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Interactive effects of 3,4-DCA and temperature on the annual killifish *Nothobranchius furzeri*

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ABSTRACT

Although aquatic organisms are increasingly exposed to pollutants and abnormally high temperatures as a consequence of climate change, interactive effects between those stressors remain poorly assessed. Especially in ectotherms, such as fish, increases in ambient temperature are expected to affect fitness-related traits and physiology. We used the turquoise killifish *Nothobranchius furzeri* to study the effects of a range of 3,4-dichloroaniline concentrations (0, 50, 100 µg/L) in combination with two temperature conditions (control and control +4 °C) during four months of exposure. As part of an integrated multi-level approach, we quantified effects on classic life history traits (size, maturation time, body mass, fecundity), critical thermal maximum and physiology (energy reserves and stress-associated enzymatic activity). While no interactive effects of 3,4-DCA exposure and increased temperature emerged, our results do show a negative effect of 3,4-DCA on thermal tolerance. This finding is of particular relevance in light of increasing temperatures under climate change. Due to increases in pest species and faster degradation of 3,4-DCA under higher temperatures, increased use of the pesticide is expected under climate change which, in turn, could result in a decreased tolerance of aquatic organisms to high temperatures.

1. Introduction

In ectotherms, body temperature mainly depends on external heat sources. Therefore, when the ambient temperature exceeds the thermal optimum of an ectothermic organism, adverse effects may occur. An increased temperature may for instance result in an increased metabolic rate due to a higher energy demand (Sokolova and Lannig, 2008) and reduced respiration efficiency (Noyes et al., 2009). In addition to such direct effects, increased temperatures may also affect the uptake, elimination and detoxification of pollutants (Heugens et al., 2001; Kimberly and Salice, 2013). Consequently, toxicants that do not affect an organism at its optimal temperature might become harmful under higher temperatures (Osterauer and Köhler, 2008). This is of particular relevance in light of climate change since it implies that predicted increases in temperature could exacerbate toxicant effects. Combined with the fact that pesticide use is expected to increase due to a higher prevalence of pests and faster pesticide degradation (Op de Beeck et al., 2017a), an improved understanding of temperature stress and pesticide exposure is needed (IPCC, 2014).

Chlorinated anilines are widespread toxicants in aquatic and terrestrial environments. They are often used as pesticides, such as phenylurea herbicides (diuron, linuron) and acylanilide herbicides (propanil) (Eijsackers et al., 1993). When they are degraded by microorganisms in the soil or water, the precursor of these herbicides, 3,4-dichloroaniline (3,4-DCA) (Crossland, 1990), is released into the environment. This results in elevated concentrations of the compound in agricultural soils (Scheil et al., 2009). In addition, the household use of diuron as a herbicide has resulted in frequent 3,4-DCA pollution in non-agricultural soils. The European Commission reports 3,4-DCA concentrations from 0.05 to 1.5 µg/L in surface water (IHCP, 2006) and states that the PNEC (Predicted No Effect Concentration) value of 0.2 µg/L is often exceeded (IHCP, 2006). Globally, concentrations of up to 567 µg/L have been measured in natural environments (Primel et al., 2007).

3,4-DCA is a highly persistent compound due to its high chemical stability and low volatility (Pubchem 2017). A biodegradation study showed only a 3% decrease of 3,4-DCA concentration in pond water after 14 days (TOXNET, 2014). Moreover, the compound accumulates

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in the sediment, which means that benthic and sediment-dwelling organisms, including insect larvae and worms, are exposed to extremely high concentrations. Since such animals are an important link in the food chain, typically eaten by fish and birds, they are a source for 3,4-DCA accumulation across trophic levels (Ware, 1983; IHCP, 2006).

In fish, 3,4-DCA toxicity results from tissue hypoxia, as it induces the formation of haemoglobin with ferric iron (Fe^{3+}) that has a lower affinity to oxygen than haemoglobin with ferrous iron (Fe^{2+}) (Crossland 1990). Early life stage and life cycle tests with four fish species have shown that the threshold concentrations (LOEC) for 3,4-DCA to affect body mass, body size, deformation, mortality and reproduction during 4–16 weeks of exposure was 200 µg/L (Scheil et al., 2009). In the common goby (*Pomatoschistus microps*), concentrations of 500–1490 µg/L were shown to induce sublethal effects after only 96 h of exposure. Specifically, the activity of two known stress-induction proteins, the biomarkers LDH (Lactate Dehydrogenase) and GST (Glutathione S-transferase), was altered. The enzyme LDH catalyses the conversion of lactate to pyruvate and is released upon tissue damage while GST plays an important function in detoxification (Aliko et al., 2018; Gobi et al., 2018). Therefore, the activity of both enzymes can serve as an indication of (toxicant) induced stress and alterations in both enzymes can also have severe fitness effects on the longer term (Monteiro et al., 2006; Scheil et al., 2009). Finally, 3,4-DCA is known to have an acute to chronic toxicity ratio (ACR) of over 1000 in fish. This implies that safety factors, which only account for an ACR of 10–100 (Ristau et al., 2015), could severely underestimate the risks of chronic exposure to this compound (Call et al., 1987; Schäfers and Nagel, 1991). This notion is supported by the fact that, for instance, size was reduced in juvenile rainbow trout (*Oncorhynchus mykiss*) that were exposed to a concentration of only 39 µg/L during four weeks (Crossland, 1990). Similarly, in an acute early life stage test on fathead minnow (*Pimephales promelas*), Call et al. (1987) showed an impaired growth and lower body mass after exposure to only 7.1 µg/L during 96 h (Call et al., 1987). Overall, these results do not only suggest that effects of chronic exposure to 3,4-DCA should be assessed, but they also imply that sensitivity to 3,4-DCA may be highly species specific. While the sensitivity range of taxa to this compound is very broad, the repeatability of the effects on fish has, for instance, led to the use of 3,4-DCA as a reference toxicant to control the quality of the brood stock in zebrafish (*Danio rerio*) embryos. For batches of larvae to be considered healthy, LC_{50} values between 1.6–4.4 mg/L should be reached (OECD Series on Testing and Assessment Fish Toxicity Testing Framework).

Although the effects of 3,4-DCA have been studied in vertebrates, there is only one study on effects of chronic exposure to this compound combined with temperature stress. Freitas et al. (2016) exposed tadpoles of the American bullfrog *Lithobates catesbeianus* to 3,4-DCA at different temperatures. Their results show that the 3,4-DCA toxicity was enhanced at higher temperatures, as evidenced by the upregulation of the thyroid hormone genes and a resulting accelerated development (Freitas et al., 2016). This is considered a mechanism to speed up metamorphosis in order to escape stressed environments in amphibians (Hooper et al., 2013). Until now, the combined effect of exposure to 3,4-DCA and temperature rise had not been studied in fish.

The turquoise killifish *Nothobranchius furzeri* is an interesting fish model to use in chronic exposure experiments. Specific populations of this fish mature in as little as three weeks and have a typical generation time of 5–6 weeks (Cellerino et al., 2016). As such, key life-history traits including maturation time and fecundity can be studied in a highly time-efficient way compared to other fish models (Philippe et al., 2018b). Furthermore, these fish produce drought-resistant eggs that can enter dormancy. The eggs remain viable for a long time when they are stored under standard conditions and a continuous culture of fish is, therefore, not needed (Polačik et al., 2016). An additional benefit for ecotoxicological studies is that replicate fish can all be hatched at the exact same moment, resulting in time synchrony for all animals.

In this study, we exposed *N. furzeri* individuals for four months to a combination of 3,4-DCA and two temperatures, a control condition at the optimal water temperature of 24 °C and a +4 °C (i.e. 28 °C) treatment. The +4 °C condition was chosen since it corresponds to an expected temperature increase by the second half of the 21th century (IPCC, 2014). As part of an integrated multi-level approach, we quantified effects on classic life history traits (size, maturation time, body mass, fecundity), critical thermal maximum and physiology (energy reserves and stress response). Overall, we expect that exposure to increased temperatures, under *ad libitum* food conditions, would speed-up timing of life events such as maturation and would increase growth rates, as long as cardiac and respiratory functions can meet the metabolic demands. However, combined with exposure to 3,4-DCA, two scenarios are possible. First of all, additive or synergistic adverse effects between both stressors could emerge, which would corroborate the results of the previously mentioned study on American bullfrog tadpoles by Freitas et al. (2016). Second, an antagonistic effect could emerge among both stressors (Kimberly and Salice, 2013), due to faster detoxification or faster degradation of the compound under higher temperatures. With regard to potential effects of the individual stressors, we hypothesise that exposure to higher temperatures will drive temperature acclimation, which would result in a higher critical thermal maximum (CTmax) of exposed individuals. Since exposure to 3,4-DCA might reduce locomotor activity (Scheil et al., 2009), we hypothesise that fecundity will be reduced with increasing exposure levels due to a reduction in the number of mating bouts. Finally, 3,4-DCA exposure has also been associated with an upregulated LDH activity and an increase in concentration of the detoxification enzyme GST in tadpoles, (Freitas et al., 2016). Therefore, we expect similar effects in *N. furzeri*.

2. Material and methods

2.1. Maintenance of the test animals and experimental set-up

Fish originated from the natural population NF414 in the Limpopo river basin in southern Mozambique (Bartáková et al., 2013) and were collected by researchers from the Czech Institute of Vertebrate Biology in 2012. Prior to the experiment, all fish were reared for three generations under optimal and standardised conditions in the laboratory. At the start of the experiment, *N. furzeri* eggs of the same age and developmental stage were hatched synchronically by inundating them in 1 cm of dechlorinated tap water at 12 °C in 2 L tanks. Afterwards, water temperature gradually converged to room temperature (22 °C). Only larvae that were able to fill their swim bladder (± 90%), which is a good proxy for health, were selected to be used in further experiments.

The experiment was performed in heated water baths to ensure a constant temperature regime of either 24 °C or 28 °C (i.e. the +4 °C treatment). Both temperatures are considered to be within the range of temperature variation that has been recorded in natural *N. furzeri* habitats and can be used for their maintenance in captivity (Polačik et al., 2016). During the entire experiment, all fish were individually housed in glass jars that were regularly randomized within their respective water baths and subjected to a 14h:10 h light-dark cycle. Exposure to the toxicant was initiated 48 h post hatching. At this time, each fish was housed in a 0.5 L jar. The experimental medium was prepared using Milli-Q and a standardised salt mix (Instant Ocean, Ocean Nutrition, Essen, Belgium) to obtain a conductivity of 600 µS/cm. Specific amounts of 3,4-DCA were added to the medium destined for the toxicity treatments. At this stage, the medium was refreshed every two days and abiotic variables (pH, % dissolved oxygen and temperature) were measured every second day in a randomly chosen control jar for each temperature treatment. After day 24, fish were transferred to aerated 2 L jars of which the medium was changed every week. Abiotic parameters were measured once a week in randomly chosen jars. The average temperature in both temperature treatments was 24.4 °C (n = 17, SE = 0.11) and 28.46 °C (n = 17, SE = 0.35), pH was 7.95

(n = 17, SE = 0.13) and dissolved oxygen was 79.60% (n = 17, SE = 4.77). The feeding regime was adapted to the age of the fish. Juveniles were fed *ad libitum* with *Artemia* nauplii (Ocean Nutrition, Essen, Belgium) twice per day until day 24. From then on, the *ad libitum* diet was complemented with chopped *Chironomus* larvae (Ocean Nutrition, Essen, Belgium). Another two weeks later, they were fed *ad libitum* twice a day with defrosted *Chironomus* larvae only.

Experimental exposure concentrations were based on the concentration range used by Scheil et al. (2009) who exposed zebrafish embryos and larvae to sublethal concentrations of 3,4-DCA (5, 10, 100, 250, 500 and 1000 µg/L). Additionally, we performed a range finding experiment at 24 °C with four concentrations (200, 1000, 5000, 25,000 µg/L) and six replicates. As even 200 µg/L 3,4-DCA resulted in 100% mortality after a week of exposure, we reduced the concentration to 100 µg/L for the highest exposure concentration. As such, the experiment consisted of two 3,4-DCA concentrations; C1 (50 µg/L 3,4-DCA) and C2 (100 µg/L 3,4-DCA) and a control treatment, fully crossed with two temperature regimes of 24 °C and 28 °C, resulting in six treatments that were replicated 16 times each. We used pure 3,4-DCA (Sigma-Aldrich, St. Louis, MO, USA). Due to the low solubility of 3,4-DCA, a stock solution 25,000 µg/L) was prepared and mechanically stirred for two days before each medium renewal. Given that the medium was only renewed on a weekly basis, mixed water samples were taken to measure the actual realised concentrations in the medium and to calculate the degradation of the compound throughout a week. Concentrations were measured at the University of Ghent (Department of Crop Protection) by means of liquid chromatography (LC/MS/MS) with ESI (Waters ACQUITY UPLC, Xevo TQD mass spectrometer). Based on the actually measured concentrations of 3,4-DCA four degradation curves were drawn-up (Fig. 1).

2.2. Life history traits

Fish were inspected daily for mortality, loss of buoyancy and other aberrant behaviour (e.g. swimming upside down). Body size was measured at day 7 (week 1), day 49 (week 7) and day 107 (week 15) by placing every fish individually in a Petri dish over millimetre paper, photographing it (top view) and analysing the images using *Analysing Digital Images* software (Pickle, 2008). Age at maturation for males was defined as the age at which the first signs of nuptial coloration were visible (Grégoir et al., 2017). Females were coupled with older non-virgin (Reichard and Polačik, 2010), non-experimental males to determine maturation as the first time the females produced eggs

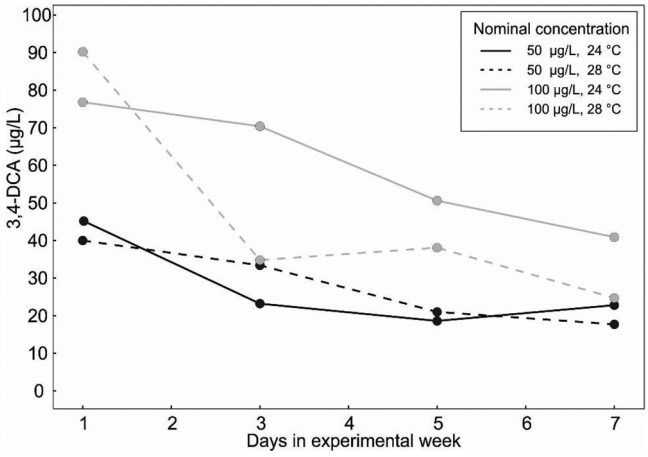

Fig. 1. Degradation of 3,4-dichloroaniline throughout a week in the Nothobranchius furzeri exposure experiment, as measured in mixed water samples taken in 2 L jars containing experimental fish. Concentrations of 3,4-DCA were determined by means of liquid chromatography (LC/MS/MS) with ESI (Waters ACQUITY UPLC, Xevo TQD mass spectrometer).

(Philippe et al., 2017). After maturation, fish were mated randomly three times per week within each condition for two hours to assess female fecundity (Polačik et al., 2011). For this, couples were transferred to individual 1 L aquaria with sand substrate for two hours (Philippe et al., 2018b). Afterwards, fish were transferred back to their individual glass 2 L jars. The sand was sieved (mesh size 500 µm) to count the eggs. All fish that survived to the last week were weighed on a weighing boat after patting them dry, four hours after their last feeding. In order to extensively measure the effect of the toxicant on fecundity, the experiment lasted 129 days. Then, all fish were euthanized by sedating them in ice water and transferring them to liquid nitrogen at −196 °C. Fish were stored at −80 °C to prevent degradation of proteins.

2.3. CTmax

Critical thermal maximum experiments were performed at day 104 in different series in a large water bath containing five parallel 1 L aquaria (each holding 1 individual) that was heated by a HETO therm heater (Anker Schmitt; Type Grant TC120), set to heat at a constant rate of +0.33 °C/min (SE 0.04 °C), in line with the heating rate used in comparable assays (Becker and Genoway, 1979; Barrett et al., 2011; LeBlanc et al., 2012), and starting from the fish's respective rearing temperature (24 °C or 28 °C). This is common practice in the measurement of CTmax of experimental animals treated at different temperatures (Op De Beeck et al., 2017b; Philippe et al., 2018b). The water was circulated and the water temperature in the 1 L aquaria was monitored using a digital thermometer (0.01 °C scale). Following standard practice, the critical thermal maximum was scored as the temperature at which fish lost their equilibrium and failed to maintain a dorso-ventrally upright position (Beitinger et al., 2000; Patra et al., 2007). At the end of each trial, fish returned to their rearing jars for recovery.

2.4. Physiology

The fish were thawed on ice for one hour prior to homogenisation. The tissue of each fish was diluted 15 times in a homogenisation buffer (0.1 M TRIS–HCl, pH 8.5, 15% polyvinyl pyrrolidone, 153 µM $MgSO_4$ and 0.2% Triton X-100), subsequently homogenised using a Ultra-turrax TP 18/10 and centrifuged for 7 min (6000 g, 4 °C). The resulting supernatant was used to measure the energy reserves (total fat, glucose, glycogen and protein contents). In addition, activity values of the two stress-associated proteins LDH and GST were determined as a proxy for induced stress and tissue damage (Aliko et al., 2018). All samples were measured in triplicate and mean values were calculated to be used in further analyses.

The fat content was quantified based on the protocol of Bligh and Dyer (Bligh and Dyer, 1959). We also determined the carbohydrate (glucose and glycogen) content using an adapted protocol from Stoks et al. (2006) based on the glucose kit by Sigma-Aldrich (USA). The protein content was measured based on the Bradford (1976) method. The activity of LDH was measured enzymatically based on a modified protocol of McPeek (1999). The activity of GST was measured based on the protocol of McLoughlin et al. (2000). All detailed protocols can be found in the supplementary methods.

2.5. Data analysis

Statistical analyses were performed in R v3.2.3 (R Development Core Team, 2016). We used the packages *survival* (differences between survival curves), *lme4* (likelihood ratio test), *multcomp* and *lsmeans* (post-hoc tests), *car* (Anova), *stats* (generalized linear models) and *mass* (StepAIC).

To assess differences in survival between 3,4-DCA and temperature treatments, we performed a full factorial survival analysis using the *survdiff* function, thereby right censoring all fish that died due to

external causes. 3,4-DCA impact on maturation time was analysed separately for both sexes as maturation was scored differently. For both sexes, maturation time was analysed using a general linear model with concentration, temperature and their interaction 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 11), using full factorial general linear models with concentration, temperature and sex (in week 7 and 15) as fixed factors. Fecundity (number of eggs per week) was analysed in two ways. First, by using a generalised linear mixed model with a Poisson distribution and concentration, temperature and their interation as fixed factors. Time (in weeks) and individual fish were included as crossed random factors. Second, total fecundity, measured as the cumulative amount of eggs a female produced throughout the experiment, was analysed using a generalized linear model with a Poisson distribution and concentration, temperature and their interaction as fixed factors. Body mass was analysed by constructing a general linear model with concentration, sex and temperature, as well as their interaction as categorical factors. Fulton's condition index was calculated using the formula $F = \frac{100 * mass}{adult\ size^3}$ (Nash et al., 2006). It was analysed by constructing a general linear model with concentration, sex and temperature, as well as their interaction as categorical factors. CTmax was analysed as a full factorial general linear mixed model with concentration, temperature and sex as fixed factors and series as a random factor. Energy reserves (glucose, glycogen, protein, total fat) and stress responses (LDH, GST) were analysed as full factorial general linear models with concentration, temperature and sex as fixed factors.

All non-significant interaction terms were left out in the final models. Tukey's Honestly Significant Difference (Tukey's-HSD) test was performed on the models outcome to test multiple pair-wise comparisons using the "multcomp" package (Hothorn et al., 2008). Equality of variance, as well as normality of residuals was checked and approved for every model.

All experiments and methods were approved by the ethical committee of KU Leuven (project number: P101/2014).

3. Results

3.1. Survival

There was no effect of exposure to 3,4-DCA ($\chi^2$2,121 = 0.332; P = 0.847) or temperature ($\chi^2$1,121 = 0.568; P = 0.451) on survival. Averaged over all treatments and the control, mortality was 29.6% at the end of the experiment after 129 days of exposure (Fig. 2). In the control condition, mortality after 129 days was around 30%. This is in line with our expectations since fish of the studied *N. furzeri* GRZ strain have a typical life span of 4–6 months. Aging related degenerative disorders within the strain can occur as of an age of ± 2 months (Reuter et al., 2019).

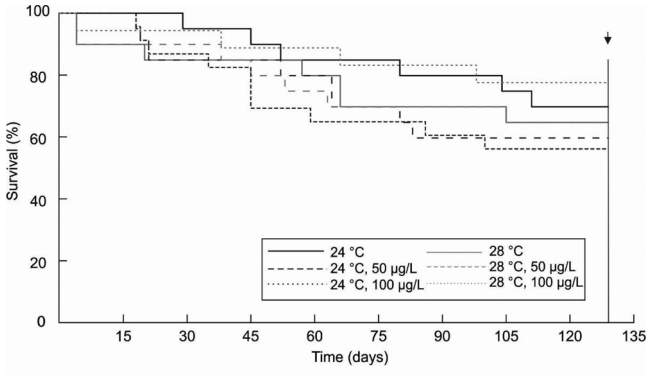

Fig. 2. Survival curves showing the proportion of surviving Nothobranchius furzeri individuals in the six experimental conditions. The experiment was terminated at day 129, as indicated by the arrow.

3.2. Maturation time

Mean male and female maturation time was 55.6 (± 8.6) and 74.2 (± 7.6) days, respectively. 3,4-DCA exposure did not significantly influence the maturation time of males ($F_{2,46}$ = 0.196, P = 0.660) or females ($F_{2,38}$ = 0.308, P = 0.737). Our results did not demonstrate a significant effect of temperature on male ($F_{1,46}$ = 0.697, P = 0.503; Figure S1A) or female maturation time ($F_{1,38}$ = 2.36, P = 0.133; Figure S1B).

3.3. Body size

Neither temperature ($F_{1,124}$ = 2.98, P = 0.087) nor 3,4-DCA exposure ($F_{2,124}$ = 2.98, P = 0.456) had an effect on body size at an age of 1 week. In week 7 (size at maturation), temperature affected body size significantly ($F_{1.77}$ = 5.21, P = 0.025) with fish exposed to 28 °C being on average 1.2 mm (4.8%) smaller than fish exposed to 24 °C. Also, there was a difference between sexes ($F_{1,77}$ = 6.04, P = 0.016) with males being on average 1.3 mm (5.8%) larger than females (Fig. 3A). 3,4-DCA concentration did not affect body size at maturation ($F_{2.77}$ = 1.20, P = 0.308).

After 107 days (15 weeks) of exposure, final body size of fish was significantly impacted by the 3,4-DCA treatment ($F_{2.74}$ = 3.42, P = 0.038; Fig. 3B). Fish that were exposed to the highest concentration (100 µg/L) were on average 1.7 mm (6.2%) larger than fish exposed to control conditions (P = 0.031). The size difference between males and females was maintained ($F_{1.74}$ = 14.04, P < 0.001) but the temperature effect was not significant ($F_{1,74}$ = 0.137, P = 0.711).

3.4. Body mass and Fulton's condition index

The total body mass of the fish was affected by temperature (F1,67 = 10.72, P = 0.002), as well as sex ($F_{1,67}$ = 89.25, P < 0.001) (Fig. 4A). Fish reared at 28 °C were on average 9% lighter than fish reared at 24 °C and females were 27% lighter than males. Furthermore, the effect of temperature on body mass differed between males and females ($F_{1,67}$ = 7.54, P = 0.008). Female body mass was comparable at 24 °C and 28 °C (P = 0.999), whereas males at 28 °C weighed on average 96 mg (16%) less than males at 24 °C (P < 0.001). There was no effect of 3,4-DCA exposure on body mass ($F_{2,67}$ = 2.68, P = 0.075). The condition index was not affected by exposure to 3,4-DCA ($F_{2,64}$ = 0.538, P = 0.586), temperature ($F_{2,64}$ = 2.85, P = 0.096) or sex ($F_{2,64}$ = 3.25, P = 0.076) (Fig. 4B).

3.5. Fecundity

Egg production (measured as number of eggs per week), was not affected by exposure to 3,4-DCA (LRT = 0.565, P = 0.754). However, temperature affected fecundity significantly (LRT = 6.279, P = 0.012), with a water temperature of 28 °C resulting in a clearly reduced egg production compared to a water temperature of 24 °C (Fig. 5A). The same pattern was found in the total egg production, that was not affected by exposure to 3,4-DCA ($F_{2,39}$ = 0.032, P = 0.968), but was affected by temperature ($F_{1,39}$ = 11.87, P = 0.001) and decreased by 61% when fish were reared at 28 °C (Figure S2).

3.6. CTmax

All fish survived the CTmax set-up and the behaviour of *N. furzeri* near the thermal maximum – erratic swimming, increased opercular movement and loss of ability to remain in a dorso-ventrally upright position – was similar to the behaviour described in other CTmax studies on fish (Beitinger et al., 2000; Patra et al., 2007). Variation in CTmax values was small (i.e., standard error < 0.4 °C). CTmax was

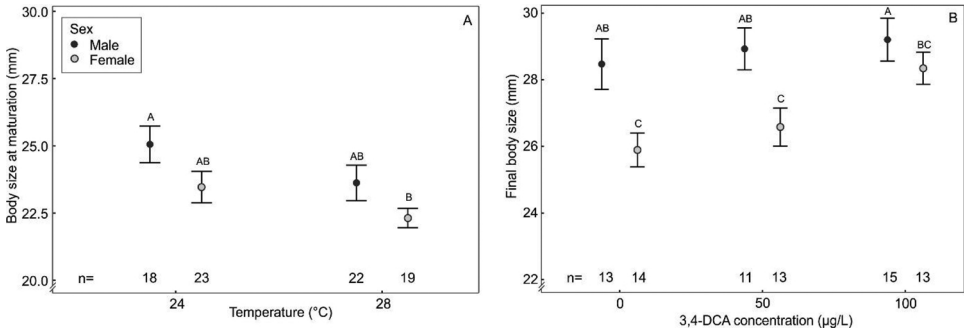

Fig. 3. Body size (in mm) of male and female Nothobranchius furzeri individuals, exposed to different concentrations of 3,4-DCA and two temperatures at A) maturation and B) after 15 weeks. Nominal concentrations are shown. Values are presented as mean ± SEM. Significant differences are indicated by letters.

affected by the rearing temperature ($\chi^2_{1,71}$ = 322.0, $P < 0.001$) and fish that were reared at 28 °C had a 1.3 °C higher CTmax compared to fish that were reared at 24 °C (Fig. 5B). Furthermore, CTmax was affected by 3,4-DCA exposure ($\chi^2_{2,71}$ = 17.65, $P < 0.001$) and fish exposed to 100 µg/L had a 0.32 °C lower thermal maximum compared to control fish ($P < 0.001$). There was no difference between CTmax of males and females ($\chi^2_{1,71}$ = 0.647, P = 0.424).

3.7. Energy reserves

The analysis of glucose content showed a different effect of temperature in males and females ($F_{1,43}$ = 18.07, P < 0.001) (Fig. 6A). In males, the same amount of glucose was found in both temperature regimes. However, females showed reduced glucose levels at 24 °C and elevated glucose levels at 28 °C, when compared to males. In general, temperature had a significant effect ($F_{1,43}$ = 16.04, P < 0.001), whereas exposure to 3,4-DCA ($F_{2,43}$ = 0.44, P < 0.650) and sex ($F_{1,43}$ = 0.004, P = 0.95) did not affect glucose levels. Glycogen content on the other hand, was affected by sex ($F_{1,44}$ = 7.13, P = 0.011), with females having a 40% lower glycogen content (Fig. 6B). Exposure to 3,4-DCA ($F_{2,44}$ = 1.05, P = 0.360) and temperature treatment did not affect glycogen content ($F_{1,44}$ = 0.282, P = 0.597).

Protein content was not affected by exposure to 3,4-DCA ($F_{2,44}$ = 1.688, P = 0.197), temperature treatment ($F_{1,44}$ = 0.188, P = 0.666) or sex of the fish ($F_{1,44}$ = 0.012, P = 0.915). Exposure to 3,4-DCA affected total fat content ($F_{2,44}$ = 4.02, P = 0.025), with fish exposed to 100 µg/L 3,4-DCA having a 25% higher fat content than fish exposed to 50 µg/L 3,4-DCA. The difference between fish exposed to 100 µg/L 3,4-DCA and control fish was, however, not significant (P = 0.27). Total fat content was not affected by temperature treatment ($F_{1,44}$ = 0.099, P = 0.754) and sex ($F_{1,44}$ = 0.175, P = 0.677) (Fig. 7A). GST activity was affected by temperature ($F_{1,44}$ = 10.71, P = 0.002), with fish reared at 28 °C having a 26% lower GST value (Fig. 7B). Furthermore, there was a difference between sexes ($F_{2,44}$ = 32.43, P < 0.001), with females having a lower GST activity compared to males. Exposure to 3,4-DCA did not affect GST activity ($F_{2,44}$ = 1.15, P = 0.325). The LDH response was similar, with reduced LDH activity at a higher temperature ($F_{1,44}$ = 11.63, P = 0.001) and in females ($F_{1,44}$ = 23.57, P < 0.001) (Fig. 7C). Exposure to 3,4-DCA did not affect the total LDH activity ($F_{2,44}$ = 2.23, P = 0.119).

4. Discussion

Exposure to a combination of stressors may result in more than additive effects. While effects of stressor combinations have been studied, at least to some extent, in aquatic invertebrates, hardly anything is known on potential effects in vertebrates. We present the first study on possible interactive effects of 3,4-DCA and increased temperature in fish. Specifically, we studied the effects of individual and combined chronic exposure to 3,4-DCA and a temperature increase of 4 °C in a multi-level approach by examining life-history along with behavioural and physiological responses. We found main effects of rearing temperature on body size at maturation, fecundity, body mass, CTmax, female glucose content, LDH activity and GST activity while 3,4-DCA exposure affected CTmax and final body size. We did not find indications of interactive effects of rearing temperature and 3,4-DCA exposure. Our findings are of particular relevance in light of increasing temperatures under climate change. Due to increases in pest species and faster degradation of 3,4-DCA under higher temperatures, increased use of the pesticide is expected under climate change which, in turn, could result in a decreased tolerance of aquatic organisms to high temperatures.

We found several indications that the temperature rise of 4 °C was stressful. For instance, fish that were kept at 28 °C were smaller at maturation compared to fish reared at 24 °C. One explanation could be that fish at 28 °C were energy deprived as a result of their higher metabolism at this temperature, resulting in a slower growth. For annual fish that are capable to grow and mature rapidly, a real *ad libitum* diet would imply that the fish would be fed several times a day. Therefore, limited food availability throughout the day could have resulted in a

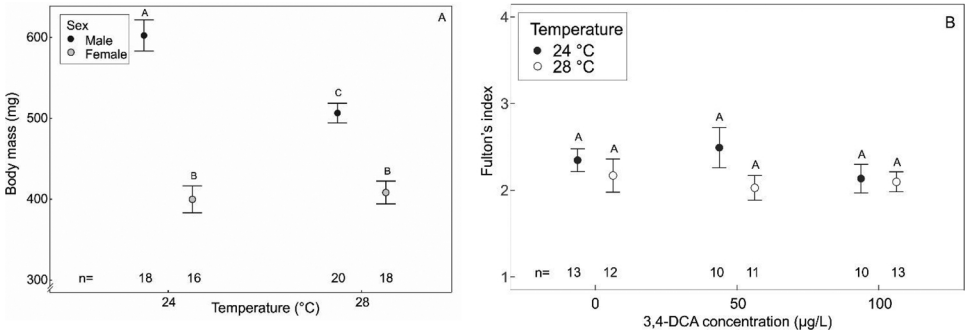

Fig. 4. A) Mean body mass of male and female Nothobranchius furzeri individuals under two different temperature treatments. B) Fulton's condition index of fish exposed to different concentrations of 3,4-DCA and two temperatures. Values are presented as mean ± SEM. Significant differences are indicated by letters.

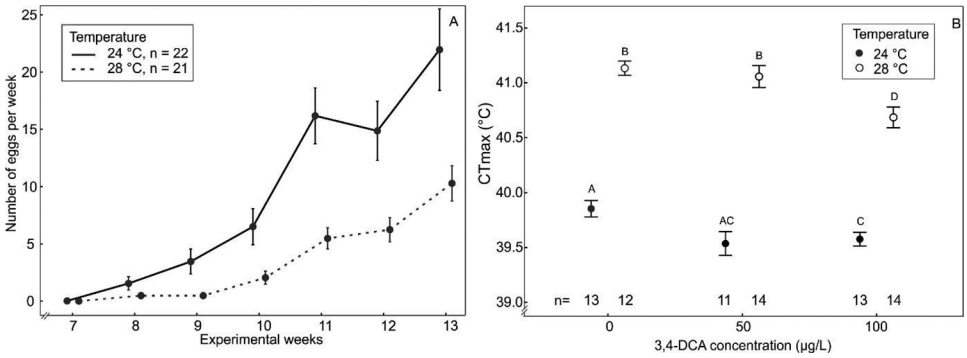

Fig. 5. A) Fecundity through time of Nothobranchius furzeri females, measured as number of eggs per week for each temperature treatment. B) Mean critical thermal maximum (CTmax) of fish exposed to different concentrations of 3,4-DCA and two temperatures. Values are presented as mean ± SEM and significant differences are indicated by letters.

smaller body size at maturation in the 28 °C treatment. The only temperature effect on energy reserves was a reduction in glucose levels in females at 24 °C and elevated glucose levels in females at 28 °C. When cortisol levels increase as a consequence of stress, glucose is also released in larger amounts (Aliko et al., 2018). As such, this could indicate that 28 °C may be higher than the optimal temperature for these fish, whose ancestors were bred at 24 °C for several generations. This temperature effect wasn't found in the glycogen reserve. In order to better interpret both energy parameters, it would be useful to measure them in different tissues, since the response to stress can be tissue-specific (Wiseman et al., 2011). The effect of temperature on body size faded with time and the body size and Fulton condition index at the end of the experiment were not significantly different between fish from the different temperature treatments. This could be an indication of life-stage specific effects of temperature on growth in killifish. Finally, fecundity was significantly lower (> 50%) at the higher temperature which may have major fitness implications. Females that were reared at 24 °C could invest more in growth (body mass) and reproduction, whereas females at 28 °C might have had to invest more in survival, resulting in elevated glucose levels, as readily available energy source, and an impaired fecundity.

Overall, our results indicate several life-stage specific effects of temperature. While larvae primarily invest in somatic growth, adults of 'extreme income breeders' such as *N. furzeri* allocate the majority of their energy towards reproduction (Vrtílek and Reichard, 2015). Therefore, every change in metabolic costs, such as the effect of a higher rearing temperature, has a high likelihood of being translated into effects that are life-stage specific. The fact that a temperature rise of 4 °C would cause such strong effects might seem counterintuitive for a fish species used to diurnal temperature fluctuations of up to 15 °C (Reichard et al., 2009). However, a continuous increase of the average temperature, even small, may have severe consequences, as was found in rainbow trout exposed to a 2 °C warming in summer (Morgan et al., 2001).

In contrast to our hypotheses, activity of LDH and GST was not increased in any of the 3,4-DCA treatments. In the study of Monteiro on the common goby, these parameters were induced in fish, stressed by 3,4-DCA exposure (Monteiro et al., 2006). However, literature shows that LDH activity can both be stimulated, as well as inhibited when organisms are exposed to stressors, depending on the tissue under study (Castro et al., 2004). Moreover, the response seems to be species specific in fish (Castro et al., 2004). Currently, the baseline physiology of *N. furzeri* is poorly documented, which complicates an accurate interpretation of this result. Potentially, the lack of response derives from a very high baseline activity of both enzymes. In nature, *Nothobranchius* fish inhabit temporary pond systems which display high daily variation in environmental conditions such as pH and temperature. Furthermore, across a limited number of days such habitats can be characterised by strong variation in dissolved salts (Polačik and Podrabsky, 2015). Potentially, high LDH and GST activity is maintained to cope with such challenges. Alternatively, the fact that *N. furzeri* individuals start displaying age-related degenerative disorders as of an age of around 2 months (Valdesalici and Cellerino, 2003) may also contribute to the lack of differences, especially for LDH. Given that aging may be accompanied with tissue damage, even control fish may have displayed upregulated levels of LDH at the evaluated time-point.

With respect to harmful effects of 3,4-DCA exposure, our results imply that we can set the NOEC at 50 µg/L (C1). Exposure to 100 µg/L resulted in a reduction of the critical thermal maximum, which is an indication of stress (Op De Beeck et al., 2017b; Sniegula et al., 2017). Because of the acute temperature rise in the CTmax trial, tissue homeostasis demands large amounts of oxygen. Exposure to 3,4-DCA, however, reduces the capacity to supply oxygen due to the induction of haemoglobin with ferric iron (Fe^{3+}). As such, tissues become hypoxic

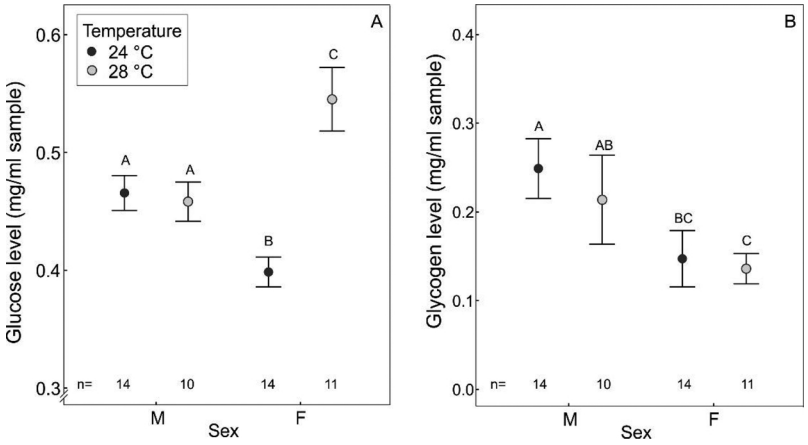

Fig. 6. A) Glucose level and B) Glycogen level in male and female N. furzeri at different rearing temperatures. Values are presented as mean ± SE. Significant differences are indicated by letters.

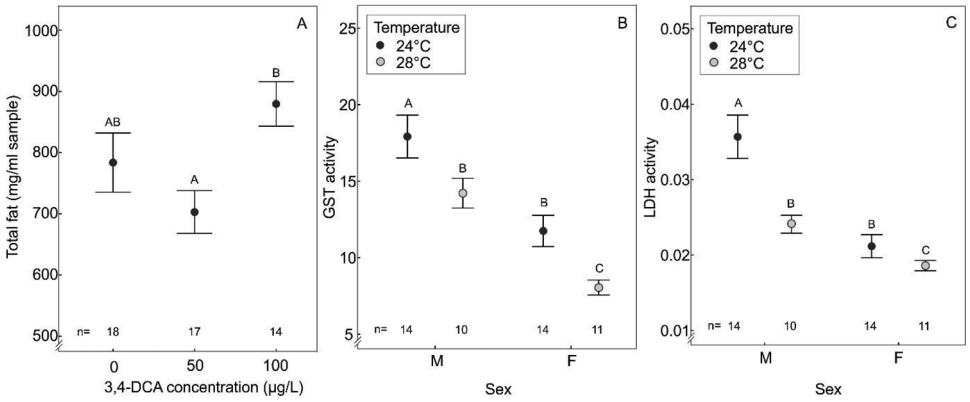

Fig. 7. A) Total fat content in male and female Nothobranchius furzeri at different rearing temperatures. Nominal concentrations are shown. B) GST activity and C) LDH activity in male and female N. furzeri at different rearing temperatures. Values are presented as mean ± SE. Significant differences are indicated by letters.

more rapidly, resulting in a lower CTmax. This decrease in critical thermal maximum is consistent with another study exposing the annual fish *Austrolebias nigrofasciatus* to a sublethal herbicide (Round-up) concentration (Zebral et al., 2018). To our knowledge, we present the first study that demonstrates effects of 3,4-DCA on CTmax. Interestingly, the standard deviance on CTmax values between the treatments was low. This supports the fact that CTmax is a reliable endpoint in stress research. Reduced thermal tolerance due to toxicant exposure will have even more severe consequences under climate change, since heat waves are expected to increase in frequency and duration (Beniston et al., 2007; Op De Beeck et al., 2017b). Moreover, as the use of toxicants such as herbicides and pesticides will increase due to global warming in order to keep the productivity of farming stable, more organisms will be exposed, possibly reinforcing the effects of global warming by reducing their thermal tolerance. Contrary to our expectations, body size was positively affected by 3,4-DCA after 107 days of exposure. However, the Fulton condition index, which is used as a general proxy for fish health by also taking into account body mass, did not differ significantly among fish from different tested exposure conditions.

When comparing the sensitivity of *N. furzeri* to 100 µg/L 3,4-DCA to that of other fish species, it appears to be relatively resistant to the compound. Nagel and colleagues did find effects of 3,4-DCA exposure on life history traits such as survival rate and growth in early life stages of zebrafish that were exposed to 100 µg/L during 4 weeks (Nagel et al., 1991). Crossland and Hillaby (1985) set the NOEC to protect an invertebrate pond ecosystem at 10 µg/L (Crossland and Hillaby, 1985), which is five times lower than the NOEC found in this study. Although life history traits such as fecundity and growth are more sensitive endpoints than survival (Philippe et al., 2017), sub-organismal endpoints may be even more sensitive. In male three-spined sticklebacks (*Gasterosteus aculeatus*), 3,4-DCA concentrations of 200–400 µg/L lowered androgen synthesis. Also, changes in secondary sex characters (male colour intensity, courtship behaviour) were induced by exposure to 100 µg/L 3,4-DCA (IHCP, 2006). We did use the onset of male colouration as a proxy for maturation time, but we did not measure the intensity of this colouration nor did we assess courtship behaviour in this study. As both of these endpoints are more sensitive than our chosen endpoints, it would be interesting to assess them in future studies, especially since color intensity and courtship behavior are strongly linked to fitness in *N. furzeri* (Reichard et al., 2009).

Using the previously identified NOEC, LOEC and LC_{50}-24 h (Philippe et al., 2018a) of 3,4-DCA in *N. furzeri,* we can calculate the acute-to-chronic ratio of the compound at both temperatures (24 °C and 28 °C) as the quotient of the LC_{50}-24 h and the MATC (Maximum acceptable toxicant concentration, geometric mean of NOEC and LOEC). This results in an ACR of 137.91 at 24 °C and 93.48 at 28 °C. Both of these values are near or higher than 100, which indicates that 3,4-DCA indeed has a high ACR in fish. Regulations should account for this to avoid underestimation of chronic effects of toxicants since safety factors typically only accommodate an ACR of 10, or in exceptional cases, an ACR of 100 (Ristau et al., 2015).

We did not find interactive effects of 3,4-DCA exposure and increased temperature. The chosen endpoints may not be sensitive enough to determine synergistic effects, or these effects might simply not exist at the increase in rearing temperature that was maintained in our study. A previous study on interactive effects between both stressors in tadpoles of bullfrogs did demonstrate effects on gene transcription (Freitas et al., 2016). The only endpoint affected both by temperature and 3,4-DCA exposure (individually, not combined) in our study was CTmax, but the individual stressors caused different effects. Increase in rearing temperature resulted in a higher thermal maximum, which is expected as fish can, to some extent, plastically adapt their thermal maximum to an average temperature rise (Healy and Schulte, 2012). In contrast, exposure to 3,4-DCA reduced thermal tolerance, which is especially relevant under global warming and increasing use of pesticides.

Although it is difficult to compare chronic studies due to the context dependency of sublethal endpoints, we believe that our study is a valuable contribution to the fields of stress ecology and ecotoxicology for several reasons. First of all, we show that even *Nothobranchius* killifish, which are exposed to large daily temperature fluctuations (> 15 °C) in nature, become accustomed to a fixed environmental temperature. Modest deviations from this temperature may entail temperature stress that induces fitness related costs. Second, our results are highly relevant in light of climate change, since we showed that a concentration of a compound that did not affect any of the other endpoints, reduced the thermal maximum of the fish, even in the control temperature treatment, making it more vulnerable to the consequences of climate change. Finally, the fact that we find clear sex-dependent differences in life-history traits and physiology in relation to temperature and toxicant stress implies that it is important to include both sexes in sensitivity ecotoxicological assays.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aquatox.2019.05.009.

References

Aliko, V., Qirjo, M., Sula, E., Morina, V., Faggio, C., 2018. Antioxidant defense system, immune response and erythron profile modulation in gold fish, Carassius auratus, after acute manganese treatment. Fish Shellfish Immunol. 76, 101–109.

Barrett, R.D., Paccard, A., Healy, T.M., Bergek, S., Schulte, P.M., Schluter, D., Rogers, S.M., 2011. Rapid evolution of cold tolerance in stickleback. Proc. R. Soc. B: Biol. Sci. 278, 233–238.

Bartáková, V., Reichard, M., Janko, K., Polačik, M., Blažek, R., Reichwald, K., Cellerino, A., Bryja, J., 2013. Strong population genetic structuring in an annual fish, *Nothobranchius furzeri*, suggests multiple savannah refugia in southern Mozambique. BMC Evol. Biol. 13, 196.

Becker, C.D., Genoway, R.G., 1979. Evaluation of the critical thermal maximum for determining thermal tolerance of freshwater fish. Environ. Biol. Fishes 4, 245–256.

Beitinger, T.L., Bennett, W.A., McCauley, R.W., 2000. Temperature tolerances of North American freshwater fishes exposed to dynamic changes in temperature. Environ. Biol. Fishes 58, 237–275.

Beniston, M., Stephenson, D.B., Christensen, O.B., Ferro, C.A., Frei, C., Goyette, S., Halsnaes, K., Holt, T., Jylhä, K., Koffi, B., 2007. Future extreme events in European climate: an exploration of regional climate model projections. Clim. Change 81, 71–95.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911–917.

Call, D., Poirier, S., Knuth, M., Harting, S., Lindberg, C., 1987. Toxicity of 3, 4-dichloroaniline to fathead minnows, *Pimephales promelas*, in acute and early life-stage exposures. Bull. Environ. Contam. Toxicol. 38, 352–358.

Castro, B.B., Sobral, O., Guilhermino, L., Ribeiro, R., 2004. An in situ bioassay integrating individual and biochemical responses using small fish species. Ecotoxicology 13, 667–681.

Cellerino, A., Valenzano, D.R., Reichard, M., 2016. From the bush to the bench: the annual *Nothobranchius* fishes as a new model system in biology. Biol. Rev. 91, 511–533.

Crossland, N., 1990. A review of the fate and toxicity of 3, 4-dichloroaniline in aquatic environments. Chemosphere 21, 1489–1497.

Crossland, N., Hillaby, J., 1985. Fate and effects of 3, 4-dichloroaniline in the laboratory and in outdoor ponds: II. chronic toxicity to *Daphnia* SPP. and other invertebrates. Environ. Toxicol. Chem. 4, 489–499.

Eijsackers, H., Heimbach, F., Donker, M.H., 1993. Ecotoxicology of Soil Organisms. CRC Press.

Freitas, J.S., Kupsco, A., Diamante, G., Felicio, A.A., Almeida, E.A., Schlenk, D., 2016. Influence of temperature on the thyroidogenic effects of diuron and its metabolite 3, 4-DCA in tadpoles of the american bullfrog (*Lithobates catesbeianus*). Environ. Sci. Technol. 50, 13095–13104.

Gobi, N., Vaseeharan, B., Rekha, R., Vijayakumar, S., Faggio, C., 2018. Bioaccumulation, cytotoxicity and oxidative stress of the acute exposure selenium in Oreochromis mossambicus. Ecotoxicol. Environ. Saf. 162, 147–159.

Grégoir, A., Philippe, C., Pinceel, T., Reniers, J., Thoré, E., Vanschoenwinkel, B., Brendonck, L., 2017. Life stage dependent responses to desiccation risk in the annual killifish Nothobranchius wattersi. J. Fish Biol. 91, 880–895.

Healy, T.M., Schulte, P.M., 2012. Factors affecting plasticity in whole-organism thermal tolerance in common killifish (*Fundulus heteroclitus*). J. Comp. Physiol. B 182, 49–62.

Heugens, E.H., Hendriks, A.J., Dekker, T., Straalen, N.Mv., Admiraal, W., 2001. A review of the effects of multiple stressors on aquatic organisms and analysis of uncertainty factors for use in risk assessment. Crit. Rev. Toxicol. 31, 247–284.

Hooper, M.J., Ankley, G.T., Cristol, D.A., Maryoung, L.A., Noyes, P.D., Pinkerton, K.E., 2013. Interactions between chemical and climate stressors: a role for mechanistic toxicology in assessing climate change risks. Environ. Toxicol. Chem. 32, 32–48.

Hothorn, T., Bretz, F., Westfall, P., 2008. Simultaneous inference in general parametric models. Biom. J. 50, 346–363.

IHCP, 2006. Summary Risk Assessment Report 3,4-Dichloroaniline (3,4-DCA). European Commission.

IPCC, 2014. Climate change 2014: synthesis report. In: Pachauri, R.K., Meyer, L.A. (Eds.), Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change [Core Writing Team.

Kimberly, D.A., Salice, C.J., 2013. Interactive effects of contaminants and climate-related stressors: high temperature increases sensitivity to cadmium. Environ. Toxicol. Chem. 32, 1337–1343.

LeBlanc, S., Höglund, E., Gilmour, K.M., Currie, S., 2012. Hormonal modulation of the heat shock response: insights from fish with divergent cortisol stress responses. Am. J. Physiol. 302, R184–R192.

Monteiro, M., Quintaneiro, C., Pastorinho, M., Pereira, M., Morgado, F., Guilhermino, L., Soares, A., 2006. Acute effects of 3, 4-dichloroaniline on biomarkers and spleen histology of the common goby Pomatoschistus microps. Chemosphere 62, 1333–1339.

Morgan, I.J., McDonald, D.G., Wood, C.M., 2001. The cost of living for freshwater fish in a warmer, more polluted world. Glob. Chang. Biol. 7, 345–355.

Nagel, R., Bresch, H., Caspers, N., Hansen, P., Markert, M., Munk, R., Scholz, N., Ter Höfte, B., 1991. Effect of 3, 4-dichloroaniline on the early life stages of the zebrafish (*Brachydanio retio*): results of a comparative laboratory study. Ecotoxicol. Environ. Saf. 21, 157–164.

Nash, R.D., Valencia, A.H., Geffen, A.J., 2006. The origin of Fulton's condition factor—setting the record straight. Fisheries 31, 236–238.

Noyes, P.D., McElwee, M.K., Miller, H.D., Clark, B.W., Van Tiem, L.A., Walcott, K.C., Erwin, K.N., Levin, E.D., 2009. The toxicology of climate change: environmental contaminants in a warming world. Environ. Int. 35, 971–986.

Op de Beeck, L., Verheyen, J., Olsen, K., Stoks, R., 2017a. Higher pesticide degradation and thermal adaptation counteract the negative effects of pesticides under global warming. J. Appl. Ecol.

Op De Beeck, L., Verheyen, J., Stoks, R., 2017b. Integrating both interaction pathways between warming and pesticide exposure on upper thermal tolerance in high-and low-latitude populations of an aquatic insect. Environ. Pollut. 224, 714–721.

Osterauer, R., Köhler, H.-R., 2008. Temperature-dependent effects of the pesticides thiacloprid and diazinon on the embryonic development of zebrafish (*Danio rerio*). Aquat. Toxicol. 86, 485–494.

Patra, R.W., Chapman, J.C., Lim, R.P., Gehrke, P.C., 2007. The effects of three organic chemicals on the upper thermal tolerances of four freshwater fishes. Environ. Toxicol. Chem. 26, 1454–1459.

Philippe, C., Grégoir, A.F., Janssens, L., Pinceel, T., De Boeck, G., Brendonck, L., 2017. Acute and chronic sensitivity to copper of a promising ecotoxicological model species, the annual killifish Nothobranchius furzeri. Ecotoxicol. Environ. Saf. 144, 26–35.

Philippe, C., Grégoir, A.F., Thoré, E.S.J., Brendonck, L., De Boeck, G., Pinceel, T., 2018a. Acute sensitivity of the killifish *Nothobranchius furzeri* to a combination of temperature and reference toxicants (cadmium, chlorpyrifos and 3,4-dichloroaniline). Environ. Sci. Pollut. Res. - Int.

Philippe, C., Hautekiet, P., Grégoir, A.F., Thoré, E.S., Pinceel, T., Stoks, R., Brendonck, L., De Boeck, G., 2018b. Combined Effects of Cadmium Exposure and Temperature on the Annual Killifish Nothobranchius furzeri. Environmental Toxicology and Chemistry.

Pickle, J., 2008. Measuring Length and Area of Objects in Digital Images Using Analyzing Digital Images Software. Concord Academy.

Polačik, M., Podrabsky, J.E., 2015. Temporary Environments. Pages 217-245 Extremophile Fishes. Springer.

Polačik, M., Donner, M., Reichard, M., 2011. Age structure of annual *Nothobranchius* fishes in Mozambique: is there a hatching synchrony? J. Fish Biol. 78, 796–809.

Polačik, M., Blažek, R., Reichard, M., 2016. Laboratory breeding of the short-lived annual killifish Nothobranchius furzeri. Nat. Protoc. 11, 1396–1413.

Primel, E.G., Zanella, R., Kurz, M.H., Gonçalves, F.F., Martins, M.L., Machado, S.L., Marchesan, E., 2007. Risk assessment of surface water contamination by herbicide residues: monitoring of propanil degradation in irrigated rice field waters using HPLC-UV and confirmation by GC-MS. J. Braz. Chem. Soc. 18, 585–589.

Reichard, M., Polačik, M., 2010. Reproductive isolating barriers between colour--differentiated populations of an African annual killifish, *Nothobranchius korthausae* (Cyprinodontiformes). Biol. J. Linn. Soc. 100, 62–72.

Reichard, M., Polačik, M., Sedláček, O., 2009. Distribution, colour polymorphism and habitat use of the African killifish *Nothobranchius furzeri*, the vertebrate with the shortest life span. J. Fish Biol. 74, 198–212.

Reuter, H., Krug, J., Singer, P., Englert, C., 2019. The African Turquoise Killifish Nothobranchius furzeri As a Model for Aging Research. Drug Discovery Today: Disease Models.

Ristau, K., Akgül, Y., Bartel, A.S., Fremming, J., Müller, M.T., Reiher, L., Stapela, F., Splett, J.P., Spann, N., 2015. Toxicity in relation to mode of action for the nematode *Caenorhabditis elegans*: Acute-to-chronic ratios and quantitative structure–activity relationships. Environ. Toxicol. Chem. 34, 2347–2353.

Schäfers, C., Nagel, R., 1991. Effects of 3, 4-dichloroaniline on fish populations. Comparison between r-and K-strategists: a complete life cycle test with the guppy (*Poecilia reticulata*). Arch. Environ. Contam. Toxicol. 21, 297–302.

Scheil, V., Kienle, C., Osterauer, R., Gerhardt, A., Köhler, H.-R., 2009. Effects of 3, 4-dichloroaniline and diazinon on different biological organisation levels of zebrafish (*Danio rerio*) embryos and larvae. Ecotoxicology 18, 355–363.

Sniegula, S., Janssens, L., Stoks, R., 2017. Integrating multiple stressors across life stages and latitudes: Combined and delayed effects of an egg heat wave and larval pesticide exposure in a damselfly. Aquat. Toxicol. 186, 113–122..

Sokolova, I.M., Lannig, G., 2008. Interactive effects of metal pollution and temperature on metabolism in aquatic ectotherms: implications of global climate change. Clim. Res. 37, 181–201.

Stoks, R., Block, M.D., McPeek, M.A., 2006. Physiological costs of compensatory growth in a damselfly. Ecology 87, 1566–1574.

TOXNET, 2014. 3,4-DICHLOROANILINE. https://toxnet.nlm.nih.gov/cgi-bin/sis/search/a?dbs+hsdb:@term+@DOCNO+1319.

Valdesalici, S., Cellerino, A., 2003. Extremely Short Lifespan in the Annual Fish *Nothobranchius furzeri*. The Royal Society, pp. S189–S191.

Vrtílek, M., Reichard, M., 2015. Highly plastic resource allocation to growth and reproduction in females of an African annual fish. Ecol. Freshw. Fish 24, 616–628.

Ware, G.W., 1983. Pesticides. Theory and Application. WH Freeman & Co.

Wiseman, S., Thomas, J.K., McPhee, L., Hursky, O., Raine, J.C., Pietrock, M., Giesy, J.P., Hecker, M., Janz, D.M., 2011. Attenuation of the cortisol response to stress in female rainbow trout chronically exposed to dietary selenomethionine. Aquat. Toxicol. 105, 643–651.

Zebral, Y.D., Lansini, L.R., Costa, P.G., Roza, M., Bianchini, A., Robaldo, R.B., 2018. A glyphosate-based herbicide reduces fertility, embryonic upper thermal tolerance and alters embryonic diapause of the threatened annual fish Austrolebias nigrofasciatus. Chemosphere 196, 260–269.