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Abbreviations:

ANOVA	analysis of variance
EEC	expected environmental concentration
H&E	hematoxylin and eosin
LC_{50}	concentration that results in death of 50% of the individuals by a given time
LOEC	lowest observable effect concentrations
MANOVA	multivariate analysis of variance

ABSTRACT

Background: Contaminants have been implicated in declines of amphibian, a taxon with similar vital systems as humans. However, many chemicals have not been thoroughly tested on or do not directly kill amphibians.

Objective: To quantify amphibian responses to chlorothalonil, the most commonly used synthetic fungicide in the US.

Methods: We reared *Rana sphenocephala* (Southern leopard frog) and *Osteopilus septentrionalis* (Cuban treefrog) in outdoor mesocosms with or without 1x and 2x the expected environmental concentration (~EEC: 164µg/L) of chlorothalonil. We also conducted two dose-response experiments on *O. septentrionalis*, *Hyla squirella* (squirrel treefrog), *H. cinerea* (green treefrogs), and *R. sphenocephala*, and evaluated the effects of chlorothalonil on the stress hormone, corticosterone.

Results: For both species in the mesocosm experiment, the 1x and 2x EEC treatments were associated with >87% and 100% mortality, respectively. In the laboratory experiments, the ~EEC caused 100% mortality of all species within 24h, 82µg/L killed 100% of *R. sphenocephala*, and 0.0164µg/L caused significant tadpole mortality of *R. sphenocephala* and *H. cinerea*. Three species showed a non-monotonic dose-response, with low and high concentrations causing significantly greater mortality than intermediate concentrations and controls. For *O. septentrionalis*, corticosterone exhibited a similar non-monotonic dose response and chlorothalonil concentration was inversely associated with liver tissue and immune cell densities (<16.4µg/L).

Conclusions: Chlorothalonil killed nearly every amphibian at the ~EEC and, at concentrations to which humans are commonly exposed, it increased mortality and was associated with elevated corticosterone levels and changes in immune cells. Future studies should directly quantify the effects of chlorothalonil on amphibian populations and human health.

INTRODUCTION

Amphibians are arguably the "poster child" of the present extinction crisis (Wake and Vredenburg 2008) with over 32% of species threatened and at least 43% experiencing population declines (Stuart et al. 2004). Chemical pollution is a concern for both the health of amphibians and humans. It is considered the second greatest threat (behind habitat loss) to aquatic and amphibious species in the US and has been linked to amphibian declines and disease (Davidson et al. 2002; Rohr et al. 2008a). Similarly, contaminants have been linked to mortality and disease in humans (Dietert et al. 2010). Importantly, many vital systems of amphibians, such as endocrine and immune systems, are similar to those in humans (Hayes 2005), and a genome analysis revealed that the amphibian *Xenopus tropicalis* has >1,700 genes with human disease associations (Hellsten et al. 2010). Thus, in addition to being of conservation concern, amphibians might be an underutilized model taxon for studying stressor effects on human health.

Although the hypothesis that contaminants are a factor in amphibian declines is plausible, most previously tested chemicals have not directly killed amphibians at or below expected environmental concentrations (EEC; but see Storrs and Kiesecker 2004; Rohr et al. 2006b), though sublethal and indirect effects can be strong (Rohr et al. 2006a). Nevertheless, many chemicals remain untested on amphibians. For example, chlorothalonil is the most commonly used synthetic fungicide in the US (Kiely et al. 2004), and is toxic to shrimp, insects, and fish at or below the EEC (164μg/L; Caux et al. 1996; Grabusky et al. 2004). It can be transported great distances and has been found in tropical mountains where most amphibian declines have occurred (Stuart et al. 2004). However, its effects on amphibians have rarely been studied.

Chlorothalonil can also affect vertebrate and invertebrate immune systems. Chlorothalonil exposure was associated with contact dermatitis (Penagos 2002) and DNA damage to leukocytes of farmers 1d after spraying (Lebailly et al. 1997). It can be immunosuppressive to oysters and fish,

reducing macrophage viability and function, immunoglobulin M, and expression of proinflammatory cytokines (Baier-Anderson and Anderson 2000; Shelley et al. 2009). These findings are a concern because pollution is often associated with wildlife disease emergence (Dobson and Foufopoulos 2001) and amphibians are being decimated by infectious disease (Daszak et al. 2003). The objective of this study was to quantify the effects of chlorothalonil on amphibian survival, immunity, corticosterone levels, and liver density.

Pesticide Background

Chlorothalonil (2,4,5,6-tetrachloroisophthalonitrile) is widely used to control fungus on peanuts, corn, and potatoes (Cox 1997). Approximately 14 million pounds are applied annually in the US with ~500,000lbs used per year in Florida (USEPA 199), the location of the present study. Chlorothalonil is typically applied during the wet season, corresponding to the breeding activity of many amphibians (Rohr et al. 2004).

Chlorothalonil binds to glutathione, which disrupts cellular respiration (Grabusky et al. 2004), a vital process to virtually every organism including humans. In water, chlorothalonil is short lived with a half-life of approximately 44h (USEPA 1999). The primary chlorothalonil metabolite (4-hydroxy-2,5,6-trichloroisophthalonitrile) is estimated to be 30x more acutely toxic than chlorothalonil and is also more persistent and mobile in soil (EPA 1988). Chlorothalonil is also contaminated during its manufacturing with hexachlorobenzene (Hung et al. 2010), a probable carcinogen with a soil half-life of 3-6 years (Cox 1997).

Shuman et al. (2000) detected chlorothalonil concentrations in runoff ≤290µg/L and chlorothalonil has been detected in groundwater ("standpipe" wells) ≤272µg/L. Nevertheless, the EEC of chlorothalonil in ponds (calculated using the US EPA GENEEC v2 software, see Table S1 for parameters) is ~164µg/L. If lowest observable effect concentrations of a chemical are near or

below its EEC, then it poses sufficient risk to warrant higher-level modeling. Hence, effects of a chemical near or below the EEC can affect the decision to approve its use.

MATERIALS AND METHODS

Additional methodological details are available in the Supplemental Methods for each of the following five sections. All animals used were treated humanely and with regard for alleviation of suffering.

Mesocosm Experiment

The mesocosm experiment was conducted at the University of Florida’s Gulf Coast Research and Education Center, Wimauma, FL from July-August, 2008 (35d total). Mesocosms consisted of cattle water tanks (1.8m dia., 60cm deep, ~1100L) containing 800L of water, 300g of leaf litter, and local zooplankton, phytoplankton, periphyton, insect, gastropod, and crayfish species (Table S2). Mesocosms were covered with 60% shade cloth to prevent overheating and entry or escape of animals. Each tank received 10 *Rana sphenocephala* (Southern leopard frog) tadpoles from eight clutches (collected at N28°06.759’ W082°23.014’) and 25 *Osteopilus septentrionalis* (Cuban treefrog) tadpoles (Gosner stages 25-28) from five clutches (collected at N28°03.537’ W082°25.410’).

Tanks were arranged in a randomized block design with four replicates of each treatment. There were two control treatments, receiving either 50ml of water or acetone solvent (used to dissolve chlorothalonil). Tanks in the remaining two treatments received chlorothalonil (technical grade, purity >98%, Chemservice, West Chester, PA) dissolved in 50mL of acetone so that nominal concentrations in the tanks were either the EEC (164µg/L) or twice the EEC. Tanks were dosed the same day as the amphibians were added and targeted nominal concentrations closely matched the

actual concentrations (1x: 172 and 2x: 351µg/L; spiked recovery efficiencies: 95%). Thus, for simplicity and consistency across the experiments, we refer to the nominal concentrations throughout this paper. Several water quality and chemistry variables were quantified at various times during the experiment (See Supplemental Methods; Table S3 &S4). Standardized dip net sampling of each tank was conducted the third day of the experiment to quantify any rapid mortality associated with chlorothalonil exposure. The number of metamorphosed frogs was noted daily and tadpole survival was determined 5wk after dosing.

Laboratory Experiment I

We obtained *H. squirella* and *O. septentrionalis* from multiple, thoroughly mixed clutches collected from two adjacent retention ponds in Tampa, FL (July, 2008; 28°0.322N 82°19532W). We employed a completely randomized design with 21 32-L glass aquaria, each filled with 10L of artificial spring water (ASW; Cohen et al. 1980)(HACH Co.: 5B Hardness Test Kit: hardness: 62.7ppm, pH~7.0) and maintained in a laboratory at the University of South Florida at 27°C and on a 14:10 light:dark cycle. Each aquarium received five *H. squirella* and 15 *O. septentrionalis* tadpoles (Gosner stages 25-28; Gosner 1960) and tadpoles were fed boiled organic spinach daily. There were five technical grade (purity >98%, Chemservice, West Chester, PA) chlorothalonil treatments (actual concentrations: 0.176, 1.76, 17.6, 176, and 1760µg/L), and two controls (water and solvent: 500 ng/L acetone) with three replicates per treatment. The targeted nominal concentration for the chlorothalonil stock was 1640µg/L, and the actual concentration was 1760µg/L (spiked recovery efficiencies: 95%). All of the other concentrations were attained through serial dilutions of this stock solution. Again, for simplicity and consistency across the experiments, we refer to the nominal concentrations. We quantified frog survival and preserved dead tadpoles 12h after the start of the experiment and then every 24h for 4d (there were no water

changes). Surviving tadpoles were euthanatized and preserved (70% ethanol) at the end of the experiment.

Laboratory Experiment II

The same protocols used in *Laboratory Experiment I* were used in this experiment, conducted in October 2008, with the following exceptions. We tested three tadpole species: *R. sphenocephala*, *O. septentrionalis*, and *H. cinerea* (all starting at Gosner stage 25). We employed a completely randomized design with 144 500-mL mason jars, each filled with 300mL of ASW and each receiving three tadpoles of a single species. Species were isolated in this experiment because *O. septentrionalis* was occasionally observed depredating *H. squirella* in *Laboratory Experiment I*. The jars received one of six chlorothalonil treatments: 0.0164, 0.164, 1.64, 16.4, 82.0, or 164µg/L, or water or solvent controls. We used the same stock solution as used in *Laboratory Experiment I*. A single water change occurred at day seven and each jar was re-dosed at that time. There were six replicates per species per treatment. The number of surviving tadpoles was noted after 4hrs, 24hrs and then every 24hrs for 10d, and all dead tadpoles were removed and preserved in formalin at those times.

To quantify the effects of chlorothalonil on tadpole livers and immune cells, one arbitrarily selected *O. septentrionalis* from each replicate at the end of the experiment was euthanatized, embedded in paraffin wax, sectioned, and stained with hematoxylin and eosin (H&E). We used *O. septentrionalis* for liver, immune, and corticosterone (see below) quantification because it had the lowest mortality of the three species and thus offered us the most survivors/tissue. To test whether chlorothalonil exposure affected liver tissue integrity, we used ImageJ software to calculate liver tissue density. We followed ImageJ's "Quantifying Stained Liver Tissue" document (Burger and Burge 2009), which reports the density of stained tissue within a designated area. To test whether

chlorothalonil exposure affected liver immune cell densities, we counted the number of melanomacrophages and granulocytes per field of view at 400x magnification. Melanomacrophages and granulocytes are leukocytes that help defend against a variety of parasites (Rohr et al. 2008b). Due to the morphological similarity among granulocytes, we conservatively categorized all granule containing immune cells as granulocytes, but most were likely eosinophils.

Corticosterone Experiment

We used *O. septentrionalis* (Gosner stages 25-28; same population as used in *Laboratory Experiment II*) tadpoles to quantify the effect of chlorothalonil exposure on frog corticosterone levels, a steroid hormone elevated in response to natural and anthropogenic stressors, including pesticides (Martin et al. 2010). We used the same general protocols as described in *Laboratory Experiment II* and had the following treatments: 0.164, 16.4, 82, and 164µg/L of chlorothalonil and water and solvent controls. These treatments were crossed with one of three chlorothalonil exposure durations: 4, 28, or 100h (n=3, 2, 3, respectively). The exception, however, was that tadpoles exposed to 164µg/L of chlorothalonil were only exposed for 4h because they do not survive for 28 or 100h of exposure. This design resulted in 43 independent replicates. After the appropriate exposure duration, tadpoles were euthanized, and individual tadpoles were weighed (to 0.0001g) and homogenized in ultrapure water. Tritiated corticosterone (2000cpm) was then added to each sample to quantify recoveries post-extraction and a corticosterone EIA kit was used to quantify hormone levels in each sample (Assay Designs: cat# 900-097, Ann Arbor, Michigan). Individual recoveries (mean: 55.3%) and tadpole mass measurements were used to estimate corticosterone per gram of tadpole tissue. Detailed methods for this EIA kit and a discussion of its potential limitations are provided in the Supplemental Methods.

Statistical Analyses

For all experiments and responses, we tested for a difference between the water and solvent controls. There was never a difference between these treatments (P>0.328), so they were pooled into one "control" treatment for all subsequent analyses.

For the *Mesocosm Experiment*, all analyses were conducted on the arcsine-square-root-transformed proportion of *R. sphenocephala* and *O. septentrionalis* surviving to the end of the experiment, controlling for the four spatial blocks. We tested whether chlorothalonil was associated with mortality relative to the control treatments by conducting a permutation-based multivariate regression analysis. For the laboratory experiment, we arcsine-square-root-transformed the proportion of tadpoles surviving until the end of the experiment, and log-transformed hours to death, mass of survivors, amount of liver damage, and melanomacrophage and granulocyte counts to meet parametric assumptions. For the liver and immune analyses, we log-transformed chlorothalonil concentration and weighted the time to death analyses by the number of animals that died per replicate. If a dose-response appeared linear, chlorothalonil concentration was treated as a continuous predictor in a regression model (liver density). If a dose-response was non-linear but relatively simple (one inflection point), chlorothalonil concentration was treated as a continuous predictor and we used polynomial regression with Type II sums of squares to fit the data (immune responses). If a response was non-linear and relatively complex (more than one apparent inflection point), chlorothalonil concentrations were treated as levels of a categorical predictor followed by Fisher's least significant difference (LSD) multiple comparison test to determine which levels were different from one another (proportion of tadpoles that survived and time to death). As an additional test for non-monotonicity (hump-shaped dose-response), we eliminated the highest concentrations, which typically caused considerable mortality, and used polynomial regression to test for a quadratic dose-response relationship with the remaining concentrations. For the immune

responses, we conducted a multivariate polynomial regression model with melanomacrophages and granulocytes as responses and followed it by univariate analyses on each response variable. We log-log transformed these relationships to improve fit and meet the assumption of the polynomial regression.

For the *Corticosterone Experiment*, we conducted polynomial regression (using least trimmed squares) with chlorothalonil concentration as a continuous predictor and log-transformed corticosterone as the response variable. All statistical analyses were conducted with Statistica v8.0 (Statsoft, Tulsa, OK). We did not calculate LC_{50} values for any responses in this paper, because all three dose-response experiments showed evidence of non-monotonicity which would violate the assumptions of LC_{50} calculations.

RESULTS

Mesocosm Experiment

The multivariate permutation test revealed a positive association between chlorothalonil concentration and amphibian mortality (P=0.005), with controls having less mortality than both the 164 µg/L (P=0.013) and 338µg/L chlorothalonil treatments (P=0.023; Figure 1). Chlorothalonil concentration was positively associated with the mortality of both *O. septentrionalis* (P=0.001) and *R. sphenocephala* (P=0.064; Figure 1).

An average of 1.5 (±1SE:0.327) live tadpoles were captured per dip netting session in control tanks, but no live tadpoles were netted from chlorothalonil tanks, which were the only tanks where dead tadpoles were netted. These results suggest that most of the mortality associated with chlorothalonil occurred within the first 72h of exposure.

Laboratory Experiment I

Survival was lower for *H. squirella* than for *O. septentrionalis*, most likely because *O. septentrionalis* was observed depredating *H. squirella* (Figure 2A). Despite this predation, time to death for *H. squirella* was shorter for tadpoles exposed to any chlorothalonil concentration relative to controls (LSD: P<0.023 for controls versus any chlorothalonil concentration, see Supplementary Materials for full ANCOVA results; Figure 2B).

For *O. septentrionalis*, survival was non-monotonic with low and high concentrations causing significantly greater mortality than intermediate concentrations and controls (Fig. 2A). Relative to controls, survival was reduced by >80% in the 0.164, 17.6, 164, and 1640µg/L concentrations, but survival was not significantly reduced by 1.64µg/L of chlorothalonil and this concentration was significantly different from both adjacent concentrations (Figure 2A). This non-monotonicity was also supported by polynomial regression, which produced a significant quadratic term for concentrations <16.4µg/L (see Supplementary Materials for statistics). Relative to controls, time to death was shorter for *O. septentrionalis* tadpoles exposed to any chlorothalonil concentration (LSD: P<0.021 for 0µg/L vs 0.164, 1.64, 164, or 1640µg/L; Figure 2B) with the exception of 16.4µg/L (LSD: P=0.190; Figure 2B).

Laboratory Experiment II

For each species, the 164µg/L killed 100% of the tadpoles by the end of the experiment (Figure 2C; see Figure S1 for mortality through time and Supplementary Materials for full statistical results). There was, however, considerable variation among species in their sensitivity to chlorothalonil. *Rana sphenocephala* appeared most sensitive, experiencing 86% mortality at 0.164µg/L and 100% mortality at 82µg/L (Figure 2C), whereas *O. septentrionalis* was least sensitive (Figure 2A).

The dose response for survival was significantly non-monotonic for *R. sphenocephala* and *H. cinerea*, with low and high concentrations causing significantly greater mortality than intermediate concentrations and controls (Figure 2C), a result similar to the non-monotonic dose response revealed in *Laboratory Experiment I* for *O. septentrionalis*. For *R. sphenocephala*, 0.164µg/L caused significantly more mortality than each adjacent concentration and there was a significant quadratic term for the dose response <82µg/L. For *H. cinerea*, 0.0164µg/L caused significantly more mortality than each adjacent concentration and, like for *R. sphenocephala*, there was a significant quadratic term for the dose response (<16.4µg/L; see Supplementary Materials for polynomial results for both species). As a reminder, each data point in Figure 2C is the mean of six data points and thus the 0.0164µg/L concentration for *H. cinerea* is not an outlier or artifact.

Osteopilus septentrionalis did not exhibit a non-monotonic response in this experiment as it did in the previous experiment (Figure 2A,C). This is possibly due to differences in tadpole densities, developmental stages, source populations, or bioaccumulation of chlorothalonil associated with *O. septentrionalis* depredating *H. squirella* in the first laboratory experiment. Chlorothalonil has been documented to bioaccumulate up to 3,000x in fish (Cox 1997; USEPA 1999).

Increasing chlorothalonil concentrations was associated with a significant decrease in liver density of *O. septentrionalis* ($F_{1,40}$=4.82, P=0.034, Figure 3A, Figure S2). Chlorothalonil concentration was also associated quadratically with both melanomacrophages and granulocytes in this species (see Supplementary Materials for statistics; Figure 3B). That is, relative to controls, tadpoles exposed to low concentrations had fewer of these immune cells, whereas tadpoles exposed to high concentrations had elevated numbers of these cells (Figure 3B). There was considerable mortality at the 82and 164µg/L concentrations that may have confounded our immune results and that might explain the increase in immune cells at these concentrations. Thus, we reanalyzed the dose response excluding these two highest concentrations and discovered that, at these lower and

more environmentally common concentrations, chlorothalonil was associated with a reduction in both melanomacrophages (Chlorothalonil: $F_{1,32}$=4.67 P=0.038) and granulocytes (Chlorothalonil: $F_{1,32}$=5.52 P=0.025; Figure 3B).

Corticosterone Experiment

Corticosterone levels increased significantly with chlorothalonil exposure duration ($F_{1,27}$=11.57, P=0.002). After 4h exposure to chlorothalonil, the relationship between log corticosterone levels and chlorothalonil concentration was significantly nonlinear (concentration^3: $F_{1,11}$=6.12, P=0.031), with low and high concentrations of chlorothalonil being associated with higher levels of corticosterone than intermediate concentrations and controls (Figure 4). Multiple comparisons tests further supported the conclusion that this dose-response curve was significantly nonlinear (Figure 4). This same general pattern persisted for up to 100h of exposure, but tadpoles were not available after the 4h exposure duration for 164µg/L because of high mortality (Figure 4). As a reminder, this study was conducted on the *O. septentrionalis* population that did not exhibit any significant non-monotonic mortality response to chlorothalonil and only exhibited significant mortality at concentrations ≥82µg/L (Fig. 2C).

DISCUSSION

Ultimately, scientists should use a weight of evidence approach to evaluate risk posed by chemicals, which is partly why we conducted four experiments to quantify the effects of chlorothalonil on amphibians: a contrived, but highly controlled, laboratory experiment (*Laboratory Experiment II*), a more ecologically relevant laboratory experiment where species were allowed to interact (*Laboratory Experiment I*), a laboratory experiment to assess whether corticosterone levels exhibited a dose response similar to that for mortality (*Corticosterone Experiment*), and a field

mesocosm experiment with a complex freshwater community (*Mesocosm Experiment*). In all these experiments, we found adverse effects of chlorothalonil on tadpoles. Although in *Laboratory Experiment I* we had low survival of *H. squirella* in the control treatment, possibly because of depredation by *O. septentrionalis*, these species regularly coexist making this interaction ecologically relevant. This experiment also reinforced the significant lethality of the EEC and lower concentrations of chlorothalonil, and provided the first indication of a non-monotonic dose-mortality response for this pesticide (Figure 2A). We conducted a follow-up experiment using three amphibian species, this time preventing heterospecific interactions. This experiment had 80-100% survival of the control tadpoles, simplifying data interpretation. It revealed that all three species were highly susceptible to chlorothalonil with the EEC causing 100% mortality of each species in <10h of exposure. Moreover, in this experiment, there was evidence of non-monotonic dose responses for mortality and full-body measurements of corticosterone, with low and high levels elevating both responses. Finally, in our mesocosm study, both the 164 and 328µg/L significantly reduced amphibian survival, suggesting that the laboratory results might be relevant to effects in nature. Together, these four experiments indicate that amphibians, in general, are susceptible to the EEC of chlorothalonil and that even low concentrations can cause amphibian mortality and physiological stress responses.

Our finding, that amphibians are sensitive to chlorothalonil, is consistent with studies examining the sensitivity of aquatic vertebrates and invertebrates to chlorothalonil. For several fish species, 48- and 96-h LC_{50} values are below 20µg/L and LOECs are near 1µg/L of chlorothalonil (Caux et al. 1996). The 48hr LC_{50} for *Bufo bufo japonicas* was 160µg/L (Hashimoto and Nishiuchi 1981). *Daphnia magna* had delayed reproduction when exposed to 32µg/L(Ernst, 1991) and ≥6.5µg/L of chlorothalonil decreased the number of eggs per spawn, egg hatchability, and fry survival of fathead minnows (as cited in Grabusky et al. 2004). The LOEC for *H. cinerea* and *R.*

sphenocephala survival in our study was 10,000x less than the EEC (0.0164µg/L; see Figure 2C) and was the lowest concentration we tested. Hence, we did not test low enough concentrations to detect a no observable effect concentration for the survival of these two species.

Three out of four amphibian species showed evidence of a non-monotonic dose-mortality response to chlorothalonil (*O. septentrionalis*: Fig. 2A; *H. cinerea and R. sphenocephala* Fig. 2C), with low and high levels causing significantly greater mortality than intermediate levels and controls. Furthermore, for all species and experiments, the low-dose increase in mortality occurred within a single order of magnitude (either 0.016 or 0.16µg/L). Although the non-monotonic dose-response for survival was only observed for *O. septentrionalis* in one of the two experiments (these experiments used different conditions and source populations), in the experiment where *O. septentrionalis* did not exhibit a non-monotonic dose response for survival, it did exhibit a non-monotonic dose response for corticosterone. Hence, the non-monotonic response was consistent and reproducible both within and across species, but whether low-dose exposure to chlorothalonil and the associated stress response cause mortality appears to be context dependent. Non-monotonic responses are important because they defy the traditional toxicological assumption that higher concentrations of a contaminant always cause greater harm. Non-monotonic patterns have been observed previously in response to chlorothalonil (Shelley et al. 2009) and other agrochemicals (Storrs and Kiesecker 2004). Non-monotonic responses can be caused by multiple mechanisms affecting responses differently at different doses, or by endocrine disruption (Welshons et al. 2003). Indeed, the Canadian Wildlife Service concluded that chlorothalonil might qualify as an endocrine disruptor because it has the potential to interfere with endogenous hormones and enzymes and is an immunomodulator (Grabusky et al. 2004). However, the mechanism or mechanisms underlying non-monotonic dose-responses in this study remain unknown.

In addition to mortality, chlorothalonil was associated with immunomodulation of the surviving *O. septentrionalis* tadpoles. This finding is consistent with DNA damage to mononuclear leukocytes of farmers 1d after spraying chlorothalonil (Lebailly et al. 1997) and with studies of chlorothalonil-induced immunosuppression of fish and marine invertebrates (Baier-Anderson and Anderson 2000). Increases in chlorothalonil concentration up to 17.6µg/L, concentrations to which humans are commonly exposed (Daly et al. 2007), were associated with reduced liver granulocytes and melanomacrophages in tadpoles, whereas further increases in chlorothalonil caused increased numbers of liver granulocytes and melanomacrophages (Figure 3b). This increase in immune cells might be in response to chlorothalonil-induced liver damage, based on our observations of decreased *O. septentrionalis* liver density at these higher concentrations (Figure S2). Alternatively, the increase in immune cells might itself have contributed to liver damage, since high levels of melanomacrophages and granulocytes can cause tissue damage (Rose et al. 1999). Though not yet studied, it is possible that exposure to chlorothalonil could reduce tolerance and resistance to parasites, which has been shown for wildlife and humans exposed to other agrochemicals (Dietert et al. 2010; Rohr et al. 2008b). If so, this could further reduce tadpole survivorship.

To our knowledge, we provide the first evidence that chlorothalonil elevates corticosterone. The significant non-monotonic dose response of corticosterone to chlorothalonil was qualitatively similar to the mortality responses also observed in this study, underlining the consistent presence of non-monotonic responses to this chemical. However, we do not know the direction of causation. Approaching mortality could have resulted in a systemic stress response that altered corticosterone and immune parameters, changes in corticosterone and immune parameters could have caused the mortality, or both of these scenarios could have occurred. Mortality at the highest concentrations of chlorothalonil seemed to occur too quickly to be mediated by corticosterone. However, it is plausible that corticosterone could have been involved in the mortality and immune cell changes

observed at low concentrations of chlorothalonil. First, corticosterone is known to cause elevations in circulating granulocytes in other animals (Davis et al. 2008), either by inducing proliferation or efflux from cell reservoirs. Second, continuously elevated corticosterone has manifold negative effects on health, including muscle atrophy, reduced neurogenesis, and immune suppression or dysregulation (Martin 2009). Lastly, glucocorticoids, including corticosterone, are commonly elevated in response to stressors, natural and anthropogenic (Martin et al. 2010), and even in cases where elevations are insufficient to cause mortality, they can generally compromise health, even in humans (Wingfield and Sapolsky 2003). Although we cannot say with certainty whether the immunological effects observed in this study were mediated by corticosterone, we strongly advocate future efforts to assess the role of chlorothalonil and glucocorticoids as potential endocrine disruptors, especially as disruptors of the immune system and disease resistance.

Although pesticides have been suggested as a cause of amphibian declines, there are few convincing cases in which pesticides cause high enough mortality at environmentally realistic concentrations to directly affect amphibian populations (Belden et al. 2010; Rohr et al. 2006b). Sometimes even high mortality of larval amphibians can have little observable effect on the population because of density-mediated compensation, in which survivors of a factor experience lower mortality than control animals after the stressor is removed because of less competition for resources (Rohr et al. 2006b). However, based on amphibian demographic models that incorporate negative density dependence (Vonesh and De la Cruz 2002), the level of EEC-induced mortality reported here would likely reduce amphibian population sizes. Given that chlorothalonil caused nearly 100% mortality at the EEC, caused significant mortality four orders of magnitude below the EEC, and caused immunomodulation in surviving individuals, exposure to this chemical has the potential to both directly and indirectly cause amphibian declines. Indeed, frog die-offs have been reported after chlorothalonil applications to cranberry bogs (Winkler et al. 1996) and, in neotropical montane regions where amphibians are declining, chlorothalonil has been regularly detected at levels causing significant mortality in this study (Daly et al. 2007). This makes chlorothalonil a plausible contributor to declines, though additional work is needed to demonstrate a causal link. Given these findings and similarities between the vital systems of amphibians and humans, we

encourage future studies to quantify the effects of chlorothalonil on amphibian populations and human health.

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Figure Legends

Figure 1. Survival of tadpoles in the mesocosm experiment. Number of *Osteopilus septentrionalis* and *Rana sphenocephala* surviving when exposed to two measured concentrations of chlorothalonil (164µg/L ~ EEC [expected environmental concentration]; 328µg/L ~ 2x EEC, single pulse) relative to the controls (water and solvent presented together). Both species had 0% survival at 328µg/L, which is why it appears as though there is only one symbol. Shown are the means ±1 SE. Refer to text for sample sizes.

Figure 2. Survival of tadpoles in the laboratory experiments. Survival (A) and time to death (B) of *Osteopilus septentrionalis* (15 tadpoles/tank) and *Hyla squirella* (5 tadpoles/tank) exposed to several concentrations of chlorothalonil (0.164, 1.64, 16.4, 164, and 1640µg/L) and controls (water and solvent presented together) for *Laboratory Experiment I.* (C) Survival of *Osteopilus septentrionalis*, *Hyla cinerea* and *Rana sphenocephala* exposed to several concentrations of chlorothalonil (0.0164, 0.164, 1.64, 16.4, 82.0, and 164µg/L) and controls (water and solvent presented together) for *Laboratory Experiment II.* Shown are the means (±SE) in log (µg/L+0.001); in panels A and B, n=3 for all chlorothalonil concentrations and n=6 for the control. In panel C, n=6 for all chlorothalonil concentrations and n=12 for the control. Different lowercase letters above points indicate that responses for a given species were significantly different (P<0.05) among treatment levels according to Fisher's least significant difference multiple comparison tests.

Figure 3. Effect of chlorothalonil on tadpole liver health and immunity. Density of liver tissue (A) and melanomacrophages and granulocytes in the liver (B) of *Osteopilus septentrionalis* tadpoles exposed to several concentrations of chlorothalonil (0.0164, 0.164, 1.64, 16.4, 82.0, and 164µg/L) and controls (water and solvent presented together); and sample sizes for the 0, 0.164, 1.64, 16.4, 82.0, and 164µg/L concentrations are 9, 4, 6, 5, 5, 3, and 6 respectively. Shown are the means (±SE) and best-fit lines or curve.

Figure 4. Effects of chlorothalonil on corticosterone per gram of *Osteopilus septentrionalis* tissue. Shown are least squares means (±1SE). Means were averaged across the three chlorothalonil exposure durations (4, 28, and 100h), except for the 164µg/L concentration, where only the 4h duration mean is shown because longer exposure kills the tadpoles. Also shown is the significant third-order polynomial function ($y=1.886571+0.035582x-0.000668x^2+0.000003x^3$) for the relationship between chlorothalonil concentration and log corticosterone adjusted for the effect of exposure duration. The corticosterone level for the 164µg/L concentration is underestimated because it is the only mean based on four, rather than an average of 44, hours of chlorothalonil exposure and corticosterone increased significantly and log-linearly with the duration of chlorothalonil exposure (coefficient for log exposure duration=0.269). Concentrations with different lower case letters are significantly different from one another based on a Fisher's LSD multiple comparison test, and sample sizes for the 0, 0.164, 16.4, 82.0, and 164µg/L concentrations are 13, 5, 7, 6, and 2, respectively.

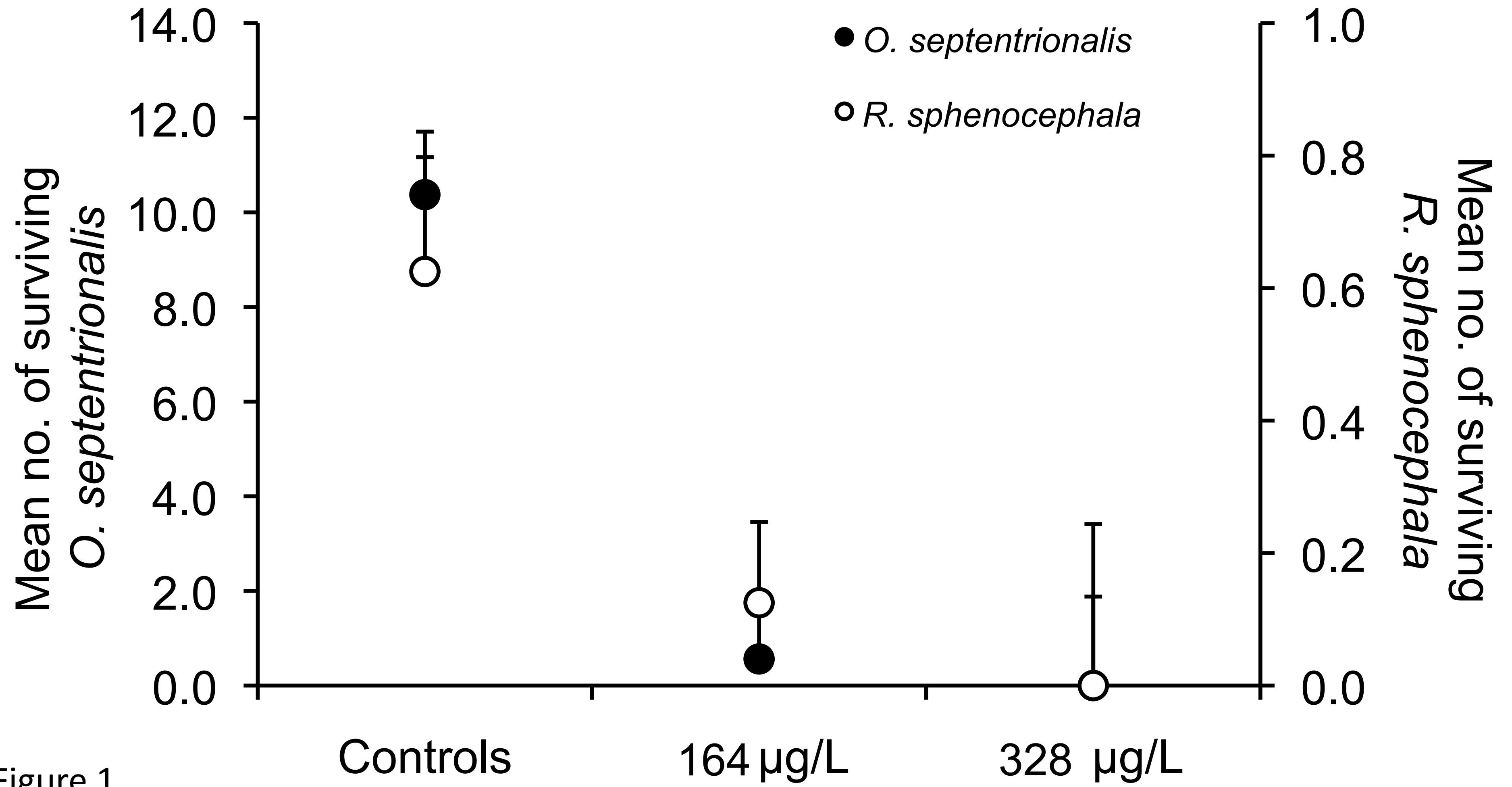

Figure 1

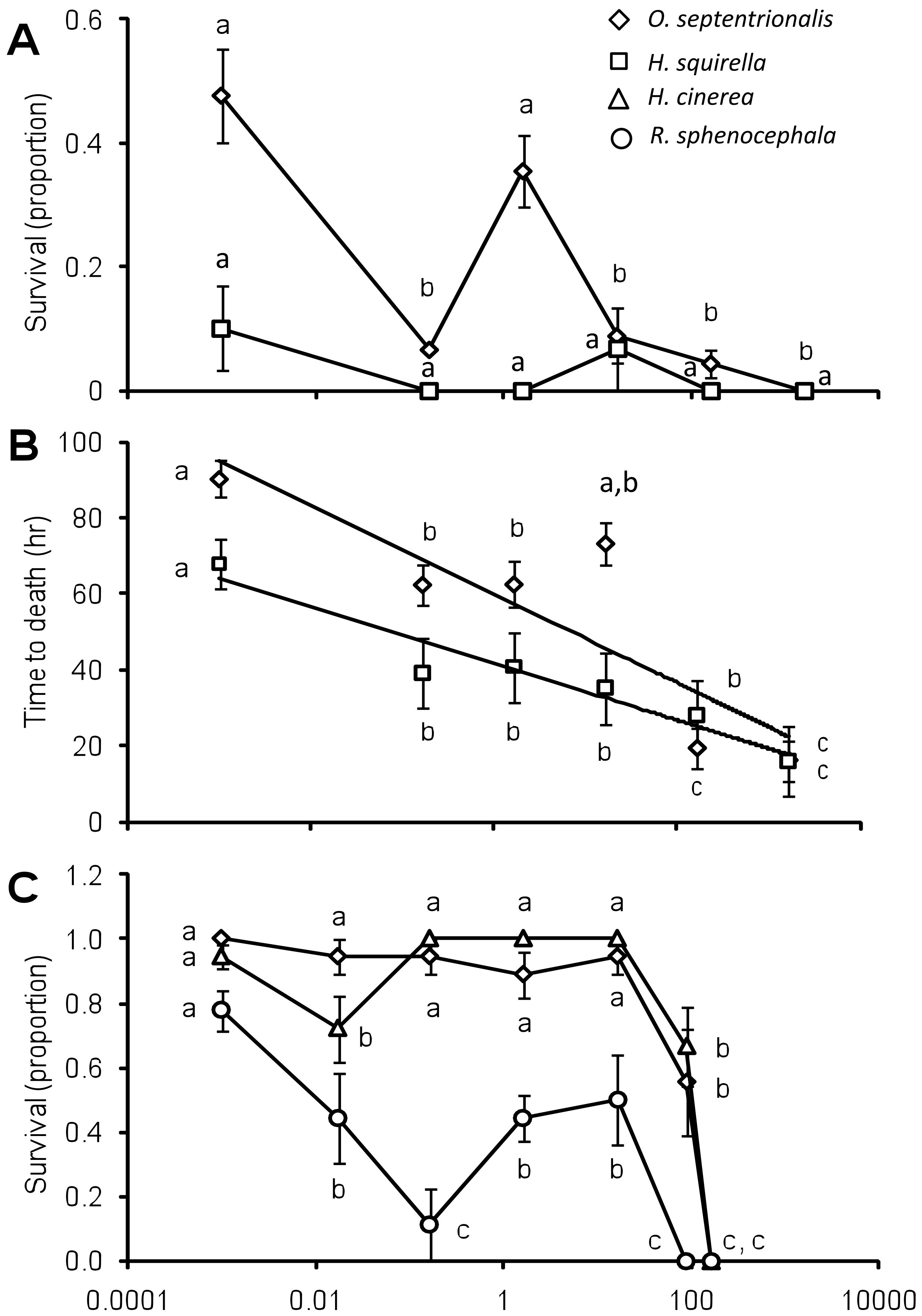

Figure 2. Chlorothalonil log (μg/L + 0.001)

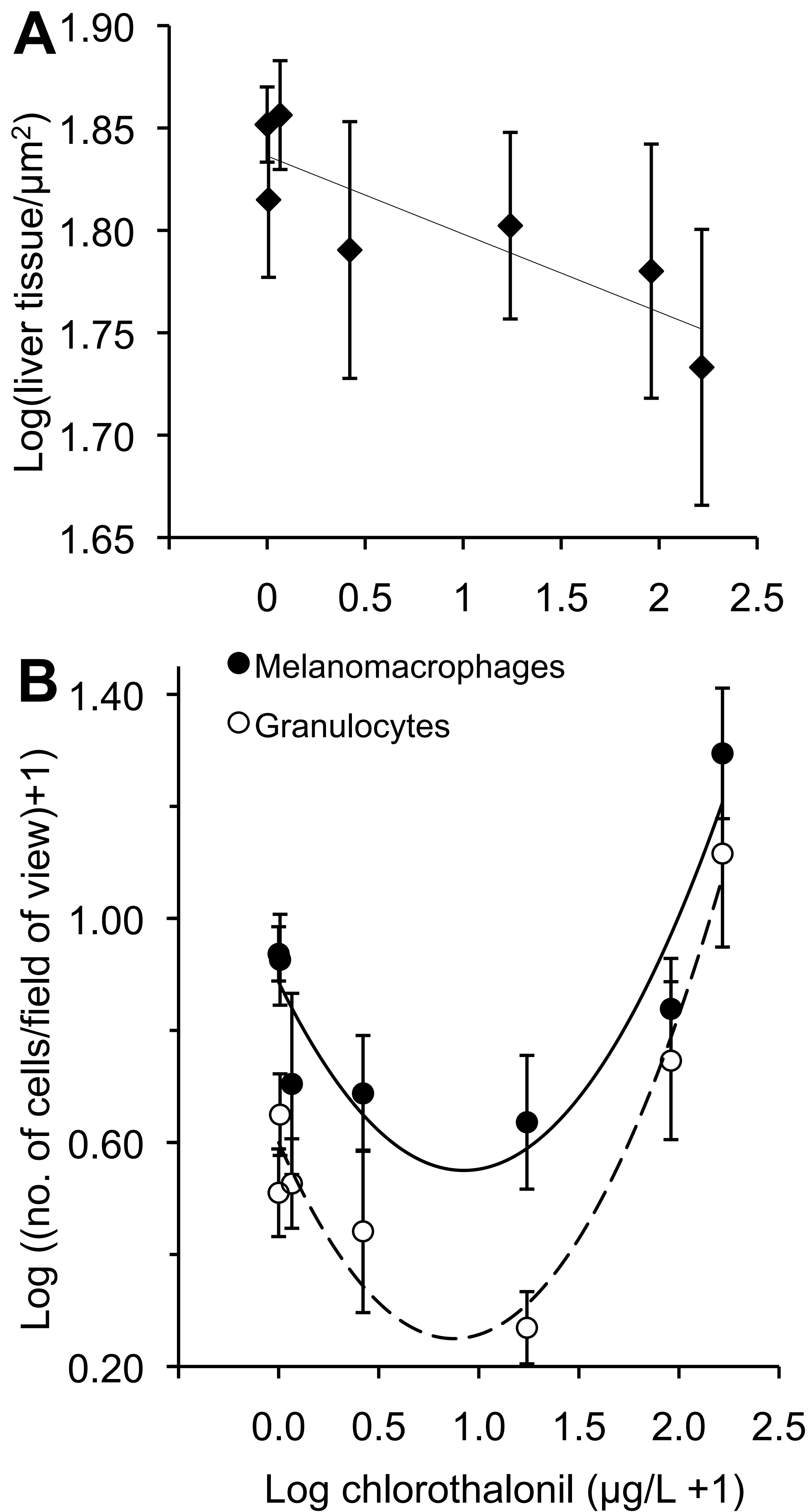

Figure 3

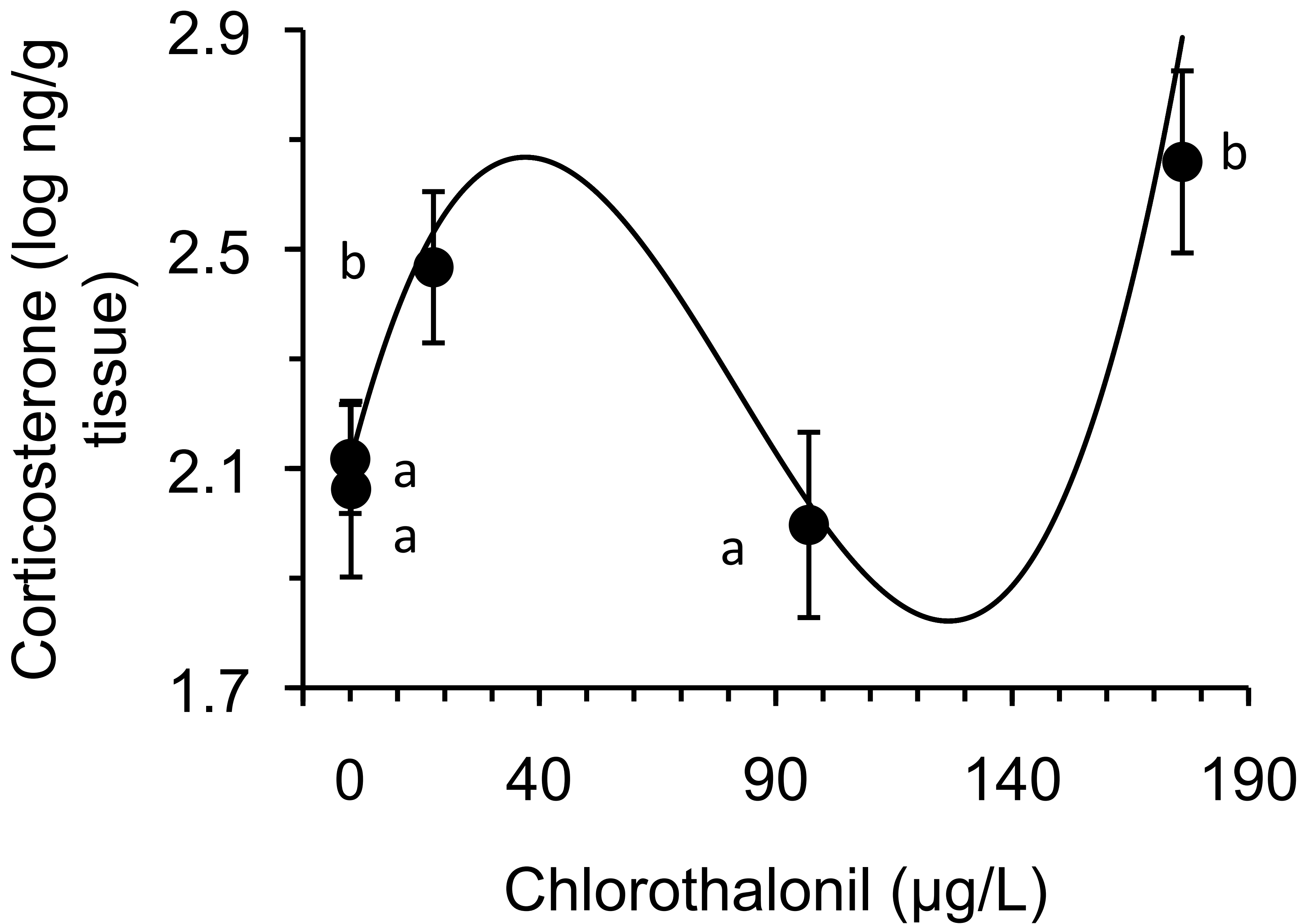

Figure 4