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THE IMPORTANCE OF EXPERIMENTAL TIME WHEN ASSESSING THE EFFECT OF TEMPERATURE ON TOXICITY IN POIKILOTHERMS

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Running title: Temperature effects of toxicity

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Abstract: Temperature is an important factor affecting toxicity, determining chemical toxicokinetics and toxicodynamics in poikilothermic organisms. As also metabolic rates are affected by temperature, interactions between the emergence of toxic effects and time are very likely. The aim of this study was to investigate how temperature affected the toxicity of copper towards the nematode *Caenorhabditis elegans* when measured during short fixed timeframes compared to during full life-cycles. Copper toxicity was tested in two experiments at four or six temperatures in the range of 11 to 24˚C, with copper concentrations spanning from one to 40 mg Cu/L agar, respectively. Reproduction and mortality were determined across the entire life-cycle, and the time to production of first egg and the population growth rate were calculated. The results showed that the 50% effect concentrations (EC50) of copper increased 1.5 to 2.5-fold with increasing temperature within the tested range, depending on endpoint. When calculating EC50 on reproduction after 24 or 96 hours, as is the typical setup for temperature/chemical interaction studies, results ranged from no temperature effect to effects much larger than for the full life cycle. Studies of temperature effects on toxicity must therefore be carefully designed in relation to the research question investigated.

Keywords: temperature, copper, acute toxicity, chronic toxicity, *Caenorhabditis elegans*

INTRODUCTION

Temperature is an important factor affecting toxicity, as it determines fate processes such as microbial degradation rates of organic compounds and processes related to biological effects, such as those influencing chemical toxicokinetics and toxicodynamics in poikilothermic organisms. As a result of these dependent effects, the toxicity of chemicals can change depending on the growth temperature [1–4].

Heugens *et al.* [5] and Cairns *et al.* [6] have reviewed the effect of changing temperature on the toxicity of a number of compounds towards aquatic organisms and found that the toxicity of metals and pesticides generally increase with temperature; particularly when the temperature approaches the upper tolerance limit of an organism. Previous studies on the effects of temperature on toxicity have often been based on fixed time point observations in acute toxicity experiments, e.g. the 48h *Daphnia* immobilization test [5,7]. This means that toxicity is actually assessed after different metabolic and physiological times for each of the different temperatures, as the rate of metabolic processes decrease with decreasing temperatures in poikilothermic organisms [3,8,9]. This difference in the physiological time experienced in exposures at different temperatures have the potential to lead to the erroneous conclusion that a chemical is more toxic at higher temperatures simply because toxicity is measured at a time where more metabolism has taken place, and toxic symptoms therefore are more developed. The “true” toxicity, defined as the effect of the chemical on the life history of the organism, could be just as severe for the organism exposed at lower temperatures if the experiment was performed at a timescale allowing for symptoms of toxicity to develop and manifest themselves.

When investigating the effect of temperature on chemical toxicity in poikilothermic organisms, it would therefore be preferable if observations could be based on full lifecycles rather than fixed short-time experiments, since these more comprehensive tests are not vulnerable to bias related to the influence of variable physiological time during exposure. One of the few articles that report a temperature/metal interaction study that takes physiological time into account is Heugens et al. [9], which showed enhanced effects of cadmium at elevated temperature. This study, however, only included three temperatures, of which the lowest (10°C) is likely to have been near the lower tolerance limit of the test organism *Daphnia magna*. This makes it difficult to extrapolate to a general trend in temperature effects on toxicity to the entire temperature range relevant for the organism. The aim of this study was therefore to investigate the effect of a larger range of growth temperatures

on the toxicity of copper towards *Caenorhabditis elegans* throughout its whole life-cycle, and to compare these results to a standard "fixed-time" setup. According to Klass [10] the egg production of *C. elegans* is significantly reduced below 10˚C and above 25.5˚C indicating direct temperature induced stress on reproductive outputs. We therefore here used four to six temperatures within this range to investigate the effect of the full relevant ambient temperature spectrum on copper toxicity. Copper effects on life-cycle traits were tracked for individual worms from egg to death, thus providing a comprehensive view of chronic exposures to copper for the full range of nematode life-stages, as well as yielding the results normally derived from time-bounded standard acute studies for comparison.

Copper was selected as the chemical for our studies for several reasons. Copper is an essential metal present in all ecosystems. It has a general toxic mode of action, catalyzing the formation of Reactive Oxygen Species (ROS) [11], which is a general type of damage of many different stressors. Because of the dual role of copper as an essential and toxic element [12,13], organisms have evolved systems for the regulation of internal copper concentrations [12–14]. This active regulation of copper uptake and excretion, together with an organism's ability to repair ROS damage is likely to depend on temperature dependent metabolic activity. It is therefore possible that, contrary to the results of the acute studies showing increased toxicity with increasing temperature [1,4], the toxicity of copper towards *C. elegans* measured over a full life cycle will be constant at temperatures within the species tolerance limits. Temperatures at the upper and lower tolerance limits of *C. elegans* are, however, expected to increase the toxicity of copper, as the combination of extreme environmental stress and chemical stress have been shown to enhance the effect of each other [15,16]. *C. elegans* was chosen as a test species because of its fast life cycle and the possibility to monitor survival and reproduction over time.

We hypothesize that copper toxicity measured over an entire lifespan will not be dependent on temperature. We further hypothesize that short term studies of temperature effects on toxicity overestimate the toxicity at warm temperatures compared to the toxicity evaluated when based on population growth rates.

METHODS

Culture conditions for test organism

C. elegans of the N2 Bristol strain (obtained from the *C. elegans* Genetics Centre, University of Minnesota) were cultivated in darkness at 18 or 20˚C on plates of a modified bacteriological agar (nematode

growth medium - NGM [17]) and fed *Escherichia coli* of the uracil deficient strain OP50. At least 2 weeks before the start of the experiments new cultures were initiated at the selected test temperatures to acclimatize the worms through several generations. The cultures were maintained by transferring a chunk of agar and associated eggs, juvenile and adult worms from an existing culture to freshly prepared NGM agar plates weekly.

Set-up of toxicity tests

To cover the full temperature range where the cumulative reproduction of *C. elegans* is relatively unaffected of 10˚C to 25.5˚C [10], Experiment 1 tested the temperatures 11, 13, 15, 18, 21 and 25˚C. Copper concentrations used were 0, 1, 2, 4, 8, and 16 mg Cu/L agar in all cases. The selected test concentrations were based on two literature EC50 values for broodsize of *C. elegans*. The first states a range 2.66-3.37 mg Cu/L measured in the water fraction from NGM agar at 15°C [18]; the second a value of 2.04± 0.19 mg Cu/L in liquid media at an unspecified temperature [19]. These concentrations equate to levels 2-3 orders of magnitude greater than those found in surface waters and soil pore waters from uncontaminated sites, but are comparable to soil pore water concentrations in heavily polluted soils [11,20].

A stock solution of $CuCl_2$ in demineralized water (2 g Cu/L) was made and used throughout the experiment. This copper stock solution was added to the NGM agar while liquid and mixed by swirling. Batches of NGM were produced once every week during the course of the experiment to minimize immobilization of copper in the agar, as shown by Álvarez *et al.* [21] for cadmium. To provide validation of the nominal concentrations used, samples of NGM from each batch were collected and stored for analysis of Cu content.

All tests were conducted using synchronized cohorts of nematodes produced from adults selected from the main stock cultures acclimatized at each temperature. To generate these cohorts, adult hermaphrodite worms were selected from the acclimatized cultures and placed on Petri dishes with copper dosed NGM and *E. coli* and left at the test temperatures for 4 hours (6 hours for 11˚C to ensure sufficient eggs were laid). After this time, the adults were removed and the eggs laid in the 4 (6) hour period left to hatch. The offspring (the test organisms), once hatched and grown to the L4 larval stage on the copper dosed plates, were transferred to 12-well plates with copper dosed NGM and *E. coli*, with one individual per well. There were 12 replicate worms for each treatment and the full set of lifecycle parameters recorded for each individual. The test organisms were

moved to new wells in fresh plates every day and the time to their first egg laying and subsequent daily egg production was determined. The daily transfer and counting were repeated through both the reproductive and senescence stages until the test organisms died (determined by the lack of response to being probed). Fertile eggs and hatched juveniles were counted as offspring, while visibly infertile eggs were excluded.

To provide further resolution of toxicity, a second experiment (Experiment 2) was conducted that included higher concentration treatments within the design. Experiment 2 was conducted at temperatures 12, 16, 20 and 24˚C with the range of copper concentrations included being 0, 1, 3, 8, 20 and 40 mg Cu/L agar. Experiment 2 followed the same procedures as Experiment 1, the only exception being that the exposure at 24˚C had 36 control individuals, instead of the 12 used in the rest of the setup.

Copper analysis

NGM samples were stored from each produced batch for Experiment 1. The samples were digested by 70% HNO_3 (Ultrapure) at 200˚C for 15 minutes within sealed microwave digestion vessels, and analyzed for copper content by Inductively Coupled Plasma-Mass Spectrometry (Perkin Elmer DRCII, Waltham, MA). Measured concentrations correlated well with nominal concentrations (slope = 0.996, R^2 = 0.95), assuming that NGM has a density of 1 g/mL, with measured concentrations deviating less than 20% from nominal concentrations. Concentrations are consequently given as nominal concentrations throughout the paper. To check for the concentration of available copper in NGM from Experiment 2, the copper content in the water fraction of the agar was determined one and five days after production. This was done to be able to distinguish between dissolved copper and copper bound to the agar and therefore not bioavailable. Samples of NGM from each produced batch were saved in centrifuge tubes and, after one or five days, centrifuged at 10000 G for five minutes to separate water and agar. A sample of the water fraction was removed, acidified and stored for later analysis. The analyses were performed on a graphite furnace AAS (Perkin Elmer Zeeman 5100, Waltham, MA).

Statistics

Broodsize and lifespan were tested for normality (Shapiro-Wilk test) and homogeneity of variance (Levene median test), and were analyzed by a two-way ANOVA (Temperature and Cu treatments), with Holm-Sidak *post hoc* analysis (chosen for its ability to compare cells of unequal size, in this case the number of

individuals per treatment). The software SigmaPlot version 11.0, which includes SigmaStat, was used. The residuals of the ANOVA were tested for normal distribution (Shapiro-Wilk and visual assessment).

The average cumulative egg production as a function of time for each treatment was described by a three parameter log-logistic sigmoid model (equation 1) using a box-cox transformation to minimize variance heterogeneity. The analyses were done in the statistical software R version 2.12.0 (http://www.r-project.org/) with the drc package [22].

$$y=d/(1+(x/e)^{b}) \quad (1)$$

In the model *d* is the maximum number of eggs produced, *e* is the time when half the maximum number of eggs is produced, *b* is proportional to the slope of the sigmoid curve at *e*, *y* is the accumulated number of produced eggs and *x* is the time. From this model the estimated time to the production of the first egg (Time to First Egg – TFE) was calculated by solving *x* for *y* equal to 1. The drc package returns a standard error with the estimate. A stage based matrix population model was constructed for each treatment, based on the daily reproduction values and lifespan. The modeling was performed with the statistical software R version 2.12.0 (http://www.r-project.org/), using the popbio package [23], which, among others, is based on the work of Caswell [24]. The models were constructed so that each day in the life of the nematodes corresponded to a life stage in the model, thus making full use of the daily reproduction data in the model. A projection of the population over 500 iterations (days) were made and after checking that the population growth had stabilized, the population growth rates (PGR) were determined. The 95%, 99% and 99.9% confidence intervals (CI) were determined by bootstrapping.

To determine EC50 values for broodsize, lifespan and PGR, these responses were described with a log-logistic three parameter concentration response model (Equation 1). To be able to compare the full life-cycle data with reproductive data from typical “fixed time setups” two acute exposure scenarios were chosen from the literature: 1) Individuals monitored for reproduction for the first 96 hours of the adult life stage [25] and 2) individuals monitored for reproduction for 24 hours at the approximate peak of reproductive output [26] (Figure 2). Short-term subsets of reproduction data were made from the full life cycle data using these scenarios, and from these we determined EC50 values for comparison with full lifecycle EC50 values.

RESULTS

The broodsize from all control treatments in both experiments are plotted in figure 1. The reproduction was slightly lower in Experiment 1 compared to Experiment 2, although maximum reproduction in both tests was within the commonly reported range for *C. elegans* [10]. The data suggest that the temperature optimum for *C. elegans* is near 20°C, with lower and higher temperatures causing a decrease in reproduction. At 25°C the reproduction decreased severely.

In Experiment 1, at the highest temperature used of 25°C, reproduction was severely limited by heat making this temperature outside the tolerable range for our *C. elegans* (Table 1). Hence data from the 25°C treatment was excluded from all further analyses. For the remaining temperature treatments used, a clear effect of increasing copper concentration on total broodsize was evident. This toxic effect was greatest for the worms kept at the higher temperature of 21°C (Table 1, Figure 2B). Similar effects of copper were evident on other measured parameters including lifespan, TFE and PGR, again with effects being more severe at higher temperature conditions (Table 1). At low temperatures, however, the toxic effects of copper was less pronounced and were not significant for effects on broodsize at 11 and 13°C (Figure 2A and Table 1).

A two-way ANOVA for the broodsize results from Experiment 1 showed significant effects of both temperature and copper treatment ($p < 0.001$, $p < 0.001$), and significant interactions between the two factors ($p < 0.001$). This significant interaction component implies that copper toxicity is dependent on exposure temperature. Broodsize data failed the normality test, but the residuals from the two way ANOVA were normally distributed ($p = 0.074$). The data also failed the test for homogeneity of variance. However, as differences between treatments were highly significant, we do not believe the failures to meet these assumptions of the tests will seriously affect the main conclusions of the study. Describing total broodsize with a log-logistic dose response model (Equation 1) produced an EC50 value of 6.0±1.9 mg Cu/L agar for the 21°C treatment. At 15 and 18°C the EC50's were 16.8±2.2 and 16.1±0.7 mg Cu/L agar and hence higher than at 21^{o}C reflecting the reduced toxicity of copper at these test temperatures, while at the two lowest test temperatures (13 and 11°C), the absence of a significant concentration response relationship did not allow calculation of EC50 values. Figure 3A shows broodsize for control treatments and the two highest copper concentrations used (8 and 16 mg/L) against the temperature treatments, which clearly illustrates the complex nature of the

relationship between temperature and the effect of copper on broodsize, identifying that while the 16 mg Cu/L caused little change from control broodsize values at 11 and 13°C it caused a greater than 80% reduction at 21°C.

Lifespan raw data passed the normality test ($p = 0.133$), as did the residuals of the ANOVA ($p = 0.133$). A two-way ANOVA for data on nematode life-span indicated a significant effects of both temperature and copper concentration ($p < 0.001$ and $p = 0.002$, respectively), but no significant interaction ($p = 0.256$). The pattern of temperature dependent effect of Copper, however, followed that seen for broodsize. Only the highest temperature (21°) yielded a significant effect of copper on lifespan compared to controls, and only at the highest copper treatment (16 mg Cu/L), thus hampering statistical identification of the interaction (Table 1, Figure 3B). From the 21°C test, it was possible to estimate an EC50 value for lifespan of 16.9±3.8 mg Cu/L agar, although the fact that this value falls outsider of the tested range limits the reliability of this estimate.

TFE decreased with increasing temperature indicating a temperature effect on this parameter. For most temperatures nematodes which were treated with 16 mg Cu/L agar used one additional day to reach adulthood (Figure 3C). The additional time taken to maturity should, however, be seen relative to the TFE of the control group for the respective temperature, which as noted above tended to reduce as temperature increased. At 13°C, the delay corresponds to a 17 % increase in time to the start of reproduction compared to the control, while at 21°C the 16 mg Cu/L agar induced a 49% delay compared to controls. At 11°C, the nematodes exposed to the highest copper concentration started reproduction slightly earlier than the controls. However, based on the standard errors there were no significant differences between the temperature treatments. Hence at 11°C no delaying effect of reproduction was evident.

Population growth rate estimates are given in Table 1 and are plotted against ambient temperature values in Figure 3D. At 11°C there was no observable difference between controls and copper treatments, while at 21°C the copper at 16 mg/L agar caused a 36% decrease in PGR. As for broodsize, the PGR estimates indicate a gradual increase in the separation of the control and copper exposed treatments as temperature increased over the range tested. From the confidence intervals derived from bootstrapping we estimated the statistical values for treatment separation (e.g. less than 5% chance of the two being equal if the 95% CI does not overlap). No significant separation of the copper treatments from the controls were seen at 11 and 13°C,

however, from 15°C up to 21°C the PGR for one or more of the copper treatments were significantly different from the control PGR (Table 1, Figure 3D).

Experiment 2 was conducted to refine the result of the first experiment, especially with respect of low temperature toxic effects. The results of this experiment largely confirmed the results of Experiment 1, although the nematodes in Experiment 2 were slightly less sensitive to copper when evaluated from EC-values (Table 2 and 3, Figure 4). Low doses of copper (1 and 3 mg/L) caused no detrimental effects; hence, in Figure 4, only results from control and 8, 20 and 40 mg Cu/L agar are plotted. EC50 values for broodsize decreased with increasing temperature in a similar pattern as for Experiment 1 (Table 3). Lifespan was also affected by copper as for Experiment 1 (Table 2, Figure 4B). However, in Experiment 2, the two-way ANOVA also found significant interactions ($p < 0.001$) between temperature and copper effect on lifespan. These interactions are most likely caused by an increase in toxicity with increasing temperature.

TFE results showed a slight non-significant decrease at low copper doses, and an increase at the highest concentration used (Figure 4C, Table 2). Of the PGR values only 24 degrees permitted the estimation of an EC50 value of 36.1±1.8, but EC10 values decreased steadily from 30.9±4.0 to 13.0±1.8 mg/L when increasing the temperature from 12 to 24°C (Table 2, Figure 4D). Several of the treatments showed signs of stimulating effects at low copper doses on the various endpoints, the so called hormesis effects, also for PGR (Table 2). Although this stimulation was rarely significant, it was consistent across the temperatures in both experiments.

The EC50 values for reproduction over the full life cycle and over the 24 and 96 hours reflecting traditional fixed time setups [25,26] from both experiments are listed in Table 3. EC50s from the 24 hour short term exposure scenario were consistently lower than EC50s for the full lifecycle for all temperatures, and the clear pattern of increased toxicity with increasing temperature is less evident. The 96 hour data showed lower EC50-values at low temperatures compared to the full life cycle data, but as temperature rose, and the 96 hour short term scenario included more and more of the total reproductive output, the EC50 values approached those obtained by using full lifecycle data. The consequence was that the consistent temperature effect on toxicity observed for the full life cycle study disappeared in the 96h study, where no significant differences between EC50 values were obtained between the four temperatures tested in the second study. Hence, while increasing temperature consistently increases copper toxicity on reproduction in both experiments when monitored over

the full lifecycle, this true temperature effect on toxicity could not be detected if reproduction was only monitored for 24 or 96 hours.

DISCUSSION

The results of both experiments showed that the toxicity of copper towards the measured whole lifecycle endpoints tended to increase with increasing temperature. This is contrary to our initial hypothesis that there is no interaction between temperature and copper toxicity within a temperature range near the optimum for *C. elegans*. For reproduction the observed temperature dependent increase in toxicity was approximately 2.5-fold in Experiment 1 between 15 and 21°C and around two fold in Experiment 2 between 12°C and 24°C (Table 3). The results of increased toxicity with increasing temperature match those found by Rathore & Khangarot [27], who showed that the toxicity of eight metals, including copper, to the sludge worm *Tubifex tubifex* increased with temperature, in the range of 15 to 30°C. Jacobson *et al*. [28] also showed a linear decrease of LC50 values for copper with temperatures increasing from 10 to 25°C for freshwater mussel *Actinonaias pectorosa*. Similarly in a review of six studies of the interactions between copper and temperature by Heugens *et al*. [5], increasing toxicity with increasing temperature was found in all cases except one (see Table 4 for summary). Contrary to these results, the toxicity of copper and cadmium to the potworm *Enchytraeus crypticus* measured on reproduction decreased approximately two-fold between 11°C and 25°C, (although LD50 values were rather constant over the same temperature range) [29]. All of these conclusions, except for that for *E. crypticus*, were based on time limited studies on mortality (Table 4). Hence, these studies may be biased by their failure to include the element of increased physiological time at higher temperatures as a component of the assessment. If we in our present study had reported mortality after a fixed time interval of 10-15 days (Fig. 4B), we would find concentration dependent mortality at 24°C, but no mortality at 12°C, even though this temperature eventually gives a clear dose-response relationship on mortality at a later time. Hence, had mortality been the chosen endpoint, as it was in all but one of the studies given in table 4, the perceived temperature effect on toxicity would have been even greater than what was found using population growth rates.

Similarly our detailed observations of reproductive output over time shows that the simulated time limited observations on nematodes would not clearly identify the true increase in toxicity of copper with increasing temperature demonstrated in the full life cycle reproductive output (Table 3). When copper toxicity

was estimated based on the values for reproduction derived during the first 96h of adult life (i.e. the four days immediately following maturation) in the second study, the effect concentrations across the experiment from 12°C to 24°C were similar. The 24h assessments showed no specific trends (Table 3). If we had chosen not only to fix the time frame of reproductive measurement after worm maturation, but also the starting point in terms of time after egg-laying, we most likely would have seen very strong temperature effects as cool treated nematodes would not yet have matured at the fixed starting time [25,30]. This type of setup might be one of the main reasons for the much higher temperature effects found in other studies (Table 4), where for example EC50 values for *Tubifex tubifex, Pyganodon grandis* and *Diaptomus clavipes* increased 7.5 to 11-fold within a temperature span of 10-20°C. Experimental design therefore affects the derived conclusions of temperature effects on toxicity substantially.

One plausible explanation for the increase in copper toxicity with rising temperature is that it results from a change in copper toxicokinetics or toxicodynamics. That is, either the uptake/exclusion processes of copper homeostasis are changing leading to an increase in the internal concentrations of Cu and/or the intrinsic toxicity of the copper taken up by the organism is increasing at increasing temperature. In a study on *Daphnia magna* and Cd both uptake and sensitivity to the internal concentrations increased with increasing temperature [7]. In a study with *E. crypticus,* where copper toxicity was shown to decrease with increasing temperature [29], the decrease in copper toxicity with increasing temperature was due to an increase in copper elimination efficiency, making the worm able to better maintain low internal concentrations at high compared to low temperatures [29]. Due to their small size, measurements of internal metal concentrations in individual nematodes are challenging and therefore we have not evaluated its importance for the increased toxicity at higher temperature. But higher uptake rates at high temperatures could potentially explain the increased toxicity with temperature. However, as the main mode of toxic action of copper is the catalyzation of reactive oxygen species, the occurrence of which increase with increasing temperature [31], the toxicodynamic processes may also play an important role for the increased toxicity at high temperatures. The increase of TFE induced both at low temperatures and high copper concentrations indicate that both parameters slowed nematode growth and development. Toxicant induced decreased growth rates have been observed for nematodes exposed to cadmium [32], but was not measured in this study.

In all ten dose-response experiments except one, copper doses as high as 8 mg Cu/L did not only result in increased reproduction, but also increased PGR's (Table 1 and 2). Such bi-phasic trends are often called hormetic responses [33] without regarding the physiological cause of the response, which might be different depending on stressor and type of organism. Seen from an evolutionary perspective, PGR should not increase in response to a stressor [34,35]. An increased fitness of treated populations compared to controls can, however, occur if the controls are deficient in some element present in the treatment [34]. As copper is an essential element, this could possibly explain the observed consistent trend. We do, however, find that this is unlikely as the population growth stimulation occurs at rather high copper concentrations compared to naturally occurring concentrations [11]. Another possible explanation for biphasic responses is if the stressor, in this case copper, is more toxic to a competitor or pathogen than to the organism on which fitness is measured [34], or if it in some way enhances the food quality [35]. We do not know of potential microbial pathogens in our nematode test-system, nor are we able to measure food quality. Hence, changes in one of these two parameters in response to the copper treatments could possibly explain the consistent increase in PGR with treatment, but this would have to be investigated further.

The comparison of reproduction EC50 values from full lifecycle and short term reproduction indicates a tendency to either overestimate the temperature effect on toxicity when using short term, time fixed, exposures, or to entirely overlook it (96h data), all depending on experimental design. These errors are mainly due to the delay in toxic response caused by the slower metabolic rates at low temperatures, giving the impression that toxic response does not occur to the same extent under low temperatures compared to high temperatures. Thus our results confirm the hypothesis that short term time fixed studies of temperature effects on toxicity underestimate toxicity at low temperatures or overestimate the toxicity at high temperatures. As very few studies so far have investigated the influence of time on the outcome of temperature effects on toxicity, we feel it is still premature to discuss the possible implications for environmental risk assessment of chemicals. We do, however, encourage studies of temperature effects on toxicity to be very carefully designed in terms of the time courses used and the endpoints measured in relation the research question investigated.

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Figure 1. The effect of temperature on the broodsize of control *C. elegans*. Dark circles represent data from Experiment 1 and white circles data from Experiment 2 (mean±SE, n=12).

Figure 2. Cumulative egg production of *C. elegans* exposed to copper. Data from experiment 1 for nematodes living at 11°C (A) and 21°C (B) (Mean± SE, n = 12). Data are described by a log-logistic model (equation 1). The shaded areas represent the 24 hour (dense shading) and 96 hour (light shading) short-term subsets of data which we compare with full lifecycle. Concentrations are in mg Cu/L agar.

Figure 3. Results of experiment 1. Broodsize (A), lifespan (B), time to first egg (TFE) (C) and population growth rate (PGR) (D) of *C. elegans* as a function of temperature for two copper concentrations and the control treatment of the first experiment. Broodsize, lifespan and TFE are given as mean±SE. PGR are given with 95% confidence intervals.

Figure 4. Results of experiment 2. Broodsize (A), lifespan (B), time to first egg (TFE) (C) and population growth rate (PGR) (D) of *C. elegans* as a function of temperature for three copper concentrations and the control treatment of the second experiment. Broodsize, lifespan and TFE are given as mean±SE. PGR are given with 95% confidence intervals.

Table 1. Broodsize, lifespan, time to first egg (TFE) and population growth rate of *C. elegans* exposed to copper (Experiment 1)[a]

Temp.	Concentration (mg Cu/L agar)	Broodsize (number of offspring)	Lifespan (days)	TFE (days)	PGR (day^{-1})	Temp.	Concentration (mg Cu/L agar)	Broodsize (number of offspring)	Lifespan (days)	TFE (days)	PGR (day^{-1})
11°C						13°C					
	0	[b]142.0±15.4^{A}	23.5±2.2^{A}	7.7±0.6	1.48 (1.45-1.50)^{A}		0	125.0±27.6^{A}	22.8±2.5^{A}	4.8±1.1	1.52 (1.47-1.57)^{A}
	1	109.3±24.5^{A}	25.0±2.7^{A}	6.4±1.3	1.44 (1.38-1.46)^{A}		1	130.8±11.7^{A}	25.1±2.1^{A}	4.4±0.5	1.56 (1.52-1.60)^{A}
	2	105.1±25.1^{A}	21.2±2.5^{A}	7.5±1.2	1.42 (1.37-1.46)^{A}		2	125.8±20.0^{A}	24.9±2.3^{A}	4.7±0.9	1.58 (1.53-1.60)^{A}
	4	153.5±29.7^{A}	25.6±1.6^{A}	6.9±1.1	1.50 (1.48-1.53)^{A}		4	170.1±14.2^{A}	25.5±1.6^{A}	4.6±0.5	1.64 (1.61-1.67)^{B}
	8	161.0±26.7^{A}	27.0±2.0^{A}	7.0±0.9	1.49 (1.46-1.51)^{A}		8	172.1±18.8^{A}	23.4±2.5^{A}	5.3±0.7	1.61 (1.57-1.66)^{AB}
	16	115.0±20.3^{A}	21.2±2.3^{A}	6.6±1.0	1.45 (1.42-1.48)^{A}		16	152.9±28.3^{A}	24.5±2.9^{A}	5.6±1.0	1.54 (1.49-1.58)^{A}
15°C						18°C					
	0	184.6±11.2^{A}	18.6±1.4^{A}	3.7±0.5	1.84 (1.81-1.88)^{A}		0	189.0±21.3^{A}	17.7±1.6^{A}	3.4±0.6	2.25 (2.18-2.32)^{A}
	1	188.7±18.8^{A}	20.3±2.4^{A}	3.8±0.5	1.90 (1.86-1.93)^{A}		1	220.1±9.6^{A}	17.9±1.5^{A}	3.1±0.2	2.46 (2.40-2.51)^{B}
	2	184.4±12.7^{A}	23.9±1.8^{A}	4.4±0.4	1.76 (1.71-1.80)^{B}		2	175.8±20.7^{A}	17.8±1.1^{A}	3.2±0.6	2.30 (2.22-2.38)^{A}
	4	177.5±26.8^{AB}	19.1±2.3^{A}	4.4±0.7	1.75 (1.70-1.80)^{B}		4	218.3±16.6^{A}	14.6±1.2^{A}	3.3±0.4	2.43 (2.38-2.50)^{B}
	8	184.3±17.4^{A}	19.4±1.7^{A}	4.3±0.5	1.76 (1.71-1.81)^{AB}		8	205.1±14.2^{A}	16.3±1.1^{A}	3.3±0.3	2.29 (2.24-2.32)^{A}
	16	106.9±20.8^{B}	17.9±1.7^{A}	4.9±1.0	1.61 (1.55-1.64)^{C}		16	103.0±25.8^{B}	13.0±1.9^{A}	4.1±1.1	1.79 (1.62-1.86)^{C}

21°C						25°C				
	0	208.7±8.9[A]	17.1±1.2[A]	2.0±0.1	2.70 (2.64-2.79)[A]	0	42.7±28.8[A]	13.2±0.5[A]	Na	2.38 (2.25-2.48)[A]
	1	136.0±11.8[B]	15.4±1.3[A]	2.3±0.4	2.38 (2.29-2.46)[B]	1	36.3±23.7[A]	12.3±0.9[A]	Na	2.34 (2.12-2.51)[A]
	2	141.1±6.3[B]	14.7±0.8[AB]	2.3±0.1	2.34 (2.29-2.40)[B]	2	55.9±28.9[A]	11.6±0.9[A]	Na	2.64 (2.34-2.80)[A]
	4	130.2±10.2[B]	15.0±0.6[AB]	2.5±0.3	2.24 (2.21-2.30)[B]	4	35.5±35.4[A]	14.6±0.8[A]	Na	2.29 (1.93-2.53)[A]
	8	117.8±12.5[B]	12.1±0.8[AB]	2.5±0.3	2.21 (2.12-2.30)[B]	8	34.3±27.2[A]	11.8±1.0[A]	2.3±0.7	2.16 (1.97-2.34)[A]
	16	35.9±18.4[C]	8.1±1.7[B]	3.0±1.6	1.73 (0.65-1.94)[C]	16	36.3±31.5[A]	8.6±1.0[A]	2.5±1.0	2.08 (1.85-2.26)[A]

[a] All parameters are given as mean ± standard error, except for PGR which is given ± 95% confidence interval.

[b] Parameters of the same temperature treatment sharing the same uppercase letter are not significantly different from each other (P>0.05), as determined by ANOVA for broodsize and lifespan or as no overlap of the 95% CI's for PGR.

Table 2. Broodsize, lifespan, time to first egg (TFE) and population growth rate of *C. elegans* exposed to copper (Experiment 2)[a]

Temp.	Concentration (mg Cu/L agar)	Broodsize (number of offspring)	Lifespan (days)	TFE (days)	PGR (day^{-1})	Temp.	Concentration (mg Cu/L agar)	Broodsize (number of offspring)	Lifespan (days)	TFE (days)	PGR (day^{-1})
12°C						16°C					
	0	[b]190.9±15.9^{A}	36.0±2.7^{A}	5.9±0.5	1.64 (1.63-1.65)^{A}		0	206.0±12.9^{A}	24.0±1.9^{A}	3.6±0.5	2.04 (2.02-2.07)^{A}
	1	236.9±6.5^{A}	33.9±2.0^{A}	5.3±0.2	1.69 (1.68-1.70)^{B}		1	254.0±9.0^{A}	25.9±1.7^{A}	3.3±0.2	2.12 (2.09-2.14)^{B}
	3	216.6±7.2^{A}	35.6±1.4^{A}	5.2±0.2	1.69 (1.68-1.70)^{B}		3	247.4±9.9^{A}	24.2±0.5^{A}	3.3±0.2	1.13 (2.11-2.15)^{B}
	8	215.9±12.5^{A}	31.9±0.9^{A}	5.2±0.6	1.65 (1.63-1.66)^{A}		8	232.5±7.9^{A}	22.6±0.9^{A}	3.7±0.2	2.01 (1.98-2.04)^{A}
	20	179.6±8.8^{A}	22.8±0.6^{B}	5.0±0.3	1.57 (1.56-1.58)^{C}		20	135.8±19.5^{B}	12.9±0.9^{B}	3.8±0.7	1.83 (1.78-1.87)^{C}
	40	76.1±8.8^{B}	17.1±0.5^{C}	6.8±0.6	1.44 (1.43-1.47)^{D}		40	71.5±18.3^{C}	12.±0.9^{B}	4.4±1.1	1.57 (1.53-1.69)^{D}
20°C						24°C					
	0	273.2±12.8^{A}	14.9±1.6^{A}	2.5±0.2	2.73 (2.68-2.78)^{A}		0	198.4±9.0^{A}	14.7±0.7^{A}	2.0±0.2	3.11 (3.05-3.17)^{A}
	1	287.3±8.6^{A}	18.4±1.7^{AB}	2.2±0.1	2.76 (2.72-2.78)^{A}		1	222.5±13.5^{A}	16.5±1.9^{A}	1.7±0.4	3.33 (3.26-3.41)^{B}
	3	285.1±8.9^{A}	16.8±1.9^{AB}	2.5±0.1	2.77 (2.75-2.80)^{A}		3	181.0±14.8^{A}	16.2±1.2^{A}	1.8±0.3	3.16 (3.04-3.27)^{A}
	8	253.5±9.4^{A}	14.1±0.4^{A}	2.6±0.1	2.65 (2.61-2.68)^{A}		8	195.1±8.9^{A}	13.8±1.2^{A}	1.8±0.3	3.11 (3.00-3.21)^{A}

	20	156.8±4.6[B]	10.0±0.3[A]	2.8±0.1	2.29 (2.24-2.34)[B]	20	93.6±19.6[B]	8.0±0.6[B]	1.9±0.7	2.50 (2.31-2.65)[C]
	40	91.7±14.6[C]	10.2±0.8[A]	2.9±0.6	1.97 (1.86-2.05)[C]	40	12.6±10.8[C]	4.7±0.6[B]	Na	1.43 (0.58-1.66)[D]

[a] All parameters are given as mean ± standard error, except for PGR which is given ± 95% confidence interval.

[b] Parameters of the same temperature treatment sharing the same uppercase letter are not significantly different from each other (P>0.05), as determined by ANOVA for broodsize and lifespan or as no overlap of the 95% CI's for PGR.

Table 3. EC50 values (mg/L) for reproduction. Values are based on data from the full life cycle and subsets of 24 hours and 96 hours.

Experiment 1				Experiment 2			
Temp.	**Full life cycle**	**24 hours**	**96 hours**	**Temp.**	**Full life cycle**	**24 hours**	**96 hours**
11°C	>16	Na	Na	**12**°C	33.1±2.1	20.6±3.1	19.4±1.7
13°C	>16	Na	Na	**16**°C	25.1±2.5	7.9±0.4	19.6±1.7
15°C	16.8±2.2	4.0±1.6	10.0±3.2	**20**°C	24.2±1.7	12.1±0.8	22.7±1.4
18°C	16.1±0.7	10.7±1.6	14.6±2.2	**24**°C	19.4±1.6	14.7±2.4	19.2±1.5
21°C	6.0±1.9	1.7±0.5	4.1±1.1				

Table 4. Examples of short term copper experiments at different temperatures extracted from the literature. The table gives: species, experimental steady state temperatures used, endpoint measured, time scale of the experiment and the fold increase in toxicity from the lowest to the highest temperature measured as the change in EC50.

Species	**Temperatures (°C)**	**Endpoint**	**Time scale of experiment (h)**	**Fold Change in toxicity**	**Reference**
Tubifex tubifex	15, 20, 25, 30	Mortality	96h	11	Rathore & Khangarot (2002) [27]
Actinonaias Pectorosa	10, 15, 20, 25	Mortality	24h	3.1	Jacobsen et al. (1997) [28]
Pyganodon grandis	10, 20	Mortality	24h	7.5	Jacobsen et al. (1997) [28]

Daphnia pulex	10, 20, 30	Mortality	48h	1.3	Boeckman & Bidwell (2005) [2]
Diaptomus clavipes	10, 20, 30	Mortality	48h	9.6	Boeckman & Bidwell (2005) [2]
Enchytraeus crypticus	11, 13, 15, 18, 21, 25	Reproduction	Variable*	-2.5	Cedergreen et al. (2013) [29,29]

* At each temperature, the experimental time was the time taken for the controls to produce an approximate average of 40 offspring.

** The study showed a decrease in toxicity with rising temperature by a factor 2.

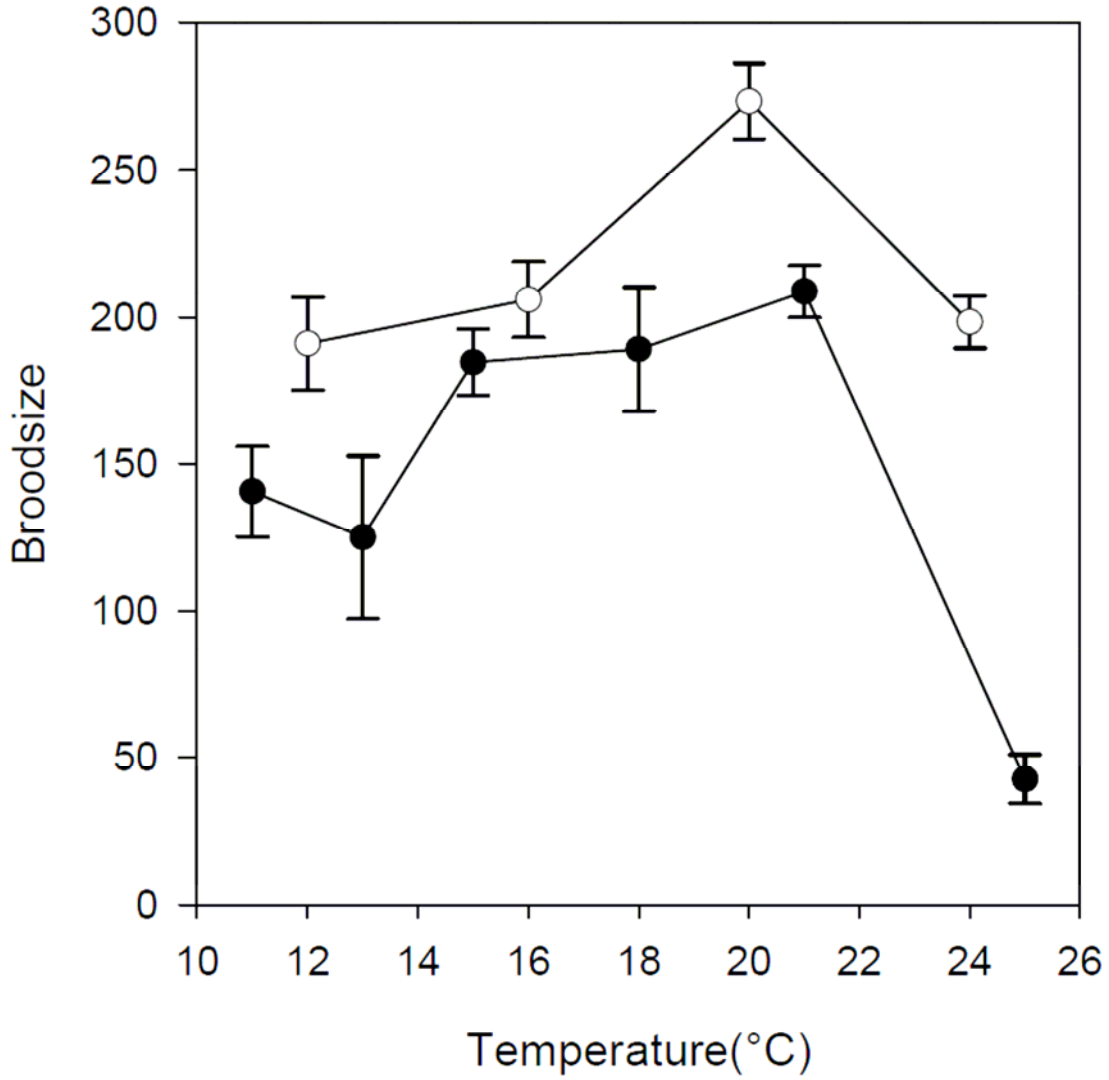

Figure 1

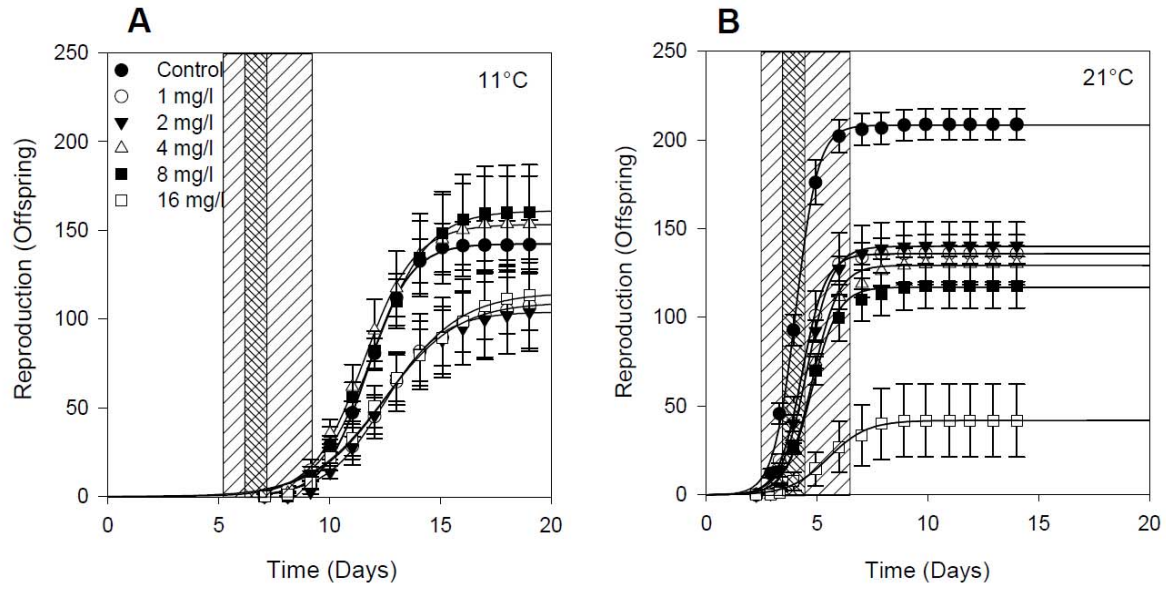

Figure 2

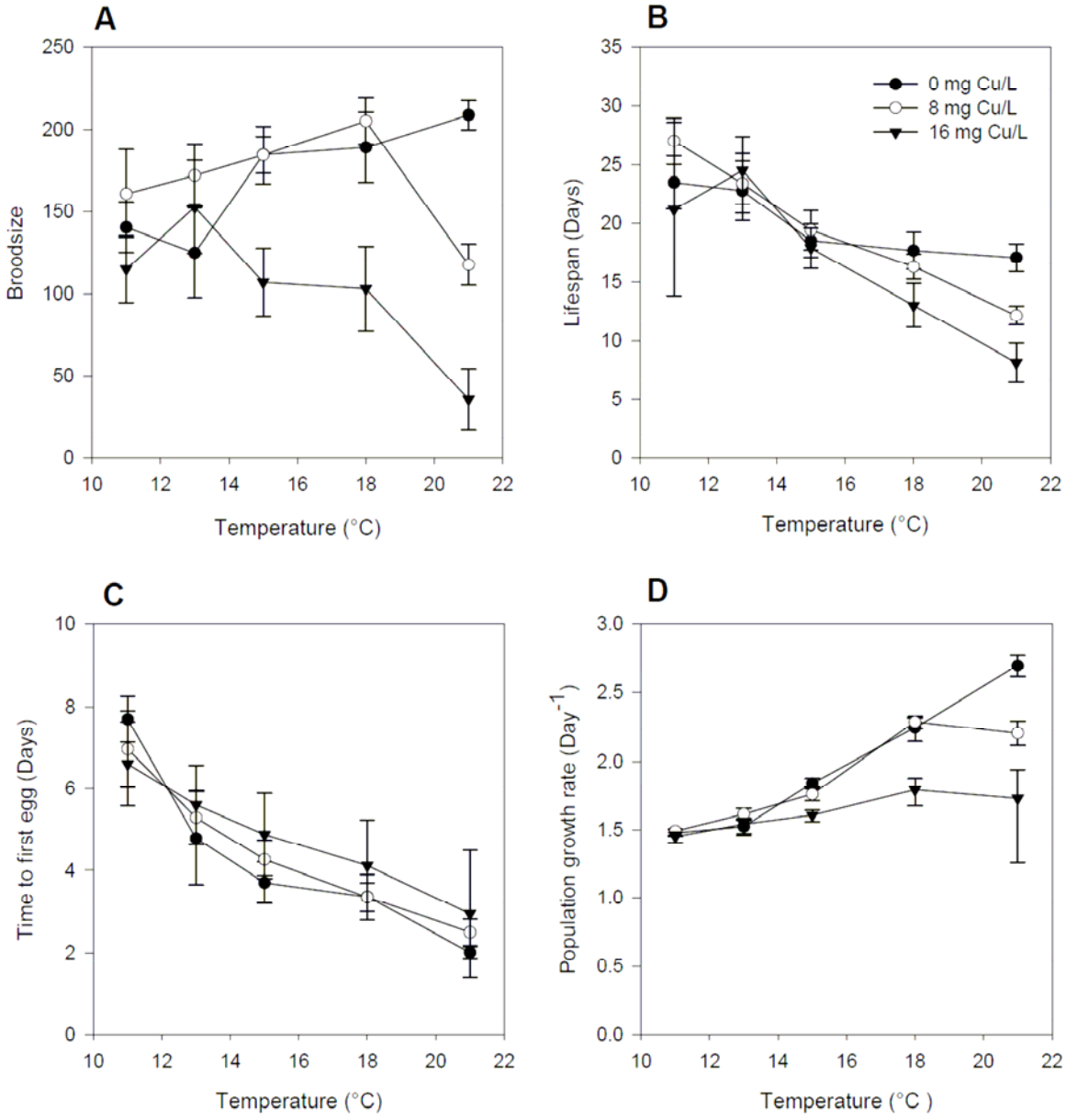

Figure 3

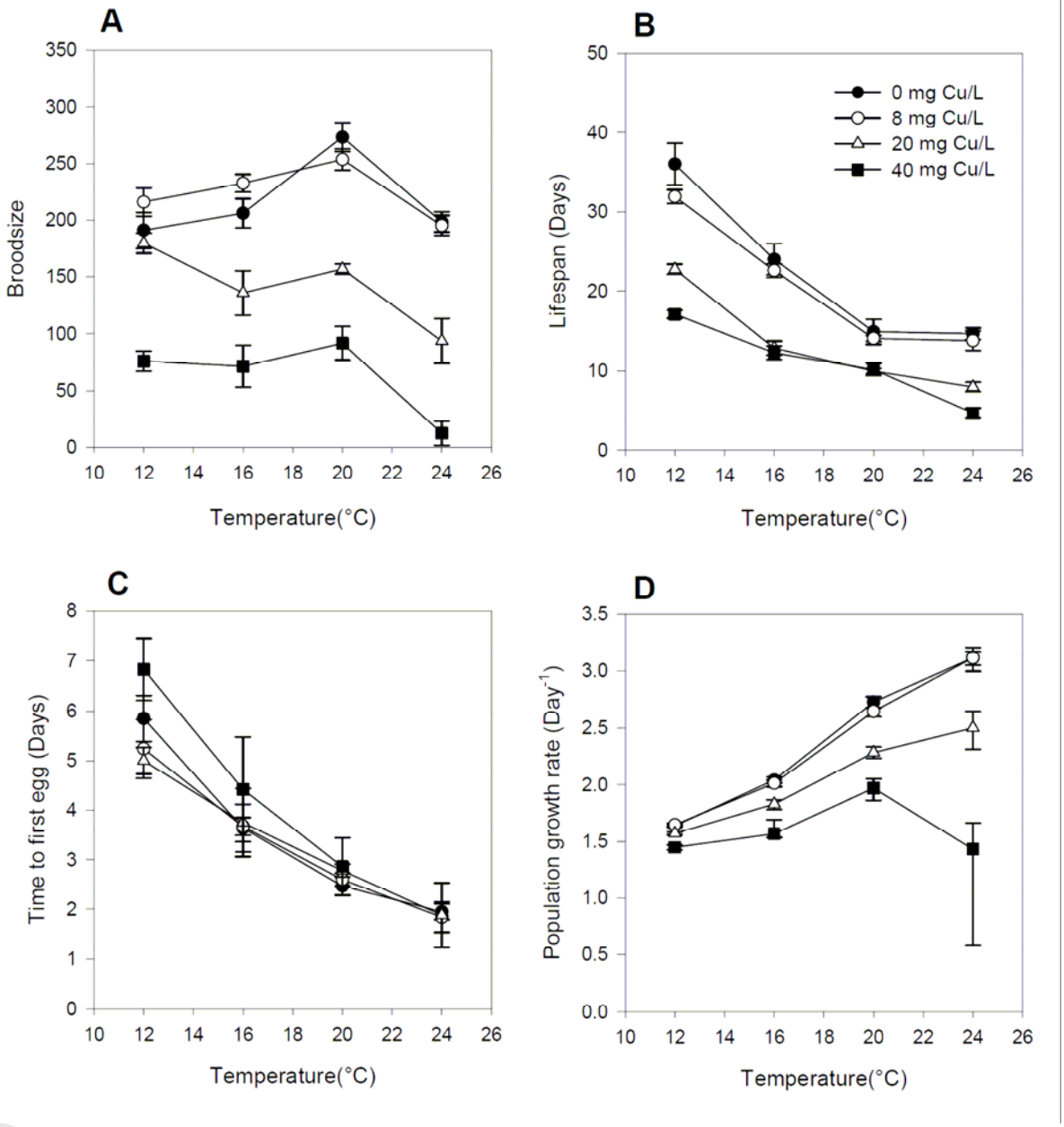

Figure 4