



Combined effects of global warming and chlorpyrifos exposure on the annual fish *Nothobranchius furzeri*

Charlotte Philippe^{a,*}, Eli S.J. Thoré^a, Sebastiaan Verbesselt^b, Arnout F. Grégoir^a,
Luc Brendonck^{a,c}, Tom Pinceel^{a,d,e}

^a Animal Ecology, Global Change and Sustainable Development, University of Leuven, Ch. Deberiotstraat 32, B-3000 Leuven, Belgium

^b Flanders Research Institute for Agriculture, Fisheries and Food, Burgemeester Van Gansberghelaan 92 box 1 9820 Merelbeke, Belgium

^c Water Research Group, Unit for Environmental Sciences and Management, North-West University, Potchefstroom, South Africa

^d Centre for Environmental Management, University of the Free State, P.O. Box 339, Bloemfontein 9300, South Africa

^e PMC Coasts Rivers and Cities, Witteveen + Bos, Posthoflei 5, B-2600 Berchem, Belgium

ARTICLE INFO

Edited by Dr. Hyo-Bang Moon

Keywords:

Freshwater

Pesticide

Ecotoxicology

Killifish

Multiple stressors

CTmax

ABSTRACT

Global warming and environmental pollution threaten aquatic ecosystems. While interactive effects between both stressors can have more than additive consequences, these remain poorly studied for most taxa. Especially chronic exposure trials with vertebrates are scarce due to the high time- and monetary costs of such studies. We use the recently-established fish model *Nothobranchius furzeri* to assess the separate and combined effects of exposure to the pesticide chlorpyrifos (at 2 µg/L and 4 µg/L) and a 2 °C temperature increase. We performed a full life-cycle assessment to evaluate fitness-related endpoints including survival, total body length, maturation time, fecundity, critical thermal maximum (CTmax) and locomotor activity. Exposure to 4 µg/L chlorpyrifos slowed down male maturation, reduced fecundity and impaired growth of the fish. While the temperature increase did not affect any of the measured endpoints on its own, the combination of exposure to 2 µg/L CPF with an increase of 2 °C reduced growth and severely reduced fecundity, with almost no offspring production. Together, these findings suggest that climate change may exacerbate the impact of environmental pollution, and that interactive effects of chronic exposure to multiple stressors should be considered to predict how populations will be affected by ongoing global change.

1. Introduction

Global warming and environmental pollution are both inherently linked to anthropogenic development and are affecting ecosystems across the globe. Interactive effects between chemicals and increased temperature on biota have been reported by several studies (Moe et al., 2013; Noyes and Lema, 2015; Marchand and Haddad, 2017). A well-studied section of environmental pollutants consists of pesticides. These compounds can enter aquatic ecosystems through spray drift, run-off or seepage; contaminating non-target land and surface waters. Aside from extensive use in agriculture, exposure of non-target organisms can also result from non-regulated household use. The use of pesticides is predicted to increase over the following decades as a result of climate change, due to an anticipated increase in pest species and faster pesticide-degradation rates at higher temperatures (Choudhury and Saha, 2020). A number of studies have predicted shifts in food web

structure, depletion of ecosystem services and biodiversity loss (IPCC, 2014), due to increased pesticide use in combination with increased temperature.

Chlorpyrifos (CPF) is an organophosphorus insecticide, commonly used to fight ticks and mites (Bhatnagar et al., 2016). Although the United States Environmental Protection Agency as well as the European Union recently announced they will no longer allow the use of CPF, it was until very recently one of the most frequently used pesticides worldwide (Eaton et al., 2008) and is well studied as a model compound for organophosphorus insecticides (Op De Beeck et al., 2017b; Philippe et al., 2018a). Chlorpyrifos can enter aquatic ecosystems (Patra et al., 2015) when contaminated water runs off from agricultural areas. Wood and Stark (2002) reported chlorpyrifos concentrations of up to 3.7 µg/L in drainage water (Wood and Stark, 2002), but in the vicinity of crop-land, concentrations higher than 100 mg/L have been measured (Moore et al., 2002; Op De Beeck et al., 2017b). The effectiveness of

* Corresponding author.

E-mail address: charlotte.philippe@kuleuven.be (C. Philippe).

<https://doi.org/10.1016/j.ecoenv.2022.114290>

Received 18 July 2022; Received in revised form 10 November 2022; Accepted 10 November 2022

Available online 17 November 2022

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chlorpyrifos as a pesticide against various organisms results from the inhibition of the enzyme acetylcholinesterase, involved in the transmission of nerve impulses at neural junctions (Humphrey and Klumpp, 2003). This leads to accumulation of acetylcholine in the synaptic cleft and induces neurotoxic changes including inhibition of the energy metabolism and overstimulation of the cholinergic reception (Bonansea et al., 2016; Topal et al., 2016). Consequently, nervous cells are damaged which results in various deleterious effects ranging from behavioural changes such as reduced swimming and feeding (Bonansea et al., 2016), to impaired growth, lower reproductive success and mortality (Philippe et al., 2018a).

Short-term exposure experiments combining pesticides and warming on different fish species have shown an increased sensitivity to pesticides at temperatures above the species' acclimation temperature (Dietrich et al., 2014; Laetz et al., 2014; Patra et al., 2015; Philippe et al., 2018a). While long-term effects of global warming on the toxicity of pesticides have been studied in several taxa, including endotherm vertebrates like mice and rats, such effect on fish have not been studied comprehensively (Rohr and Palmer, 2013). The thermal tolerance of ectothermic organisms such as fish is mainly determined by oxygen limitation (Noyes et al., 2009). In ectotherms, environmental warming directly increases the metabolism and feeding activity, which implies that, when oxygen levels are limited, trade-offs between oxygen-dependent traits may result in a lower energy fraction available for other traits such as detoxification (Ferreira et al., 2015), growth and reproduction (Pörtner and Farrell, 2008). Furthermore, while an increased temperature can stimulate juvenile growth as the metabolic rate increases, growth can also decline when cardiac function and respiration cannot be maintained (Neuheimer et al., 2011).

In this study, we exposed turquoise killifish (*Nothobranchius furzeri*) to chlorpyrifos in weekly pulses and to a continuous temperature increase (+2 °C), to each stressor individually and to a combination of both stressors, over a period of four months. *Nothobranchius furzeri* has recently been introduced as a model to study the long-term effects of toxicants (Philippe et al., 2019; Thoré et al., 2020, 2021d). Since *N. furzeri* has an extremely fast life cycle, with a typical lifespan of around 6 months and a maturation time of just 18 days, full life-cycle studies are possible within < 4 months (Philippe et al., 2019; Thoré et al., 2021c). This is a major advantage of working with this species compared to traditional fish models such as zebrafish or medaka, which only mature three and four months after hatching, respectively (Roper and Tanguay, 2018; Sun et al., 2020). We hypothesise that exposure to chlorpyrifos will impair growth and reduce fecundity, as previous studies on *N. furzeri* have shown that these endpoints are sensitive to pollutants (Philippe et al., 2018b). Furthermore, we expect to find interactive effects of both stressors for several reasons. First, warming will likely increase the uptake of chlorpyrifos by increasing respiratory rates and feeding activity (Noyes et al., 2009; Kimberly and Salice, 2013). Second, elimination and detoxification rates of pesticides are expected to increase with higher temperatures, resulting in higher concentrations of toxic metabolites, such as chlorpyrifos-oxon, diethylphosphate and 3, 5, 6-trichloro-2-pyridinol (Noyes et al., 2009; Kimberly and Salice, 2013; Kharabsheh et al., 2017). Besides temperature-induced toxicant sensitivity, also toxicant-induced temperature sensitivity can drive the synergetic effects between temperature and chlorpyrifos exposure on fish (Patra et al., 2007; Moe et al., 2013). Since chlorpyrifos toxicity may increase with temperature, and sensitivity to thermal stress may increase after chlorpyrifos exposure, we expect these stressors to interact synergistically.

2. Material and methods

2.1. Experimental setup and fish maintenance

In this experiment, fish from the homozygous GRZ (Gona-Rhe-Zhou) laboratory strain were used. All fish came from our culture facility at the

university of Leuven, Belgium (KU Leuven), where parental fish are reared at a standard temperature of 28 °C. As the strain has been reared for > 10 generations at 28 °C, we opted to impose temperature stress by increasing the water temperature to 30 °C (i.e. +2 °C, after RCP 4.5 projections (Representative Concentration Pathway; (Flato et al., 2014)).

In a previous study, the acute sensitivity of larval (2–7 dph) *N. furzeri* to chlorpyrifos was calculated to be 14.2 (± 1.05) µg/L (measured as 96 h-LC₅₀ at 28 °C) (Philippe et al., 2018a). In the current study, we aimed at stressing the fish sub-lethally. Therefore, the experiment entailed a full-factorial crossing of two temperature treatments (28 °C and 30 °C) and three sublethal chlorpyrifos concentrations (C0 = 0, C1 = 2 and C2 = 4 µg/L), to a total of 6 experimental conditions. 100% Ethanol was used as solvent for the preparation of the chlorpyrifos stock solution (1 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA). The control therefore contained 0.0004% ethanol to match the ethanol concentration in the highest exposure treatment. Fish were exposed from two days post hatching (2 dph) until 106 dph, as this enabled us to measure fecundity across ± 10 weeks (Philippe et al., 2019). Due to the nature of the compound, a degradable organophosphate, we aimed for a design in which the complete medium was renewed each week, resulting in a weekly pulse of chlorpyrifos. Mixed water samples (4 jars) of each treatment were taken to measure the actual concentrations in the medium and to calculate the degradation of the compound after 48 h. 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). The actual start concentrations for C1 (2 µg/L) were 2.34 µg/L at 28 °C and 2.32 µg/L at 30 °C. For C2 (4 µg/L), start concentrations were 4.63 µg/L at 28 °C and 5.00 µg/L at 30 °C. Based on the measured concentrations after 48 h, degradation rates after 48 h were 80–85% in the C1 treatment and 91–93% in the C2 treatment. The time-weighted mean according to OECD test guideline No. 211 (OECD, 2012) over the 7 day period was 0.33 (at 28 °C) and 0.30 µg/L (at 30 °C) in the C1 treatment and 0.50 µg/L at both 28 °C and 30 °C in the C2 treatment. This exposure setup aimed at mimicking an exposure to weekly pulses. This is a realistic exposure pattern, as chlorpyrifos also rapidly degrades in natural ecosystems and farmers apply pesticides at regular intervals, resulting in peaks in pesticide concentrations in nearby freshwater ecosystems (Op de Beeck et al., 2017a).

Experimental fish were reared in individual transparent glass jars (0.5 L until 14 dph, then 2 L) which were kept in temperature-controlled, heated water baths throughout the experiment. The jars were continuously aerated and subjected to a 14:10-h light:dark cycle. Jar positions in the water baths were randomised twice a week throughout the experiment.

The experiment started with 96 fish that were divided over the six treatments, resulting in 16 replicates in all treatments (Fig. 1). The protocol for chronic exposure of *N. furzeri* of Philippe and co-workers (2018a) was adopted.

The feeding scheme during the first three weeks post hatching (i.e. 0–21 dph, juvenile stage) consisted of *Artemia franciscana* nauplii (Ocean Nutrition) until satiation twice a day. Afterwards, nearly-adult fish were fed a mixture of *Artemia* and *Chironomus* larvae (Ocean Nutrition) until 35 dph, and from then on were fed entirely on frozen *Chironomus* larvae. Fish were always fed until satiation and leftover food was removed from the jars by pipetting.

Average temperature and conductivity values during the whole experiment are given in Table S2. pH varied between 7.2 and 7.4 and dissolved oxygen levels always exceeded 80%. Both were measured with a multimeter (Hanna Instruments, Temse, Belgium). Glass jars were cleaned weekly after use in a dishwasher at 60 °C without soap.

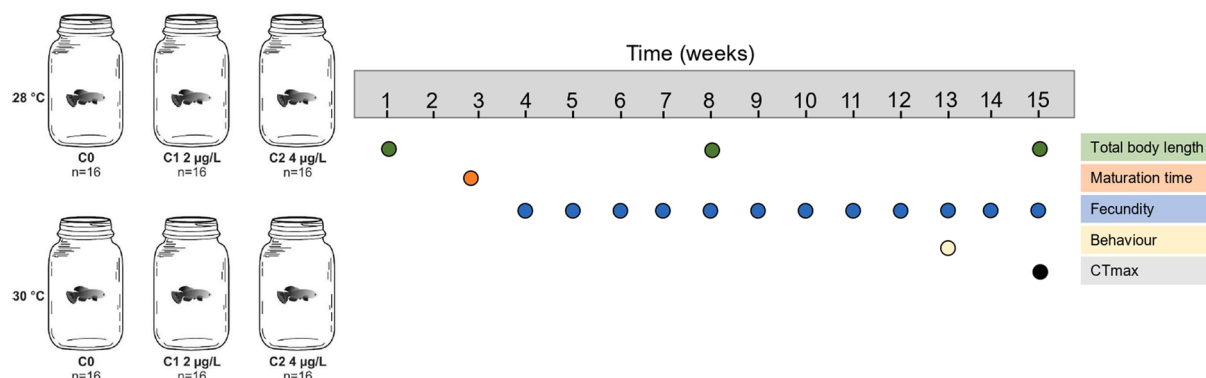


Fig. 1. Schematic representation of the experimental setup, the numbers of replicates (n) and when the endpoints were scored. Nominal concentrations are shown. When setting up the experiment, sex of the fish was not yet known.

2.2. Endpoints

2.2.1. Life history and morphological traits

Male maturation time was assessed as the day the first signs of nuptial coloration appeared. From 40 dph, fish were randomly paired (one female and one male) once per week and allowed to spawn for 120 min in a spawning tank. After spawning, fish were returned to their individual jars and eggs were counted manually as a measure of fish fecundity (i.e. productivity per female per week). For details on the spawning protocol, see Philippe et al. (2018a). Female maturation time can be approximated as the age at which a fish produces the first eggs. Unfortunately, and by coincidence, the sex ratio was biased towards males, leaving too few females to reliably assess female maturation time.

Total body length of fish was measured three times during the experiment (day 5, day 47, day 102, Fig. 1) by photographing fish in a petri dish containing 1 cm of medium over millimetre paper and analysing the pictures digitally using the programme “Analysing Digital Images” (Philippe et al., 2018b) (accuracy up to 1 mm).

2.2.2. Behavioural endpoints

At 92 dph, fish were subjected to a diving test (Fig. 1) to assess 1) spontaneous activity level and 2) anxiety-related behaviour. Prior to the test, fish were abstained from food for 6 h to motivate fish to explore the test arena and to prevent disinterest in food (Thoré et al., 2018, 2019). At the onset of the test, fish were individually transferred to a 2 L glass test arena (d: 12 cm, h: 25 cm) and allowed to acclimate for 5 min, after which fish movements were manually tracked (one observer, treatment-blind) during a period of 10 min. Opaque screens around the test arena prevented disturbance and confounding social interaction among individuals (Thoré et al., 2021a). Spontaneous activity level was assessed after the method of Thoré et al. (2019). Briefly, individual fish were scanned every 15 s for a total of 40 times during the 10-min observation period, each time scoring fish as either active (score = 1, when swimming) or passive (score = 0, when fish remained motionless during the first two seconds of observation). Afterwards, a total activity score was calculated for each fish by summing the collected scores (max. score = 40, min. score = 0) as a proxy for spontaneous activity level. In addition, we assessed the mean depth in the water column, since the natural tendency to dive to the bottom of the water column can be used as a measure for anxiety-related or risk-averse behaviour (Parker, 2016), i.e. benthic behaviour is considered to be more risk-averse than pelagic behaviour (Thoré et al., 2021b). For this, we virtually divided the water column in 3 horizontal layers of equal size (height of 8 cm each), and the virtual position (lower, middle, upper) of each fish was scored alongside its activity score (i.e. every 15 s for a total of 40 times in the 10-min observation period). After Thoré et al. (2021b), a value was assigned to each position (lower = 0, middle = 1, upper = 2), and a mean depth score was calculated for each fish as the sum of all

values during the observation period (max. score = 80, min. score = 0). Therefore, a higher score indicates that the individual on average resides closer to the surface (i.e. risk-prone behaviour), and vice versa.

After the 10-min observation period, fish were fed 5 frozen *Chironomus* larvae and given 10 min to feed. After this, leftover feed was removed from the test arena by gentle pipetting, and fish were allowed 5 more minutes to acclimate. Subsequently, above behavioural observations were repeated for another period of 10 min in order to account for potential context-dependency of behaviour (Martin et al., 2019). After the test, fish were transferred back to their respective housing jars.

2.2.3. Critical thermal maximum

Critical thermal maximum experiments were performed at 102 dph by gradually heating the aquarium (+ 0.33 °C / min) by means of a water bath (detailed protocol in (Philippe et al., 2019) and starting from the fish's respective rearing temperature (28 °C or 30 °C). 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 housing jars for recovery.

At the end of the experiment (106 dph), fish were sedated in ice water and euthanized using liquid nitrogen at − 196 °C to ensure immediate death and to avoid interference of anaesthetic agents with potential postmortem physiological analysis for subsequent research.

2.3. Animal welfare note

All experimental procedures and methods were conform the legal requirements for animal research in Belgium and were approved by the ethical committee of KU Leuven (file number: P173/2016). C. Philippe and S. Verbesselt monitored the mortality, stress and condition of each fish daily. Optimal water conditions were maintained (7.2–7.4 pH, ammonium < 0.2 mg/L, nitrate < 25 mg/L, nitrite < 0.2 mg/L, dissolved oxygen > 80%). Fish were neither disturbed nor handled unnecessarily.

2.4. Data analysis

Statistical analyses were performed in R v3.2.3 (R-Core-Team). We used the packages *survival* (differences between survival curves), *lme4* (linear mixed-effect modelling), *multcomp* and *lsmeans* (post-hoc tests), *car* (Anova), *stats* (generalised linear models) and *mass* (StepAIC).

To assess differences in survival of fish between chlorpyrifos and temperature treatments, we performed a full-factorial survival analysis using the *survreg* function (survival package). Unreliable datapoints (e. g., fish that escaped the jars) were excluded from the final dataset. Male maturation time was analysed using a generalised linear model with a Gamma distribution and concentration, temperature and their interaction as fixed factors. Total body length was analysed at the start of the

experiment (day 5), at maturation (day 47) and at the end of the experiment (day 103). To analyse total body length at day 5 and 103, we used full-factorial generalised linear models with Gamma distribution and concentration, temperature and sex (at day 103) as fixed factors. At day 47, we used a full-factorial linear model with Gaussian error distribution and concentration, temperature and sex as fixed factors.

Fecundity (total number of eggs per week) was analysed by using a generalised linear mixed-effects model with a Poisson distribution and concentration, temperature and their interaction as fixed factors, and time (in weeks) as well as fish identity as crossed random effects. Mean vertical position in the water column was analysed using a generalised linear mixed-effects model with a Quasipoisson distribution (to accommodate overdispersion) and concentration, temperature and feeding (before feeding, after feeding) as fixed factors, and fish identity as random effect. Activity was analysed using a generalised linear mixed-effects model with a Quasipoisson distribution and concentration, temperature and feeding status as fixed factors, and fish identity as random effect. CTmax was analysed as a full-factorial general linear mixed-effects model with concentration, temperature and sex as fixed factors, and series as a random effect. All non-significant interaction terms were left out in the final models. Post-hoc differences were assessed by means of Tukey-corrected pairwise comparisons (lsmeans package, Lenth and Lenth, 2018). Model assumptions were verified for every model.

3. Results

3.1. Survival

There was no effect of exposure to chlorpyrifos ($\chi^2_{2,89} = 0.775$, $P = 0.679$) or warming on survival ($\chi^2_{1,89} = 0.967$, $P = 0.325$) (Fig. S1). Mean survival at the end of the experiment was 46%, which is as expected for a short-living species such as *N. furzeri* (Thoré et al., 2021c).

3.2. Total body length

Total body length of the fish at an age of one week was affected by exposure to chlorpyrifos ($\chi^2_{2,72} = 15.72$, $P < 0.001$), with fish exposed to the 4 µg/L CPF being smaller than fish exposed to 2 µg/L ($P = 0.002$) and control fish ($P = 0.001$) (Fig. 2A). Total body length in the first week was not affected by rearing temperature ($\chi^2_{1,72} = 0.020$, $P = 0.886$).

At day 47, chlorpyrifos exposure affected body length differently for males compared to females ($F_{2,45} = 3.44$, $P = 0.041$). Males that were exposed to 4 µg/L (C2) chlorpyrifos were 7% smaller compared to those in the control condition ($P = 0.006$) and 8% smaller than males that were exposed to 2 µg/L (C1) ($P < 0.001$). In contrast, body length of females was unaffected. There also was a main effect of exposure to chlorpyrifos ($F_{2,45} = 18.00$, $P < 0.001$) (Fig. 2B). Finally, temperature significantly affected body length at day 47 ($F_{1,45} = 4.50$, $P < 0.039$), with fish reared at 30 °C being 0.4 cm smaller.

At day 103, the difference in total body length between males and

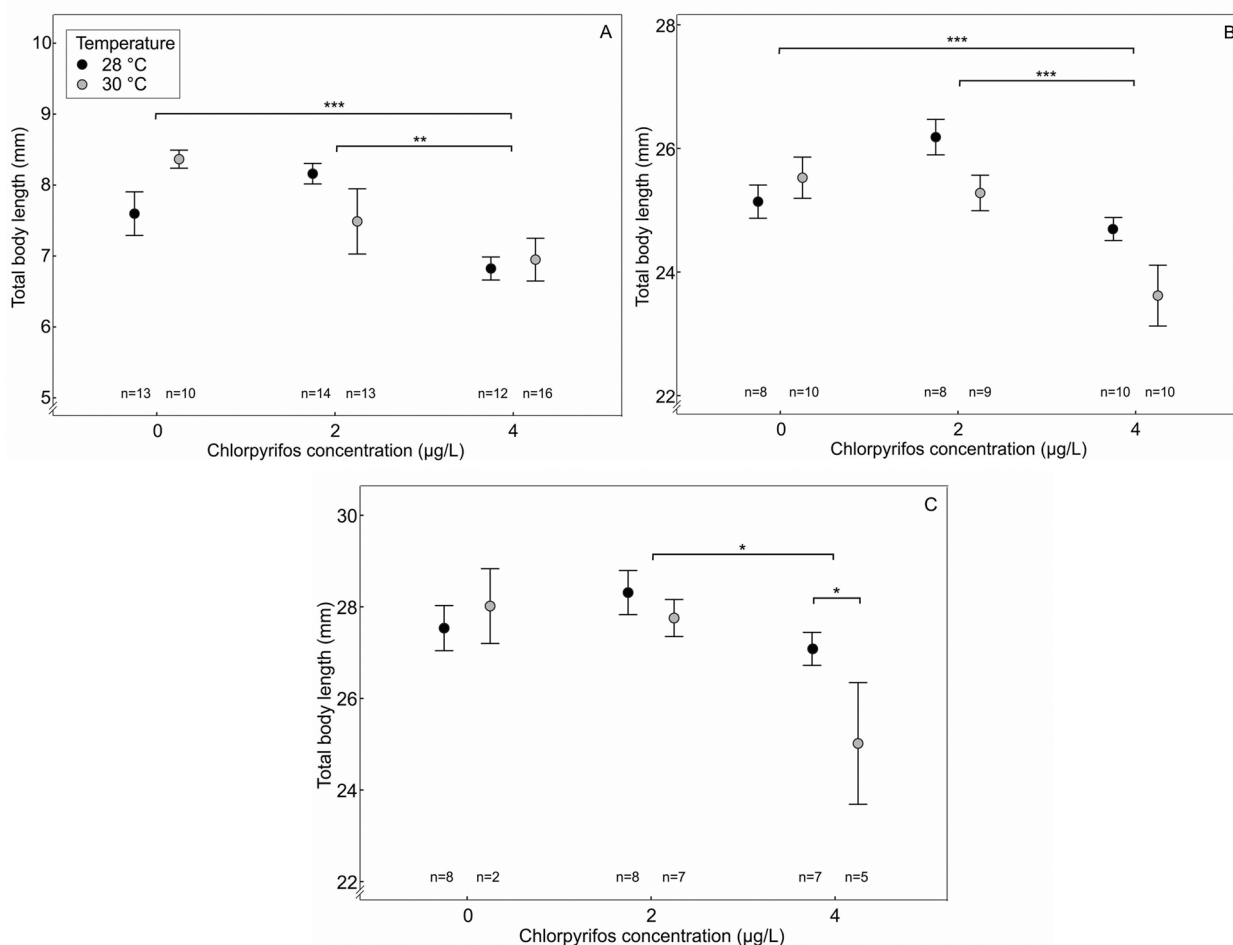


Fig. 2. Total body length (in mm) of *N. furzeri* exposed to different concentrations of chlorpyrifos and two temperatures at A) day 5, B) day 47 and C) day 103. Nominal concentrations are shown. Values are presented as mean $\pm$ SE. Asterisks indicate statistical differences between and within chlorpyrifos groups (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$).

females was no longer significant ($\chi^2_{1,29} = 3.06$, $P = 0.080$). Body length was still affected by exposure to chlorpyrifos ($\chi^2_{2,29} = 7.43$, $P = 0.024$). A posthoc comparison between fish from different chlorpyrifos treatments revealed that fish exposed to 4 $\mu\text{g/L}$ were statistically smaller compared to fish exposed to 2 $\mu\text{g/L}$ ($P = 0.032$) (Fig. 2C). We did not find an effect of rearing temperature on body length at day 103 ($\chi^2_{1,29} = 2.122$, $P = 0.145$).

3.3. Age at maturation

Male maturation time was affected by exposure to chlorpyrifos ($\chi^2_{2,41} = 11.79$, $P = 0.003$), with males exposed to 4 $\mu\text{g/L}$ CPF (C2) taking 18% longer to mature compared to control males (Fig. 3). There was, however, no effect of warming on male maturation time ($\chi^2_{1,41} = 0.707$, $P = 0.401$).

3.4. Fecundity

We found a significant interaction between increased temperature and chlorpyrifos exposure on fecundity ($\chi^2 = 25.5$, $P < 0.001$). At 28 °C, fish exposed to 4 $\mu\text{g/L}$ produced less eggs compared to fish exposed to 2 $\mu\text{g/L}$ and control fish (posthoc Tukey within 28 °C: both $P < 0.001$) (Fig. 4). At 30 °C, control fish produced more eggs compared to all chlorpyrifos exposed fish (posthoc Tukey within 30 °C both $P < 0.008$). Both the main effect of exposure to chlorpyrifos ($\chi^2 = 60.1$, $P < 0.001$) and the main effect of temperature ($\chi^2 = 6.87$, $P = 0.008$) significantly reduced fecundity.

3.5. CTmax

There was no effect of rearing temperature ($\chi^2_{1,23} = 0.632$, $P = 0.427$), exposure to chlorpyrifos ($\chi^2_{2,23} = 2.48$, $P = 0.290$) or sex ($\chi^2_{1,23} = 1.104$, $P = 0.294$) on CTmax (Fig. S2).

3.6. Spontaneous locomotor activity and anxiety-related behaviour

There was no effect of exposure to chlorpyrifos ($\chi^2_{2,75} = 3.6$, $P = 0.165$) or warming ($\chi^2_{1,75} = 0.204$, $P = 0.652$) on the vertical position of the fish in the aquarium. However, fish generally maintained a higher position in the water column before feeding ($\chi^2_{1,75} = 12.5$, $P < 0.001$) (Fig. S3A). The activity level of the fish was not affected by exposure to chlorpyrifos ($\chi^2_{2,75} = 0.866$, $P = 0.649$), temperature ($\chi^2_{1,75} = 2.81$, $P =$

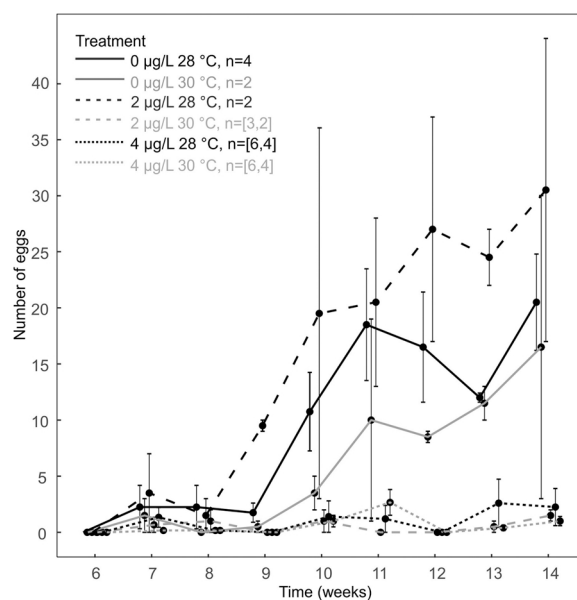


Fig. 4. Fecundity through time, measured as number of deposited eggs per week for each treatment. To improve the readability and interpretability of the figure, error bars are not shown on the graphs. Values represent the mean. The number of females (n) in each treatment at the beginning and end of the egg laying period is indicated.

0.093) or feeding ($\chi^2_{1,75} = 3.21$, $P = 0.073$) (Fig. S3B).

4. Discussion

In this study, we used the annual killifish *N. furzeri* to assess the impact of chronic exposure to chlorpyrifos and increased temperature. We found negative effects of pulsed chlorpyrifos exposure on male maturation time and total body length of both males and females. Furthermore, we found interactive effects of exposure to chlorpyrifos and warming on fecundity, with severely reduced fecundity in fish exposed to both stressors combined compared to fish exposed to only one stressor. Our results are suggestive of exacerbated, synergistic impacts of multi- compared to single-stressor exposure. Overall, our study demonstrates the necessity for chronic testing in a multi-stressor environment since effects were both life-stage specific and interactive effects between pesticide exposure and warming occurred.

Consistent with our expectations, male maturation was delayed when fish were exposed to chlorpyrifos. A number of non-mutually exclusive explanations can be given for this effect. Firstly, the exposure could trigger detoxification mechanisms, which require energy. This may limit energy availability for other processes (Calow and Sibly, 1990). Secondly, delayed maturation may at least partly have been caused by endocrine disruption. In male *Tilapia*, exposure to 5–15 $\mu\text{g/L}$ CPF decreased testosterone levels (Oruç, 2010). Also, in zebrafish, exposure to 200 $\mu\text{g/L}$ CPF for a period of 96 h resulted in reduced 11-ketotestosterone levels, an essential sex-hormone in males (Manjunatha and Philip, 2016). Both of these hormones are involved in the maturation of the testes and the expression of secondary sex characteristics in male fish such as nuptial colouration (Schade and Stallsmith, 2012). The chlorpyrifos concentrations applied in our study (2 and 4 $\mu\text{g/L}$) were much lower than those in the previously-performed studies on *Tilapia* and zebrafish. This is in line with earlier studies showing that *N. furzeri* is a highly sensitive model organism (Philippe et al., 2018a; Thoré et al., 2021c).

Total body length was affected by exposure to 4 $\mu\text{g/L}$ chlorpyrifos at all analysed time points. Although the reduction in body length could

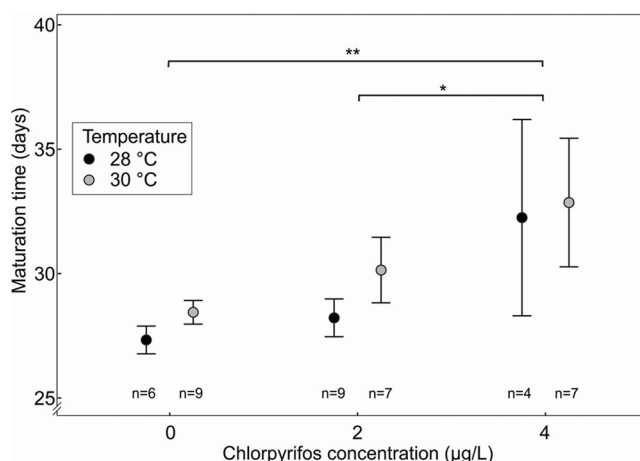


Fig. 3. Mean maturation time of male *N. furzeri* exposed to different chlorpyrifos concentrations and two temperatures. Nominal concentrations are shown. Values are presented as mean $\pm$ SE. Asterisks indicate statistical differences between chlorpyrifos groups (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$).

have been a consequence of hypo-activity and reduced feeding due to the inhibition of acetylcholine (Levin et al., 2003; Bonansea et al., 2016), this is unlikely since we did not find effects on any of the scored activity endpoints. While appetite-suppressing effects could also appear without a loss of activity, the effect is more likely part of an energy allocation trade-off between growth rate and pesticide tolerance (Sibly and Calow, 1989). The amount of energy that can be spent is limited and when an organism needs to allocate energy to defence mechanisms, trade-offs with other energy demanding processes such as growth and fecundity may emerge (Congdon et al., 2001).

Finally, our results indicate that fecundity was reduced by both chlorpyrifos and increased temperature. However, these results should be interpreted with care as the sample size of fecund females was low. Chlorpyrifos exposure may have reduced fecundity due to its impact on endocrine steroid hormones such as testosterone and oestrogen in fish. Such impact is known to impair oocyte development (Oruç, 2010; Schade and Stallsmith, 2012). In zebrafish, for instance, chlorpyrifos exposure damages gonads by vacuolisation, formation of atretic follicles, degeneration of oocytes and elongation of seminiferous tubules (Manjunatha and Philip, 2016). Chlorpyrifos may also have interfered with the expression of mating behaviour (e.g. courtship) by disturbing neuroendocrine pathways. This was previously shown for guppies (De Silva and Samayawardhena, 2005). Likewise, exposure to neuroactive chemicals was already shown to affect fecundity through changes in mating frequency in *N. furzeri* (Thoré et al., 2020). Warming may have caused a temperature-dependent delay in oocyte and sperm development, and may have reduced sperm motility (Bombardelli et al., 2013). At 28 °C, fecundity of fish that were exposed to 2 µg/L CPF was higher compared to that of control fish. This possibly represents a hormetic effect of 2 µg/L CPF on fecundity. The hormetic effect of pesticides on fecundity was already shown in a variety of insects (beetles, mosquitos, cicadas and butterflies) (Cohen, 2006; Cutler, 2013; Guedes and Cutler, 2014; Ayyanath et al., 2015). Hormesis in fecundity due to neuroendocrine stress was also described as a general phenomenon in fish (Schreck, 2010).

Interestingly, chlorpyrifos exposure combined with a temperature increase of 2 °C almost entirely obstructed offspring production. This synergistic effect of exposure to a combination of both stressors potentially originates from an increased chlorpyrifos uptake at higher rearing temperatures and from an upregulated metabolic transformation to more toxic metabolites (Hooper et al., 2013). This sudden drop in fecundity could be seen as a threshold or tipping point, described by Noyes et al. (2009), at which major synergistic effects in species, populations, and communities are triggered. Regardless of the underlying mechanisms, our data suggest detrimental effects of combined exposure to increased temperatures and chemicals. This is especially relevant in a context of global climate change since both pesticide use and temperatures are forecasted to increase (Noyes and Lema, 2015).

Combined, our results show an effect of chronic chlorpyrifos exposure in *N. furzeri* at 4 µg/L, while no effects were found when exposed to 2 µg/L at 28 °C. However, due to the interactive effect with temperature increase, we did find effects after exposure to 2 µg/L at 30 °C. Using this information and the LC₅₀-24 h reported in Philippe et al. (2018a), the calculated acute-to-chronic ratio (ARC) of *N. furzeri* for chlorpyrifos is 4.6 at 28 °C. As current regulations use a factor 10 to predict the ACR (Ockleford, 2013), chronic effects of chlorpyrifos appear to not be underestimated in predictions. However, ACR's are highly temperature dependent, especially in the case of toxicant effects associated with tipping point concentrations. The acute sensitivity to chlorpyrifos should be tested at 30 °C, to calculate this temperature-specific ACR, and to check if it accounts for the strong synergistic effect of both stressors on fecundity.

Overall, our results illustrate the need for multi-stressor studies to obtain an ecologically relevant risk-assessment of exposure to chemicals. Furthermore, this study serves as a proof of principle of the usefulness of the killifish *N. furzeri* as a model for time-efficient chronic vertebrate

exposure tests.

CRediT authorship contribution statement

Charlotte Philippe: Conceptualization, Methodology, Data analysis, Visualization, Writing. **Eli Thoré:** Writing and conceptual reviewing and editing. **Sebastiaan Verbesselt:** Methodology. **Arnout Grégoir:** Conceptualization, Methodology. **Luc Brendonck:** Conceptualization, Methodology, Supervision. **Tom Pinceel:** Conceptualization, Methodology, Writing and conceptual reviewing and editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

We are grateful to the Department of crop protection of the UGent for analysis of water samples. Support during this project was provided by the Excellence Centre 'Eco and socio-evolutionary dynamics (PF/10/007) of the KU Leuven Research Fund. AFG (11Q0516N) and EST (SB151323) were funded as doctoral and TP (12F0716N) as post-doctoral fellow by FWO Flanders (Fonds Wetenschappelijk Onderzoek).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2022.114290.

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