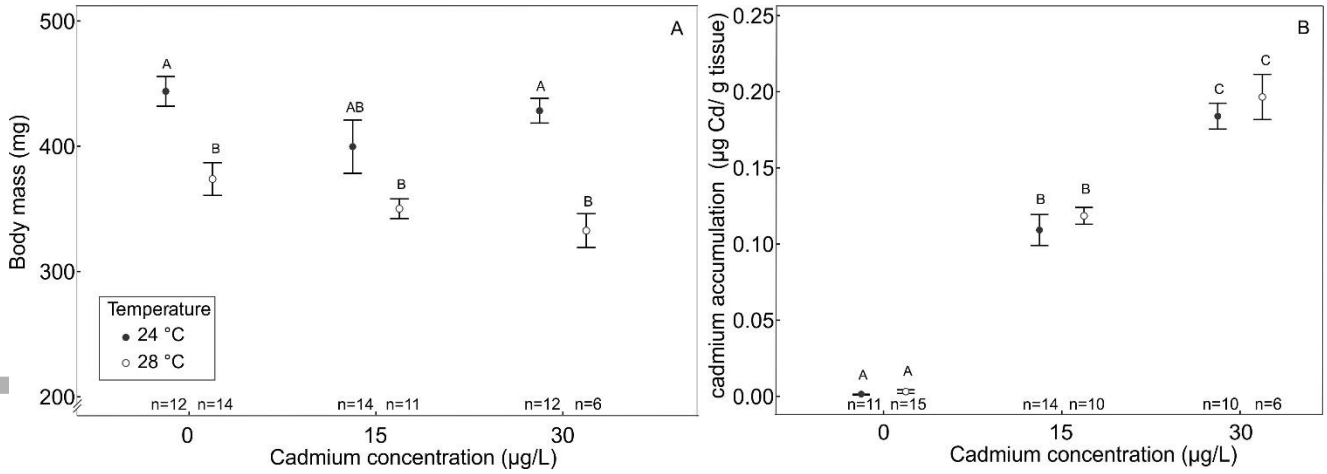

Figure 5

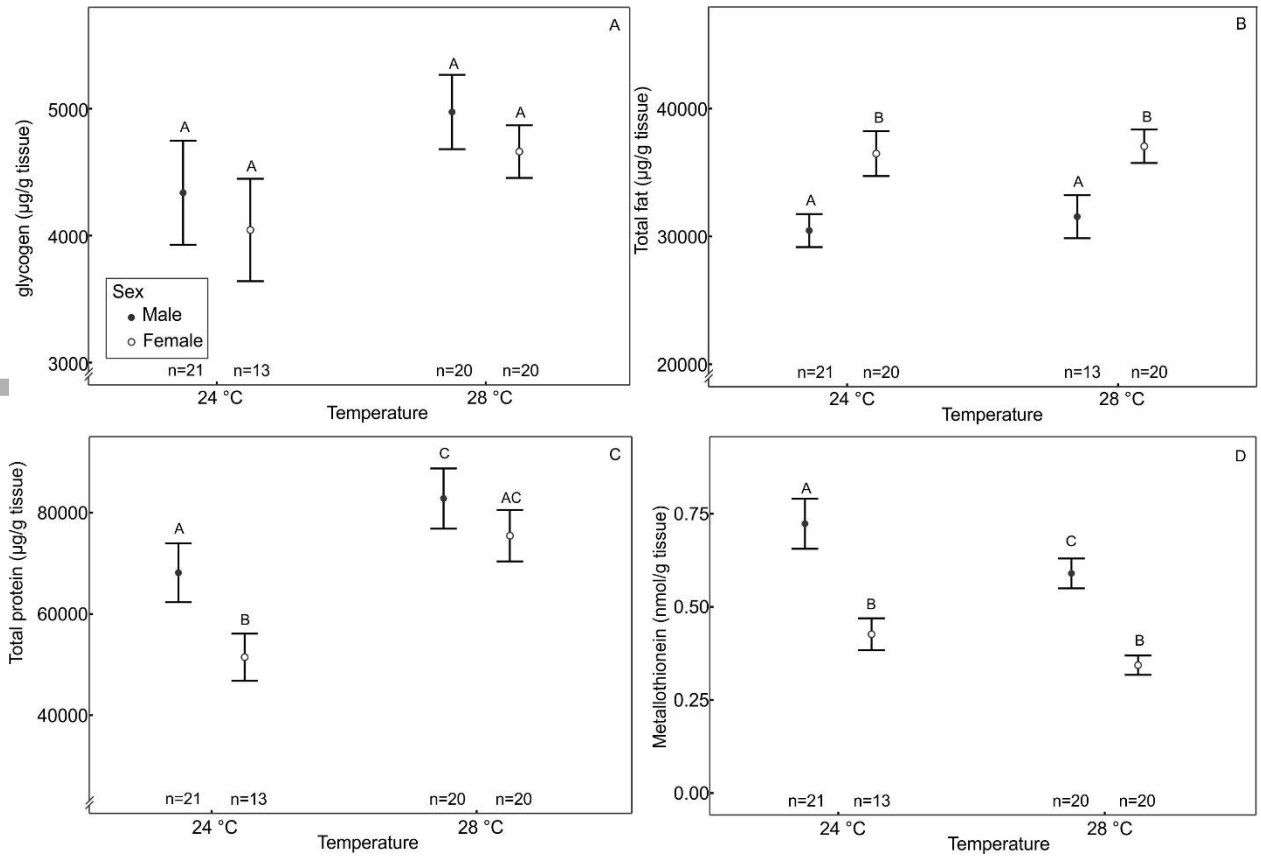

Figure 6

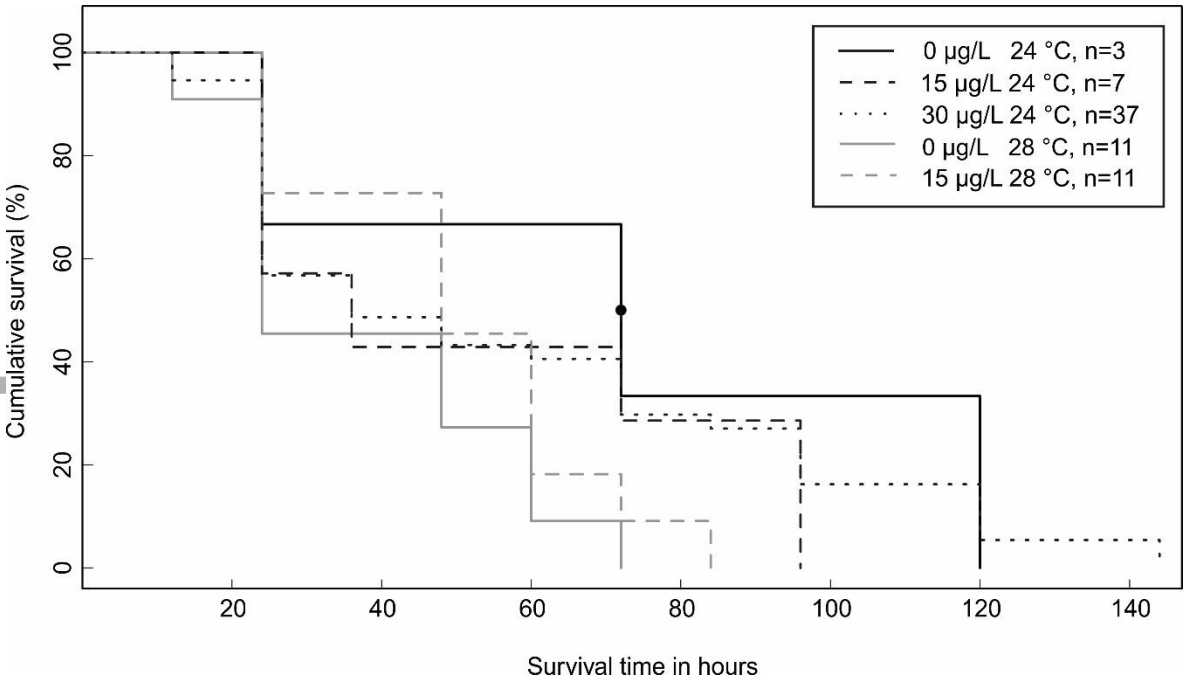

Figure 7

Table 1: Sample size for each treatment in the multigenerational exposure experiment. Replicates consist of the offspring of each parental treatment that developed to the hatchable DIII stadium.

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