INVESTIGATING THE EVOLUTIONARY ORIGINS AND MECHANISMS UNDERLYING XENOBIOTIC SENSITIVITY USING RESURRECTION ECOLOGY

By

ADAM MARTIN SIMPSON

Bachelor of Science Zoology
Oklahoma State University
Stillwater, OK
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Dissertation Approved:

Dr. Jason Belden

Dissertation Adviser

Dr. Punidan Jeyasingh

Dr. Polly Campbell

Dr. Janette Steets

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Name: ADAM MARTIN SIMPSON

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Abstract:

Sensitivity to an organophosphate insecticide, chlorpyrifos, was evaluated in a resurrected population of *Daphnia pulicaria*; this population originated from a lake that has experienced over a century of cultural eutrophication. Acute toxicity tests demonstrated that ancient clones (1301-1646 A.D.) were 2.7x more sensitive to this chemical when compared to the contemporary clones (1967-1977, 2002-2008 A.D.). Such changes might have arisen from historical exposure to chlorpyrifos, other organic contaminants, or by selection driven by stressors associated with cultural eutrophication.

To determine the physiological mechanisms of tolerance, a series of biochemical assays was performed on the three most tolerant and three most sensitive genotypes from the population, resurrected from 1967-1977 and 1301-1646 A.D., respectively. *In vitro* acetylcholinesterase assays were conducted to examine the potential for target-site tolerance, but yielded no significant differences in constitutive enzyme activity or sensitivity to inhibition. To evaluate the potential for metabolic tolerance, acute toxicity tests were conducted using *i*) the toxic metabolite chlorpyrifos-oxon (CPF-O) and *ii*) CPF-O co-applied with piperonyl butoxide (PBO), a known inhibitor of Phase-I biotransformation. Both series of toxicity tests reduced the mean variation in sensitivity between tolerant and sensitive genotypes. Most significantly, exposure to CPF-O and PBO reduced the disparity between the clones from a 4.7-fold to 1.2-fold difference in sensitivity. This strongly suggested that pathways involving Phase-I detoxification and/or bioactivation of chlorpyrifos play a significant role in dictating tolerance in this population.

An *in vivo* assay was developed to quantify biotransformation of metabolic substrates in invertebrates via gas chromatography/mass spectroscopy. The assay was highly successful for *Physa* snails, but was unable to detect activity in response to most substrates in *Daphnia magna*. The assay shows promise, but further optimization is needed to effectively evaluate xenobiotic metabolism in *Daphnia*.

Finally, the evolutionary origins of shifts in xenobiotic sensitivity are discussed. The role of metabolism in dictating sensitivity to natural and anthropogenic stressors suggests that adaptive shifts in response to one stressor might indirectly influence susceptibility to others. This phenomenon could have important implications for the success of wild populations facing rapid environmental change.

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INTRODUCTION

RESURRECTION ECOLOGY AND ITS UTILITY IN ECOTOXICOLOGY

The following chapter appears as published in *Integrated Environmental Assessment and Management*:

Simpson, A.M., P.D. Jeyasingh, and J.B. Belden (2017). "Resurrection ecology and its utility in ecotoxicology." Integrated Environmental Assessment and Management. **13**(4): 790-792.

The central goal of ecotoxicology is to understand how natural populations and communities respond to contaminants in the environment. Current research in ecotoxicology builds upon decades of work describing the toxicity of natural and anthropogenic contaminants to non-target species. From this knowledge base, it is understood that populations harbor variation in sensitivity to contaminants; however, it is often difficult to empirically investigate the evolutionary forces that act on this variation.

The most common approach to characterize population-level variation involves a comparison between multiple populations across a spatial scale. Such a study usually samples populations from several contaminated and uncontaminated (i.e., reference) sites. If significant variation in sensitivity is detected among populations, then one can readily evaluate the physiological underpinnings of this variation. Spatial comparisons, however, are

often forced to assume the evolutionary histories of their study systems. Specifically, it is necessary to assume that the mean phenotype of the reference population is reflective of the ancestral state of populations from contaminated sites. The significance of relying on this assumption is of course dependent upon the aim of the research and the study system, and does not necessarily detract from its impact. It is important, however, to be cognizant of the potential implications of this assumption when addressing evolutionary questions.

Clearly, the best way to determine the validity of ancestral state (i.e., evolutionary history) assumptions is to empirically examine changes in sensitivity across a temporal scale; however, it is exceedingly difficult to design such an experiment unless long-term environmental and genetic data are available for the system of interest. The life histories of certain plants and animals enable rigorous temporal investigations. Such organisms utilize a dormant life stage, wherein preserved embryos enter diapause and can remain viable indefinitely. This reproductive strategy functionally preserves the population's genetic information within seed (Franks et al. 2008) or egg banks (Brendonck and De Meester 2003).

Resurrection ecology is a field that hatches dormant embryos of these unique organisms to address novel ecological and evolutionary questions (Kerfoot et al. 1999; see Orsini et al. 2013 for a recent review). Due to the resiliency of these dormant eggs, it is possible to successfully hatch and manipulate living representatives of centuries-old populations (Frisch et al. 2014). As a consequence, this approach enables one to: empirically evaluate ancestral phenotypes; and, develop informed hypotheses about the ancestral environment (**Figure 1**). Our perspective describes the approach using examples from a familiar model system within ecotoxicology.

Class Branchiopoda comprises many macro- and micro-crustacean species that are distributed worldwide. Among this sizeable class of invertebrates, cladocerans (e.g., *Daphnia* spp.) are the best studied. These organisms are frequently used in ecotoxicological research. *Daphnia* thrive in laboratory conditions, are sensitive to most environmental contaminants, and commonly reproduce via cyclical parthenogenesis. The primary benefit of an asexual organism is the ability to maintain a clonal lineage indefinitely. Manipulations using clonal replicates of a genetically distinct individual can definitively elucidate links between genotype and phenotype (Shaw et al. 2008).

While daphniids produce genetically identical daughters via parthenogenesis under optimal conditions, females of most species are also capable of producing males when environmental conditions are poor. In these stressful situations, sexual reproduction is adaptive because genetic diversity increases as a result of sexual recombination; and, the embryos are encapsulated within a protective egg case (ephippium) that allows them to recolonize a habitat (e.g., overwintering). The embryos remain within the ephippium until environmental conditions improve, after which some of the embryos hatch, allowing the population to persist. The majority of these embryos sink to the sediments of the water body, where they become buried over a period of time. Buried beneath the sediment, the embryos are not exposed to the environmental triggers for hatching, and thus remain in diapause.

Studies in resurrection ecology isolate these egg banks by coring the sediments of lakes and ponds. Within these cores one can often find preserved ephippia, from which dormant embryos can be extracted. In systems where there is negligible sediment turnover or disturbance (e.g., glacial lakes), it is also possible to acquire a conservative age estimate of the individuals that were found in each sediment layer using dating techniques (e.g., ^{137}C or

^{210}Pb; Kerfoot et al. 1999). The embryos can be hatched by extended exposure to light and/or freeze-thaw cycles; these treatments simulate the cues that would trigger hatching in their natural environment.

If hatching is successful, the female neonate can be grown in the laboratory until it produces parthenogenetic clones, at which point the clonal line can be cultured indefinitely. Once cultures of several individuals hatched from multiple sediment layers have been established, phenotypic assays can be performed on different genotypes. Consequently, it is possible to experimentally compare the phenotypes expressed by ancestral and contemporary representatives of a population following exposure to historical or novel contaminants.

In addition to acquiring valuable information about ancestral phenotypes, resurrection ecology practices can also inform speculations about the ancestral environment. After sieving sediment layers for preserved resting eggs, it is common practice to quantify a number of environmental variables within the temporally explicit layers following standard paleolimnological protocols (Kerfoot et al. 1999). Variables such as nutrient composition or contaminant concentration can yield information about the ancestral environment. When combined with phenotypic data, estimates of ancestral environmental conditions permit the detection of relationships between changes in the environment and changes in phenotype.

Several resurrection ecology models have been used successfully in ecotoxicological studies. One such model involved *Daphnia pulicaria* resting eggs, which were hatched from ephippia found within lake sediment cores (Frisch et al. 2014). This lake has a history of cultural eutrophication, evidenced by a drastic increase in sediment phosphorus concurrent with European colonization of the area. Simpson et al. (2015) used this system to evaluate how susceptibility to an insecticide has changed over time in this population. They tested

Daphnia clones resurrected from three time points: one pre-colonization (1301-1646), and two post-colonization (1967-1977, 2002-2008). They found that the pre-colonization clones were significantly less tolerant to the insecticide than post-colonization clones (particularly 1967-1977), indicating a shift in sensitivity in response to a strong selection pressure (e.g., pesticide exposure or eutrophication).

Turko et al. (2016) established a unique system involving zooplankton resting eggs found within the sediment of two lakes with historical lead pollution. *Daphnia galeata* clones were hatched from eggs representing three distinct time periods: 1977-1981 (intense lead exposure), 1989-1992 (moderate lead exposure), and 2001-2004 (low lead exposure). They discovered that the individuals hatched from the intense exposure period exhibited significantly higher growth rate and survivorship when subjected to lead contamination than the more contemporary representatives. Consequently, Turko et al. (2016) were able to experimentally demonstrate the progressive loss of resistance over several decades.

Both of these examples employed resurrection ecology to examine population-level responses to environmental contaminants. The ability to experimentally detect shifts in sensitivity across substantial temporal gradients provided unique insight regarding the mechanisms and evolutionary origins of tolerance to environmental stressors. Despite its strengths, however, resurrection ecology has its limitations. Importantly, the process of acquiring resting eggs is labor- and time-intensive, so these resources are usually devoted toward studying a single population. While this approach can provide a wealth of information about this population over time, it would be costly to have a replicated study comparing several populations. Also, resurrection ecology is limited to a small number of taxa that produce egg or seed banks. Finally, it has been argued that resurrected individuals might not

be adequate representatives of ancestral sub-populations, and that extended dormancy could confound evolutionary inferences (but see Orsini et al. 2016 for an experimental rebuttal). As such, the limitations of resurrection ecology should be carefully considered in the context of the research aim.

Resurrection ecology studies provide unprecedented insight into the evolutionary origins of contemporary phenotypes. They are an excellent complement to spatial approaches, and can yield valuable information regarding the justification of assumptions inherent to spatial studies. Additionally, they have the potential to inform predictions of how a population might respond to future challenges (Orsini et al. 2013; Simpson et al. 2015). We posit that the unique and powerful approach of resurrection ecology will be useful for environmental scientists interested in understanding the eco-evolutionary consequences of contaminants, and aid in forecasting the fate of biota.

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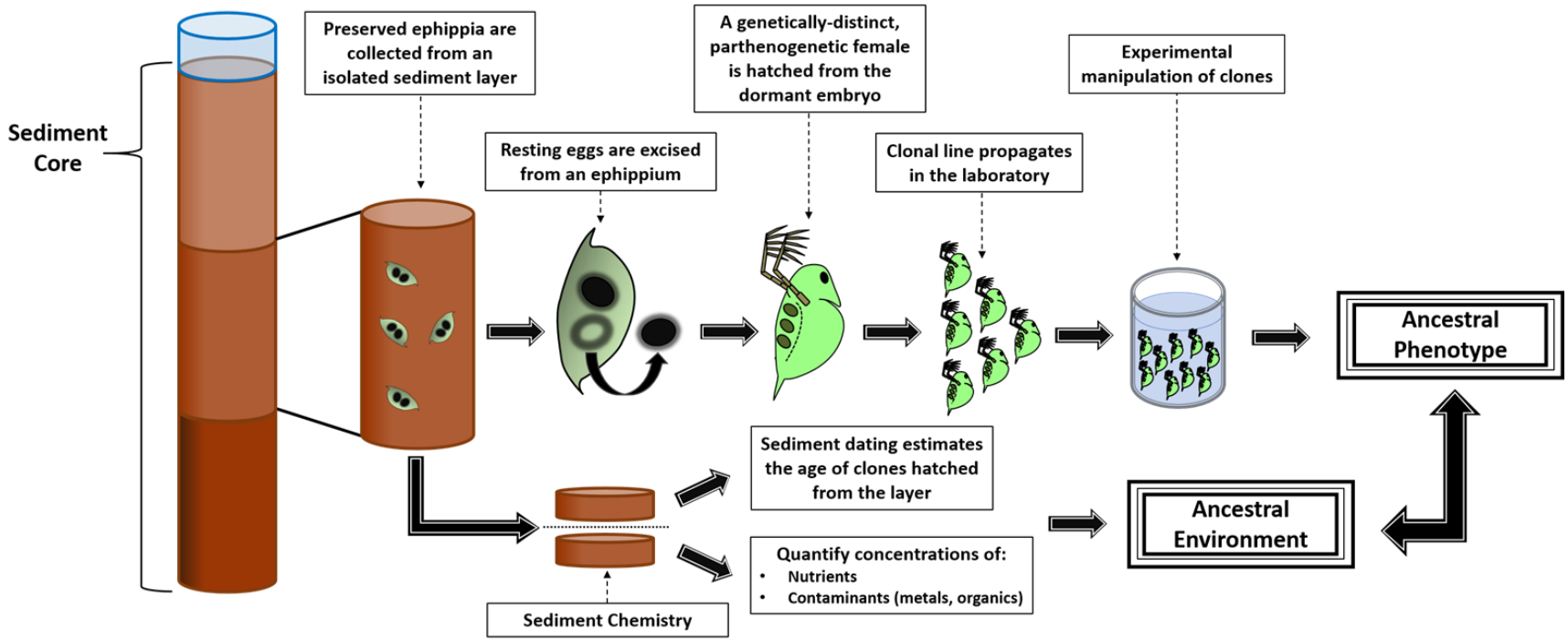

Figure 1. The resurrection ecology approach. Experimental manipulation of dormant embryos (resurrection ecology) enables experimental assessments of ancestral phenotype, while analysis of temporally divergent sediment layers (paleolimnology) facilitates an informed reconstruction of the ancestral environment. The integration of these data permits an innovative approach for describing evolutionary responses to environmental contaminants.

CHAPTER II

VARIATION IN TOXICITY OF A CURRENT-USE INSECTICIDE AMONG RESURRECTED *DAPHNIA PULICARIA* GENOTYPES

The following chapter appears as published in *Ecotoxicology*:

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ABSTRACT

This study examined how genotypes of *Daphnia pulicaria* from a single population, separated by thousands of generations of evolution in the wild, differ in their sensitivity to a novel anthropogenic stressor. These genotypes were resurrected from preserved resting eggs isolated from sediments belonging to three time periods: 2002-2008, 1967-1977, and 1301-1646 A.D. Toxicity of the organophosphate insecticide chlorpyrifos was determined through a series of acute toxicity tests. There was a significant dose-response effect in all genotypes studied. Moreover, significant variation in toxicity among genotypes within each time period was detected. Importantly, a significant effect of time period on sensitivity to chlorpyrifos was found. Analysis of the median effect concentrations (EC50s) for genotypes within each time period indicated that the 1301-1646 genotypes were 2.7 times more sensitive than the 1967-1977 genotypes. This trend

may be partially explained by microevolutionary shifts in response to cultural eutrophication.

Key Words: Chlorpyrifos, Eutrophication, Evolutionary Toxicology, Genetic variation, Microevolution, Resurrection Ecology.

INTRODUCTION

Over the past 20 years, the realization that contaminants can change the genetic composition of exposed populations resulted in the formation of a new discipline, evolutionary toxicology (Bickham 2011). Studies in evolutionary toxicology have frequently demonstrated the potential for pesticides to act as strong sources of selection in both target and non-target organisms. For example, natural populations of amphipods (*Hyalella azteca*) have been shown to exhibit dramatic differences in sensitivity to pyrethroids, due to point mutations in voltage-gated sodium channels (Weston et al. 2013). Similar examples can be found in vertebrates as well: Whitehead et al. (2012) demonstrated that populations of Atlantic killifish (*Fundulus heteroclitus*) have adapted to polychlorinated biphenyl (PCB) contamination through common genetic mechanisms in urban estuaries. Development of resistance to pesticides and other organic contaminants requires persistent or frequent exposure, and genetic variability of relevant traits in the population that enable some genotypes to survive exposure (Wirgin and Waldman 2004).

Evolutionary change in aquatic organisms has also occurred as a result of anthropogenic stress not directly related to exposure to pesticides and organic contaminants. There is mounting evidence that anthropogenic environmental change is causing rapid evolutionary shifts in a variety of traits such as metabolism (Dillon et al. 2010), heat tolerance (Sørensen et al. 2003), and nutrient-use physiology (Frisch et al.

2014), most likely due to multifarious selection (Johnson and Kliman 2002). Whether such correlated evolutionary shifts interact with key toxicological traits such as tolerance remains to be rigorously addressed.

The effect of metabolism on toxicity can vary greatly depending on the toxicokinetics of the compound of interest. Pesticides that decrease in toxicity after biotransformation (ex: pyrethroids, carbamates) would likely be less toxic to an organism with a higher metabolism (Coats et al. 1989), while pesticides that are bioactivated (increase in toxicity) through biotransformation (e.g., dithio-organophosphates) would be more toxic (Clark and Di Giulio 2012). However, extrinsic factors such as temperature can also play important roles in dictating chemical toxicity. Increased temperature has been shown to dramatically increase toxicity of the organophosphate chlorpyrifos to chironomids; this increase in toxicity was likely due to an increase in biotransformation rates, a trait directly linked to metabolism (Lydy et al. 1999). Clearly, such correlated factors should be considered when investigating the interaction between acquired traits associated with environmental change and xenobiotic tolerance.

Most studies in evolutionary toxicology focus on investigating differential sensitivity among spatially distinct populations inhabiting contaminated and reference sites (Meyer and Di Giulio 2003, Nacci et al. 2010). Comparatively, resurrection ecology enables the observation of a single population over long periods of time, and enables the integration of genetic data from resting eggs with phenotypic observations on individuals of revived genotypes from the past (Hairston et al. 1999; Decaestecker et al. 2007). Organisms belonging to the arthropod class Branchiopoda possess unique life histories that provide rare insight into the ancestral genetics of populations (Lampert 2011). One group in particular, *Daphnia* spp., are frequently used as model systems in ecotoxicology due to their high fecundity (via parthenogenesis) and their ability to form egg banks (via

sexual reproduction) (Lampert 2011). Several recent studies have used egg banks to determine population-level changes over time (Bailey et al. 2004, Van Doorslaer et al. 2009, Derry et al. 2010, Orsini et al. 2013), but few have exploited this unique system to answer toxicological questions (Barata et al. 2002, Wyn et al. 2007, Navis et al. 2013).

Here, we utilize the unique *Daphnia* system to test whether previously reported microevolutionary changes (Frisch et al. 2014) influence susceptibility to new anthropogenic stressors. Specifically, we determined whether there is variation in toxicity to a common-use insecticide within a "resurrected" population of *Daphnia pulicaria*, and we examined whether genotypes isolated from different sediment strata, spanning several centuries differed in their susceptibility to a new environmental stressor. Previous work in this system has demonstrated significant shifts in key environmental parameters, population genetics, phosphorus-use efficiency (Frisch et al. 2014), and metabolism (Roy Chowdhury 2014), the latter being an important component in evaluating the toxicity of xenobiotic compounds. These shifts could potentially change this population's sensitivity to xenobiotics that it has never encountered before, through altered metabolism of toxic compounds. To our knowledge this is the first toxicological study that evaluates the toxicity of an insecticide to a single population before, and during anthropogenic environmental change.

MATERIALS & METHODS

Study organisms

Organisms for this study were established using individuals from the original cultures described in Frisch et al. (2014), arising from South Center Lake, Minnesota, USA. Each genotype studied was hatched from a single resting egg and propagated in the

laboratory. ^{210}Pb dating was used to age each sediment layer (4cm), indicating the relative age of each daphniid genotype (Frisch et al. 2014) (Table 1). Given that these organisms reproduce asexually under optimal laboratory conditions, genetic integrity was maintained across generations (verified periodically using molecular markers, L.J. Weider and P.D. Jeyasingh, unpublished data). Twenty individual genotypes were chosen and divided into three groups based on the sediment layers from which they were isolated: 4-8cm (7 genotypes), 20-24cm (10 genotypes), and 52-64cm (3 genotypes). The number of genotypes from each sediment layer varied because hatching success of resting eggs isolated from the various layers varied (Frisch et al. 2014). Analysis of microsatellite data indicated that the genotypes were genetically distinct. The approximate age of each sediment layer (i.e. age of genotypes) is presented in Table 1. The 52-64cm group is a combination of one genotype from 52-56cm and two genotypes from 60-64cm. The time spread in this group is not ideal; however, we were limited to a small sample size due to poor hatching success of the older genotypes. Each culture was initiated with six gravid females chosen at random and stored in 1L jars. We used the same culture medium as the original cultures: low-nutrient COMBO (no nitrogen or phosphorus; pH: 7.3-7.7) (Kilham et al. 1998). All cultures were stored in an environmentally-controlled room set at 21°C (±1°C) and a 16:8 photoperiod (light:dark). Organisms were fed 3mL of *Selenastrum capricornutum* algae ($3x10^7$ cells/mL) acquired from Aquatic Biosystems, Inc. (Fort Collins, CO) every other day. Partial water changes and culling were performed when population density exceeded ~50 adults/1L.

Table 1. Acute toxicity EC50 values (with confidence intervals) for each genotype by age class. Dates acquired using ^{210}Pb dating performed by the St. Croix Watershed Station in Minnesota.

Sediment Depth (cm)	Date (A.D.)	Chlorpyrifos EC50 (µg/L)
4-8	2002-2008	0.50 (0.35-0.65)

		0.26 (0.20-0.32)
		0.20 (0.16-0.24)
		0.22 (0.17-0.27)
		0.15 (0.10-0.20)
		0.31 (0.27-0.35)
		0.25 (0.21-0.29)
20-24	1967-1977	0.31 (0.25-0.37)
		0.70 (0.60-0.80)
		0.33 (0.29-0.37)
		0.29 (0.25-0.33)
		0.46 (0.39-0.53)
		0.48 (0.41-0.55)
		0.40 (0.33-0.47)
		0.32 (0.27-0.37)
		0.31 (0.26-0.36)
		0.25 (0.20-0.30)
52-64	1301-1646	0.07 (0.01-0.13)
		0.21 (0.17-0.25)
		0.14 (0.06-0.22)

Model contaminant

The toxicity of our chosen pesticide, chlorpyrifos, is directly influenced by the metabolism of an affected organism. As a pro-insecticide, the parent compound (chlorpyrifos) is relatively non-toxic until it is oxidized into its metabolite form, chlorpyrifos oxon (Giesy et al. 1999). Consequently, we would predict that the documented differences in metabolism between these genotypes will be accompanied by differences in sensitivity to this compound. Chlorpyrifos is a suitable compound for several reasons. As a common-use insecticide, chlorpyrifos (and its toxic effects) has been studied extensively in numerous vertebrate and invertebrate species (Jarvinen et al. 1983, Atterberry et al. 1997, Moore et al. 1998, Palma et al. 2008). As a result, information regarding environmental fate, toxicity, metabolism, and even mechanisms of resistance are readily available (Barron et al. 1991, Hemingway et al. 1993, Kamrin et al. 1997, Carvalho et al. 2013). Analytical grade chlorpyrifos (99.5% purity) was purchased

from Sigma Aldrich. High grade acetone (99.6% purity - Fisher Scientific) was used as the pesticide carrier in all chlorpyrifos standards.

Toxicity Testing

We performed 48-hour acute effect toxicity tests in static test systems for each of the twenty genotypes. Ten days before each test, 12 adult females were separated from the main culture and stored in four 250mL jars filled with culture medium (COMBO). Six neonates were randomly selected from a pool of <48 hour-old neonates and assigned to each experimental unit. Experimental units consisted of 100mL glass beakers, filled with 80mL of COMBO medium. Although the EPA suggests that toxicity testing should use <24 hours old neonates, the lower fecundity of *D. pulicaria* (compared to *D. pulex*) did not generate a sufficient number of <24-hour-old neonates for experimental tests. The EPA standardized toxicity test protocols are designed for risk assessment, to evaluate toxicity at an organism's most sensitive life stage, and to maintain consistency among laboratories. Our toxicity tests are not being performed to address such questions; therefore, we widened the age range by 24 hours for our toxicity tests. The five treatments of chlorpyrifos were replicated three times each, ranging nominally by a factor of two: 0.075 µg/L, 0.15 µg/L, 0.30 µg/L, 0.60 µg/L, and 1.2 µg/L. The treatment range was determined by preliminary range-finder tests with randomly selected genotypes from each age group. Our estimates of EC50 for the range-finder tests were derived from *Daphnia pulex* data in the EPA Ecotox Database. *Daphnia pulex* is a sister species of *D. pulicaria*, so the reported 48-hour EC50 range (0.21 µg/L-0.42 µg/L) was useful in determining the treatment range for this study (Van Wijngaarden and Leeuwangh 1989, Van der Hoeven and Gerritsen 1997). The same treatment range was used for all toxicity tests in this study. Chlorpyrifos was introduced to each experimental unit via 40 µL with acetone as a carrier. Each test had two negative controls: COMBO media only and

solvent spike (40 µL acetone). Our acute effect, immobility, was recorded after 48 hours. Tests were duplicated for 20% of the genotypes, with treatments also replicated in triplicate.

Verification of Exposure

In order to determine the stability of chlorpyrifos in the test system, a stability test was performed using the highest and lowest spiking concentrations, extracted both immediately after spiking, and after 48 hours (n=5 for each concentration/time combination, 20 total samples). Water samples (150mL) were extracted using 1000mg C18 SampliQ Solid Phase Extraction (SPE) cartridges (Agilent Technologies-Palo Alto, CA). Each cartridge was activated using 5mL of methanol and rinsed with 5mL of distilled water prior to extraction. After extraction, the cartridges were centrifuged for 2 minutes at 4 *g*, in order to remove any water. Chlorpyrifos was eluted using 8mL of ethyl acetate, which was evaporated down to 0.5mL using a gentle flow of nitrogen gas. Additionally, stock dosing solutions were checked against analytical standards to verify that spiking was accurate. Acetone spike solutions were transferred to a 2mL vial, evaporated to dryness, and reconstituted in ethyl acetate.

Analysis of extracts was conducted using gas chromatography-mass spectrometry (Agilent 5975c). The gas chromatography inlet was set at 290°C, while the oven temperature started at 110°C, increasing 12°C/minute until it reached a final temperature of 290°C. Select ion monitoring identified chlorpyrifos (197, 314) and the internal standard, atrazine D5 (178). Sample concentrations were adjusted based on expected extraction efficiency. Additionally, we spiked four blank replicates with the intermediate test concentration (0.3 µg/L), then extracted and analyzed the samples as described above to evaluate the efficiency and precision of the technique.

Data Analysis

A Shapiro-Wilk test for normality indicated that the data was not normally distributed ($p<0.05$). Thus, we logit transformed the dependent variable (% immobility) prior to analysis (Warton and Hui 2010). A univariate general linear mixed model (IBM SPSS 21) was used to determine analysis of variance (ANOVA) for our data, with genotypic age and chlorpyrifos treatment as the fixed factors. This test also included a nested ANOVA to test for genotypic variation within age classes. In order to calculate the EC50 (median effect concentration) for each genotype, we used Probit Analysis (Society of Toxicology and Chemistry: *Hazard Assessment Tools v1.0*). Following rank transformation of the EC50 values, the residuals were normally distributed (Shapiro-Wilk: $p>0.05$) with equal variances (Bartlett's: $p>0.05$). As the one-way ANOVA for the individual factor (Age) was significant, we then performed a test for multiple comparisons (Tukey's Honestly Significant Difference Test; $\alpha=0.05$) to determine relationships between mean EC50 and age class.

RESULTS

Quality Control

Control mortality never surpassed 10% in any individual test. Toxicity tests were duplicated for four randomly chosen genotypes, at least one from each age class (two were duplicated from 1301-1646 class). All duplicated tests yielded EC50s that were less than 10% apart, except one genotype—Age class = 1301-1646 A.D.; EC50 = 0.07 µg/L (Table 1)—which differed by 30%. The differences in this genotype's EC50s (0.07 vs. 0.10 µg/L) were likely higher due to its EC50 value being at the low end of the tested concentrations. Based on the quad study, the extraction efficiency of the technique was 89% (S.D. = 4.4%). The 1.2 µg/L standard (mean = 82%; S.D. = 2.3%) and 0.075 µg/L

standard (mean = 94%; S.D. = 2.1%) extracted immediately after spiking were stable within the experimental units. After 48 hours, the stability of chlorpyrifos was maintained for both the high standard (mean = 93%; S.D. = 2.7%) and the low standard (mean = 104%; S.D. = 5.7%). All dosing solutions were within 10% of expected concentrations.

Variation in Sensitivity to Chlorpyrifos

Acute toxicity tests demonstrated significant variation in susceptibility across all age classes. Variation in sensitivity among genotypes was most evident at the intermediate treatments, ranging from 100% immobility to no effect (Figure 1). Similarly, there was variation between individual genotypes within each specific age class, indicating significant differences in susceptibility within each time period (Table 2). After converting toxicity results into EC50 values, variation can still be seen across all 20 genotypes (Table 1). The most sensitive and least sensitive genotypes differed in their EC50s by a factor of 10, ranging from 0.70 to 0.07 µg/L.

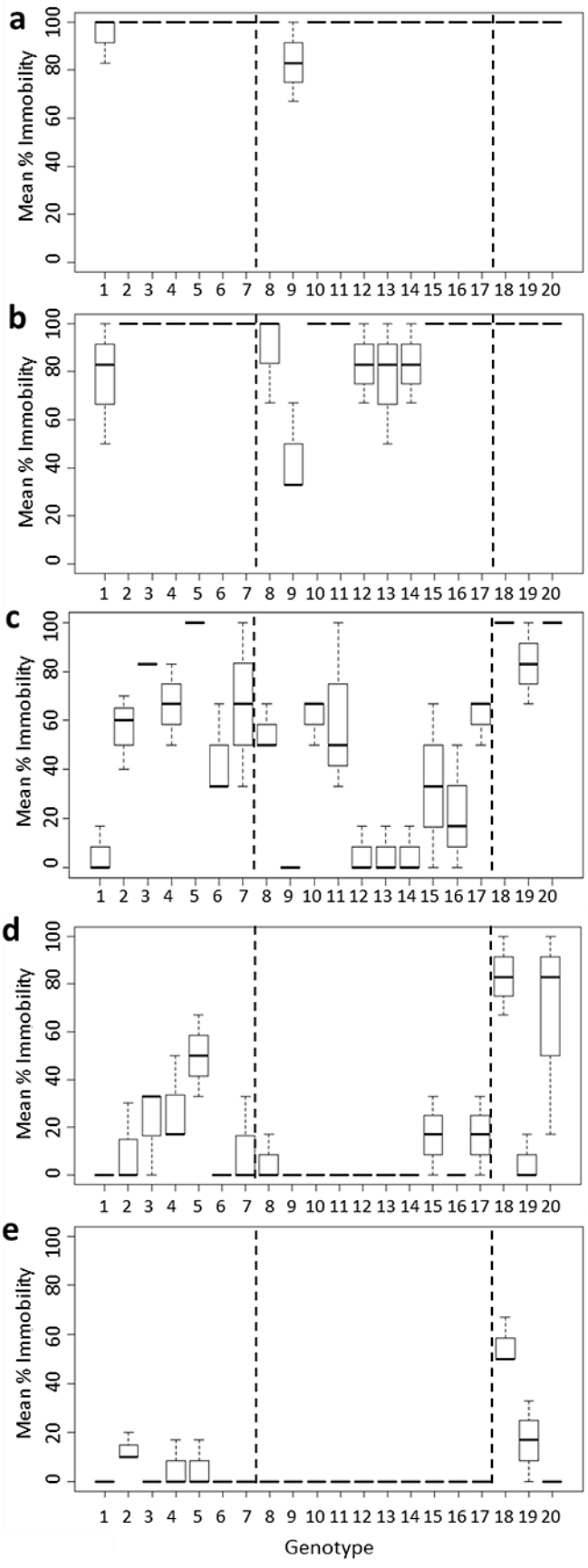
a
b
c
d
e
Mean % Immobility
0 20 40 60 80 100
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Genotype

Fig.1 Individual clonal variation in toxicity to chlorpyrifos by treatment. Treatment concentrations 1.2, 0.60, 0.30, 0.15, and 0.075 µg/L correspond to Plots a, b, c, d, and e respectively. Each number (1-20) corresponds to an individual genotype. Genotypes 1-7 = 2002-2008 A.D.; genotypes 8-17 = 1967-1977 A.D.; genotypes 18-20 = 1301-1646 A.D. Control mortality never surpassed 10% in any individual test.

Temporal Variation in Susceptibility to Chlorpyrifos

Variation present within this population appears to be temporally-directed. The relationship between age class and percent immobility was highly significant, as well as the interaction between age class and chlorpyrifos treatment (Table 2). Specifically, the 1301-1646 genotypes were consistently more sensitive to chlorpyrifos at all treatment levels when compared to the 1967-1977 and 2002-2008 genotypes (Figure 2). Furthermore, analysis of EC50 values yielded significant differences between age classes (Figure 3). When the EC50 values were averaged within each age class, the 1301-1646 genotypes differed in their mean sensitivity to chlorpyrifos by a factor of 2.7 when compared to the 1967-1977 genotypes (p=0.003); all other comparisons were not statistically significant (Figure 3).

Table 2. Results of Logit-Transformed General Linear Mixed Model and Nested ANOVA.

Dependent Variable: Logit Percent Immobility

Source	df	F	Sig.
Corrected Model	31	59.3	<0.001
Intercept	1	49.6	<0.001
Age	2	60.1	<0.001
Treatment	4	269.6	<0.001
Age * Treatment	8	6.88	<0.001
Genotype ID(Age)	17	6.83	<0.001
Error	268		
Total	300		
Corrected Total	299		

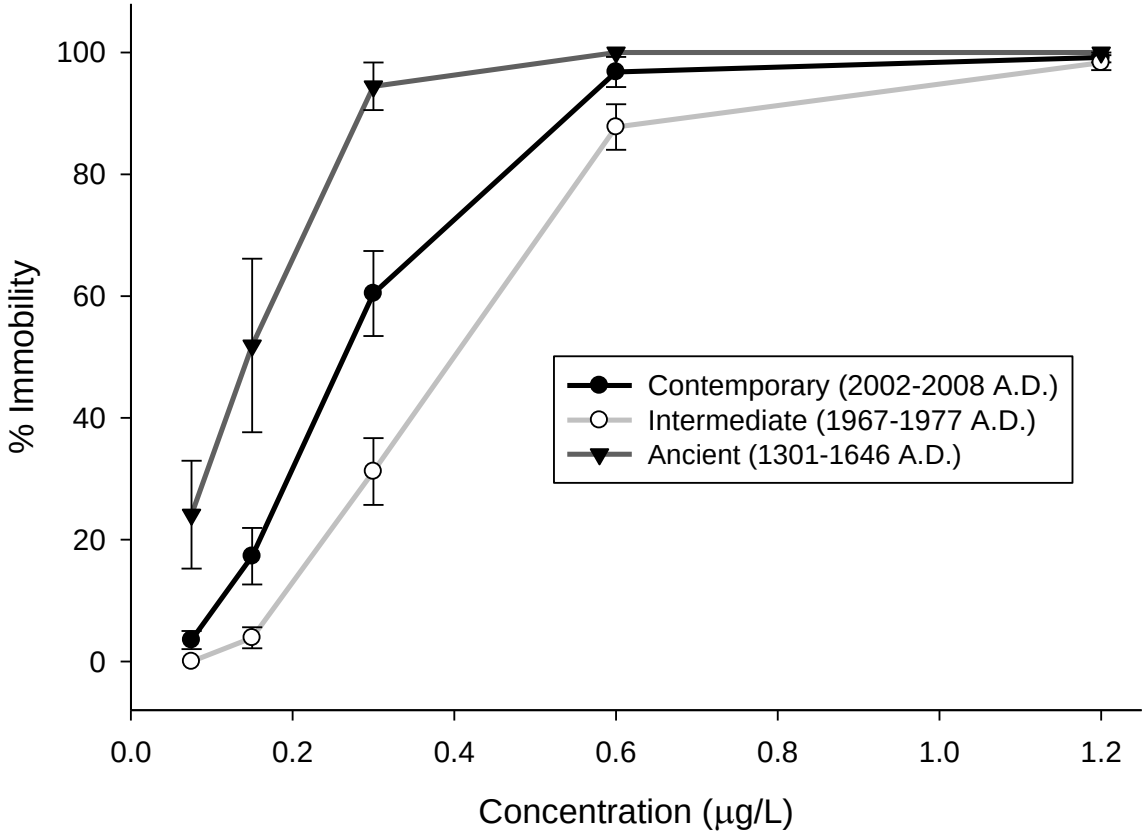

Fig.2 Mean immobility (%) by concentration treatment; error bars represent standard error. Control mortality never surpassed 10% in any individual test.

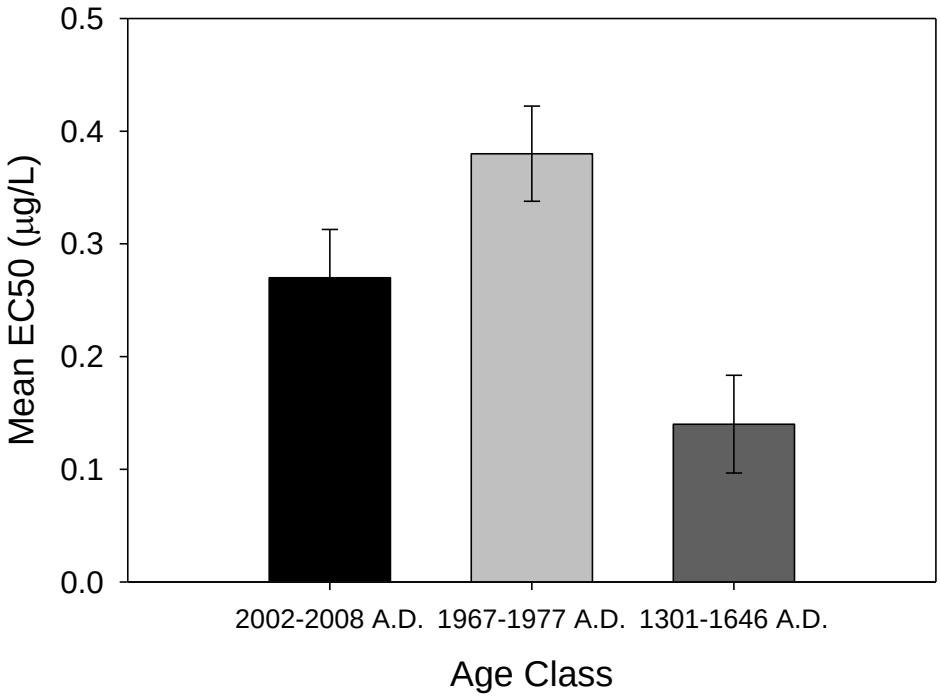

Fig.3 Mean EC50 values for each age class; error bars represent standard error. Categorical letters represent statistical differences between EC50 and age class ($p<0.05$). Control mortality never surpassed 10% in any individual test.

DISCUSSION

We found significant variation in toxicity to a common-use organophosphate insecticide in genotypes from a single population of *D. pulicaria*, established from resting eggs spanning several centuries. Specifically, ancient genotypes were more sensitive to chlorpyrifos than the more contemporary genotypes. Given previous genetic and physiological work that indicates genetically-based phenotypic shifts (Frisch et al. 2014),

the decreased sensitivity to chlorpyrifos is likely a result of evolutionary change. We propose three potential mechanisms that could be responsible for these observed differences in sensitivity to chlorpyrifos.

Evolutionary change in response to historical exposure to chlorpyrifos is one potential mechanism that could explain the results of this study. When chlorpyrifos was first introduced as a primary agricultural insecticide in 1965, its use was largely unregulated and undocumented (Giesy et al. 1999), due to the fact that the Environmental Protection Agency was not created until 1970 (United States Environmental Protection Agency 2014). Thus, it is possible that the 1967-1977 A.D. genotypes experienced years of acute exposure to the insecticide, resulting in selection for tolerance in this population of *D. pulicaria*. This could potentially explain why the genotypes in this age class are significantly more tolerant to chlorpyrifos. Consequently, we hypothesize that the contemporary genotypes (2002-2008 A.D.) are more sensitive to chlorpyrifos than the 1967-1977 A.D. genotypes because the presence of chlorpyrifos, and therefore its potential as a selective pressure, has disappeared from South Center Lake. However, as we have no water quality documentation for South Center Lake from the 1960s-1970s, this hypothesis is merely plausible, and cannot be fully supported.

South Center Lake is one of many lakes included in the Minnesota Sentinel Lakes Program (MSLP). This program, maintained by the Minnesota Pollution Control Agency in collaboration with the Minnesota Department of Natural Resources, focuses on monitoring water quality in each of its sentinel lakes (Minnesota Department of Agriculture 2013). The monitoring region that contains South Center Lake has been sampled 120 times during the last six years with no detections of chlorpyrifos or other

organophosphate insecticides (Minnesota Department of Agriculture 2013). Chlorpyrifos has relatively low persistence in the water column and likely partitions to sediment prior to runoff to the lake (Kamrin et al. 1997). Thus, there is no direct evidence that chlorpyrifos, or other organophosphate insecticides have occurred in this lake. However, the lack of a long-term monitoring history and potential for pulsed exposures that would not be detected during infrequent monitoring suggest that direct selection cannot be discounted.

Selection in response to other organic contaminants is another potential mechanism. While many pesticides have been recently detected in the area, only a few have been quantified at concentrations of concern: all of them are common-use herbicides in Minnesota (atrazine, acetochlor, and metolachlor) (Minnesota Department of Agriculture 2013). Additionally, it is possible that South Center Lake has experienced years of exposure to other persistent organic contaminants, including polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs), as these compounds are ubiquitous by-products of anthropogenic activity (Baker et al. 1991). Whenever persistent chemicals are present in an ecosystem, there is the potential for exposure and ultimately acquired resistance. Therefore, it is possible that the *D. pulicaria* population in South Center Lake could have been exposed to other contaminants, and even could have experienced evolutionary selection for tolerance. However, even if this did occur, it should be noted that persistent organic contaminants generally do not elicit acute neurotoxic effects. Cross-resistance—the evolution of tolerance to one compound through a mechanism that also confers resistance to another compound—can occur if the contaminants share a component of their modes of action (Golenda and Forgash 1985).

Chlorpyrifos, as an organophosphate, has a very specific mode of action (cholinesterase inhibitor) (Giesy et al. 1999), one that no known PCB or PAH possesses (Safe et al. 1984). Some herbicides have been shown to exhibit general neurotoxic effects, although none directly inhibit cholinesterase (Costa et al. 2007). Therefore, it is unlikely that cross-resistance occurred in this manner. Cross-resistance can also potentially occur through another scenario: exposure to one compound could induce an evolutionary change in a key biotransformation or detoxification pathway that decreases the toxicity of a second compound. Such resistance pathways have been demonstrated in *Fundulus heteroclitus* populations, specifically involving aryl hydrocarbon receptor repression, which inhibits cytochrome P-450 induction (Clark and Di Giulio 2012, Whitehead et al. 2012). It is possible that evolution in response to other organic contaminants has altered this *D. pulicaria* population's constitutive or inducible responses to exposure (e.g. upregulation or downregulation of cytochrome P-450 enzymes), ultimately affecting the metabolism of chlorpyrifos.

Finally, selection imposed by non-anthropogenic environmental stressors could be a potential mechanism for variable chlorpyrifos toxicity presented in this study. The interaction of rising temperature (global warming) and increased nutrient loads (eutrophication) promotes the growth of a number of phytoplankton species, including toxin-producing cyanobacteria (Steinberg and Hartmann 1988). Species such as *Microcystis aeruginosa* and *Anabaena affinis* pose a threat to primary consumers in freshwater ecosystems, particularly zooplankton grazers. Thus, frequent exposure to blue-green algal toxins in eutrophic water bodies has the potential to serve as a selective pressure for the evolution of tolerance to organic chemicals. Indeed, recent findings

demonstrate the ability of zooplankton species to evolve and adapt to the presence of cyanobacterial toxins (Hairston et al. 1999, Hairston et al. 2001, Sarnelle and Wilson 2005).

Cyanobacterial colonies primarily harm daphniids in two ways: causing blockages near the mouth and feeding appendages, and exerting toxicity following consumption (Gilbert 1990). Adaptations in behavior and physiology can alleviate these two negative effects. Behavioral adaptations such as higher selectivity in feeding or general avoidance of toxic strains could be one evolutionary response to the increased prevalence of blue-green algae (DeMott et al. 1991). However, it is not expected that these mechanisms would change sensitivity to a waterborne chemical contaminant.

On the other hand, *Daphnia pulex*, a close relative of *D. pulicaria*, exhibit physiological adaptations to *A. affinis* filaments (Gilbert 1990). Analysis of two populations of *D. pulex* yielded significant intraspecific differences in tolerance to *A. affinis*. Because individuals from both populations successfully ingested the algal filaments, Gilbert (1990) concluded that the variation in susceptibility was likely due to differential physiological responses to the toxin. These physiological responses could include changes in toxicokinetics within the organism, such as distribution and biotransformation. Changes in toxicokinetics have been frequently reported to result in resistance to organic contaminants (Oi et al. 1992, Siegfried and Ono 1993). Thus, variation in toxicity to chlorpyrifos demonstrated in this study could have resulted from such adaptive physiological changes due to selection imposed by algal toxins.

Alternatively, environmental change could have exerted other selective forces on this population. Recent work conducted on the same daphniid genotypes used in this

study investigated the potential for evolved physiological responses due to cultural (anthropogenic) eutrophication. In these two studies, the contemporary genotypes (2002-2008) exhibited significantly lower nutrient efficiency, as well as a lower metabolism, when compared to the 1301-1646 genotypes (Frisch et al. 2014; Roy Chowdhury 2014). It was hypothesized that these differences are due to evolution in response to dramatic changes in nutrient composition at South Center Lake (Frisch et al. 2014). As the supply of limiting nutrients such as phosphorus increases, the strength of selection for carbon use efficiency could be relaxed (Jeyasingh 2007), potentially resulting in microevolutionary shifts in metabolic rate (Jeyasingh et al. 2009; Roy Chowdhury 2014). Therefore, it is possible that the lower sensitivity of younger genotypes to chlorpyrifos could be a correlated outcome of relaxed selection on efficient metabolism. Lydy et al. (1999) measured toxicity of chlorpyrifos across a temperature range and found differential toxicity, likely due to changes in metabolism related to temperature, demonstrating that fluctuations in metabolic rate can change the toxicity of chlorpyrifos to aquatic arthropods. Possessing a slower metabolism could decrease the rate at which chlorpyrifos is bioactivated into its toxic metabolite chlorpyrifos-oxon, thus decreasing toxicity. In order to evaluate potential variation in metabolism and increased activity of biotransformation enzymes, the next logical step would be to investigate the toxicokinetics of chlorpyrifos within these daphniid genotypes.

While the unique biology of *Daphnia* enabled this first observational study over such a vast time scale, one must be careful when interpreting its results. This study was performed using a population from one lake; it is possible that the trends observed in South Center Lake are not representative of other lakes. Furthermore, the low number of

genotypic replicates, especially from older sediment strata, limits robust inferences. This is an unavoidable issue in all resurrection ecology studies. However, these obstacles do not diminish the importance of the data generated from successfully hatched older eggs.

Despite these limitations, our study clearly shows that there is genetic variation in tolerance to chlorpyrifos within this population. Further, the temporally explicit data on genotypes of a population separated by thousands of generations of evolution demonstrate strong differences in toxicity based on genotype age, possibly indicating evolutionary shifts in xenobiotic tolerance. Identifying the microevolutionary mechanisms underlying xenobiotic tolerance is a central challenge in evolutionary toxicology. Temporally explicit approaches such as resurrection ecology enable the exploration of such frontiers.

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CHAPTER III

ASSESSMENT OF BIOCHEMICAL MECHANISMS OF TOLERANCE TO CHLORPYRIFOS IN ANCIENT AND CONTEMPORARY *DAPHNIA PULICARIA* CLONES

ABSTRACT

Previous research has demonstrated that "resurrected" clones from a population of *Daphnia pulicaria* express temporal variation in sensitivity (median effect concentration – EC50) to the insecticide chlorpyrifos. Ancient clones (1301-1646 A.D.) were on average more sensitive to this chemical compared to the contemporary clones (1967-1977 A.D.). To determine the physiological mechanisms of tolerance, a series of biochemical assays was performed on three ancient and three contemporary clones; these six clones exhibited the most sensitive and most tolerant phenotypes within the population, respectively. Metabolic tolerance mechanisms were evaluated using acute toxicity testing, while target-site tolerance was assessed via *in vitro* acetylcholinesterase (AChE) assays. Acute toxicity tests were conducted using *i*) the toxic metabolite chlorpyrifos-oxon (CPF-oxon) and *ii*) CPF-oxon co-applied with piperonyl butoxide (PBO), a known Phase-I metabolic inhibitor. Both series of toxicity tests reduced the mean variation in sensitivity between tolerant and sensitive genotypes. Exposure to CPF-O reduced the disparity from a 4.7-fold to 1.6-fold difference in sensitivity. The addition

of PBO further reduced the variation to a 1.2-fold difference in sensitivity. *In vitro* acetylcholinesterase assays yielded no significant differences in constitutive activity or target-site sensitivity. These findings suggest that pathways involving Phase-I detoxification and/or bioactivation of chlorpyrifos play a significant role in dictating tolerance in this population.

1. INTRODUCTION

Populations face a variety of environmental stressors, which can impose strong selection pressures that drive evolutionary change. Among these, acute and chronic exposure to common-use pesticides remains a considerable threat to non-target species, despite the continued effort to synthesize compounds with high target specificity and low environmental persistence. Exposure to these organic contaminants can trigger intense, direct selection events favoring individuals possessing adaptive physiologies (Oziolor et al. 2016). Studies investigating the evolution of pesticide tolerance have historically focused on crop-pests and disease vectors, given the severe economic and human-health repercussions of pesticide tolerance in these species (Katz et al. 1973, Yu 1991, Hemingway and Ranson 2000). Such studies have generated a wealth of physiological data describing the mechanisms that underlie tolerance to several legacy and current-use pesticides.

Tolerance to pesticides is most commonly mediated by adaptive alterations of pathways involving *i*) the disposition of the toxicant through the organism (i.e., toxicokinetics), and *ii*) the interaction between the toxicant and its target receptor (i.e., toxicodynamics) (Soderlund and Bloomquist 1990). While several toxicokinetic

parameters, such as uptake (Balabanidou et al. 2016) and elimination (Sun et al. 2017), have been associated with tolerance, far more attention has been directed toward biotransformation mechanisms. Biotransformation encompasses a broad range of reactions, ranging from simple functional group substitutions (Phase I) to secondary conjugation involving endogenous molecules (Phase II) (Livingstone 1998). These metabolic reactions are catalyzed by numerous taxonomically conserved enzyme families, including mixed function oxidases (MFOs) and glutathione S-transferases (GSTs) (Ranson et al. 2002). Among the MFO enzymes, cytochrome P450 monooxygenases (CYPs) are most frequently implicated in pesticide tolerance, as they exhibit broad substrate specificities, and perform the Phase I metabolic reactions that comprise an organism's first line of defense against organic xenobiotics (Scott 1999). Furthermore, CYPs are highly inducible by nature, as they are up-regulated in response to both natural (e.g., dietary exposure to secondary metabolites) and anthropogenic stressors (e.g., toxicant exposure) (Li et al. 2007, Schuler and Berenbaum 2013). As a result, tolerant phenotypes often manifest via the constitutive overexpression and/or enhanced induction of CYP genes, both of which increase the detoxification of organic contaminants (Liu et al. 2011).

In addition to toxicokinetic mechanisms, modifications to toxicodynamic pathways can also significantly reduce toxicant sensitivity by interfering with its mechanism of toxicity. Although the mechanism of action varies by pesticide, common mechanisms include the inhibition of critical enzymes (esterases) and receptors (voltage-gated sodium channels, gamma-aminobutyric acid receptors) (Casida 2009). For example, organophosphate and carbamate insecticides bind to acetylcholinesterase and

other esterase proteins, which impairs the hydrolysis and subsequent recycling of neurotransmitters at post-synaptic terminals, resulting in excessive cholinergic signaling (Hemingway et al. 1993). Toxicodynamic (i.e., target-site) tolerance mechanisms usually involve either 1) enhanced constitutive and/or induced synthesis of the target enzyme or receptor, or 2) point-mutations that reduce the toxicant's affinity for binding to their enzyme/receptor substrates (Hemingway et al. 2004). In some cases, individuals possessing the mutations responsible for these target-site mechanisms exhibit phenotypes that are orders of magnitude more tolerant than naïve individuals (Weston et al. 2013, Wu et al. 2015, Bacca et al. 2017).

Research investigating the aforementioned tolerance mechanisms has traditionally characterized tolerance via spatial comparisons of populations originating from contaminated and reference sites. Assessments of temporal shifts in pesticide sensitivity, however, are much less common due to the limited availability of long-term datasets and other logistical constraints. Resurrection ecology—an approach that utilizes dormant propagules to reconstruct and experimentally manipulate ancestral lineages—offers a rare opportunity to conduct longitudinal studies (Kerfoot et al. 1999). This approach has proven its utility in numerous interdisciplinary fields, including evolutionary ecology (Frisch et al. 2014, Geerts et al. 2015, Stoks et al. 2015, Henning-Lucass et al. 2016), functional ecology (Roy Chowdhury et al. 2014, Roy Chowdhury et al. 2015, Frisch et al. 2017), and recently ecotoxicology (Piscia et al. 2015, Simpson et al. 2015, Turko et al. 2016). Furthermore, resurrection ecology facilitates informed predictions regarding the origins of evolutionary change. For example, one can examine the chemical composition of sediment cores to infer characteristics of the ancestral environment that might have

influenced contemporary phenotypes, such as historical nutrient and pesticide concentrations. The ability to associate characteristics of the ancestral environment with concomitant changes in phenotype offers a unique perspective from which to assess the evolution of tolerance to pesticides (Simpson et al. 2017).

Simpson et al. (2015) employed resurrection ecology to investigate how sensitivity to an insecticide (chlorpyrifos) changed over time in a population of *Daphnia pulicaria* originating from a Minnesota lake experiencing progressive eutrophication (see Frisch et al. 2014). Simpson et al. (2015) discovered significant intra-population variation in sensitivity to chlorpyrifos: clones resurrected from a time period pre-dating the advent of pesticides (1301-1646 A.D.) were 2.7-fold more sensitive than their more derived counterparts (1967-1977 A.D.). Simpson et al. (2015) attributed this phenotypic shift to several possible evolutionary origins, including direct selection from pesticide exposure or indirect selection in response to cultural eutrophication.

The current study aims to elucidate the mechanism(s) that underlie the variation in chlorpyrifos sensitivity observed by Simpson et al. (2015). Here, we evaluated the potential contribution of metabolic and target-site tolerance mechanisms by performing a series of biochemical assays using a subset of the *D. pulicaria* population, which included clones from the most sensitive and most tolerant time periods (1301-1646 and 1967-1977 A.D.). Chlorpyrifos, an organophosphate pro-insecticide, requires metabolic bioactivation to become a potent cholinesterase inhibitor (Giesy et al. 1999); thus, we hypothesized that metabolic tolerance would involve 1) enhanced detoxification of the parent compound, and/or 2) reduced bioactivation of the parent into its toxic metabolite, chlorpyrifos-oxon (CPF-oxon). Alternatively, we hypothesized that target-site tolerance

would involve 1) enhanced constitutive cholinesterase activity, and/or 2) insensitive cholinesterase enzymes. We tested these hypotheses using acute toxicity testing and *in vitro* cholinesterase assays.

2. MATERIALS & METHODS

2.1 Study Organisms

The organisms used in this study were derived from the *D. pulicaria* population described by Frisch et al. (2014). Six clones were selected based on their age class and sensitivity to chlorpyrifos (Simpson et al. 2015). The six clones consisted of three ancient clones (1301-1646 A.D.) and the three most tolerant constituents of the 1967-1977 A.D. age class, ranked according to their respective EC50 estimates (Simpson et al. 2015; see Figure 1). Hereafter, these six clones will be referred to according to their relative sensitivities (S1, S2, S3, T3, T2, T1), ranging from most sensitive (S1) to most tolerant (T1). Cultures of each clone were established with 12 genetically identical gravid females stored in 5 L jars. Low-nutrient COMBO (no nitrogen or phosphorus; pH 7.3-7.7) was used as the culture medium (Kilham et al. 1998). All cultures were maintained in a temperature-controlled room (21 °C ±1 °C) with a 16:8 (light:dark) cycle. The organisms were fed 6 mL of *Scenedesmus acutus* (3×10^7 cells/mL) daily. For all of the assays in this study, *Daphnia* neonates (≤48-hours old) were harvested from their stock cultures as they became available (Simpson et al. 2015).

2.2 Chemicals

Analytical grade chlorpyrifos-oxon (98.1% purity) and technical grade piperonyl butoxide (90% purity) were purchased from Sigma Aldrich (St. Louis, MO, USA).

Reagent grade acetone was used as the carrier. For the AChE assays, acetylthiocholine iodide (ATC; ≥98% purity), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB; ≥98% purity), and bovine serum albumin (BSA; ≥98% purity) were also purchased from Sigma. ATC, DTNB, and BSA stocks were prepared in Millipore ultrapure water, while CPF-oxon was administered via reagent-grade methanol.

2.3 Evaluation of Metabolic Tolerance

To test for mechanisms of metabolic tolerance, a series of acute toxicity tests were performed on each of the six clones. These tests were designed to systematically inhibit the metabolic capabilities of the clones. A subsequent reduction in the variation of clonal sensitivity (EC50s) was interpreted as evidence of the inhibited pathway's contribution to chlorpyrifos tolerance. The first test (Experiment I) exposed the clones to the bioactivated form of chlorpyrifos, chlorpyrifos-oxon (CPF-oxon). Bioactivation occurs via oxidative desulfuration of the parent compound, resulting in a bioactive metabolite possessing a high affinity for cholinesterase enzymes (Tang et al. 2001). Administering this compound in its most toxic form factors out any potential clonal differences in metabolic bioactivation pathways. The second test (Experiment II) exposed the clones to a mixture of CPF-oxon and piperonyl butoxide (PBO). PBO is a potent inhibitor of cytochrome P450 monooxygenases (CYPs), which are the primary enzymes recruited for the biotransformation (and bioactivation) of exogenous organic compounds (Ankley and Collyard 1995). Exposure to this mixture similarly factors out bioactivation, while additionally inhibiting the organism's ability to detoxify CPF-oxon.

All toxicity tests (Experiments I & II) were conducted in a static test system for a duration of 48 hours. Each experimental unit consisted of 80 mL of culture media in a

100 mL glass beaker containing 10 neonates, which were harvested from clone-specific stock cultures. The endpoint for all tests was immobility, which was scored at the conclusion of the 48 hour exposures. For Experiment I, the six clones were exposed to five CPF-oxon treatments: 0.10, 0.25, 0.40, 0.55, and 0.70 µg/L. In Experiment II, the six clones were exposed to five CPF-oxon concentrations (0.01, 0.025, 0.05, 0.25, 0.50 µg/L) co-applied with one of two PBO concentrations (100 or 200 µg/L). Two PBO treatments were used to test for dose-dependence (i.e., whether higher concentrations = greater CYP inhibition). In both experiments, CPF-oxon and PBO were introduced to the experimental units via 50 µL spikes, with methanol as the solvent carrier. Negative control treatments included 50 µL spikes of methanol and culture media. All treatments were replicated in triplicate.

2.4 Evaluation of Target-Site Tolerance

Target-site tolerance was assessed by quantifying AChE activities *in vitro* using a modified Ellman method (Ellman et al. 1961, Guilhermino et al. 1996). In short, the Ellman method quantifies the hydrolysis of an acetylcholine mimic (ATC) by AChE; the hydrolyzed substrate reacts with a chromagen (DTNB) to produce a yellow color, which is detected via UV spectrometry. It is important to note that *Daphnia* possess a variety of esterases; however, these enzymes do not significantly contribute to the hydrolysis of our substrate, ATC (Barata et al. 2001). Thus, the reported enzyme activity from our assays most accurately represented that of AChE. Prior to the assay, neonates (<48-hours old) of each clone were harvested from their respective stock cultures, flash-frozen in liquid nitrogen, and stored at -80˚C until sufficient biomass was acquired (~1500 neonates/clone). Seven groups of 250 neonates were individually homogenized in 600 µL

of 0.1 M phosphate buffer (pH = 7.2) using a Pyrex pestle homogenizer, after which the samples were centrifuged at 8,000 x *g* for 5 minutes. The supernatant was transferred to a new microcentrifuge tube and temporarily stored on ice.

From each 600 µL volume of supernatant, four aliquots (100 µL) were subjected to either a CPF-oxon treatment (2.8, 1.6, 0.87, 0.49, 0.27, 0.15 µg/L) or a control treatment (solvent or water); the remaining 200 µL were saved for protein analysis. The CPF-oxon treatments were replicated in triplicate, while the solvent and water treatments had six replicates each. The samples were incubated in 2 mL amber vials (VWR) with their respective treatments at room temperature for 45 minutes on a shaker table. Following the incubation, the volume of each vial was transferred to a 96-well plate, and 250 µL of reaction solution (0.1 M phosphate buffer, 1.4 mM ATC, 0.46 mM DTNB) was added to each well. Absorbance was measured kinetically for five minutes on a microplate spectrophotometer (SPECTRAmax® 190 – Molecular Devices, California) set at 405 nm. Activity (nmol of substrate hydrolyzed/minute) was calculated using Beer's Law and the known extinction coefficient of the hydrolyzed substrate (Ellman et al. 1961). The activity of each replicate was standardized by the protein content of the original homogenate, which was quantified using the Bradford method (Bradford 1976). Thus, specific activity was expressed in nmol of substrate hydrolyzed/minute/mg of protein.

2.5 Quality Control

A series of extractions was performed to verify the stability of CPF-oxon in the test system. Eighteen beakers containing 200 mL of COMBO media were spiked with one of three CPF-oxon spiking solutions to make the following concentrations: 0.7 µg/L

(7 replicates), 0.4 µg/L (7 replicates), or 0.1 µg/L (4 replicates). Twelve of these beakers were extracted immediately, while the remaining six (0.7 and 0.4 µg/L) were extracted 48 hours later. Both groups of water samples were extracted in Cleanert Solid Phase Extraction (SPE) columns containing 500 mg of C8 (Agela Technologies – Wilmington, DE, USA). Each column was activated with 5 mL of methanol and rinsed with 5 mL of distilled water prior to extraction. Following the extraction, each cartridge was centrifuged for 3 minutes at 5 x *g* to remove any water. CPF-oxon was eluted with 8 mL of ethyl acetate, which was evaporated down to 1 mL by a gentle flow of nitrogen gas.

Analysis of all extracts was conducted using gas chromatography-mass spectrometry (GC-MS; Agilent 5975c). The inlet temperature was set at 260 ˚C, while the oven temperature started at 100 ˚C and increased by 11 ˚C/minute until it reached 295 ˚C. Select ion monitoring identified CPF-oxon (197, 270) and the deuterated internal standard, anthracene-D10 (188). Sample concentrations were corrected by the extraction efficiency (determined through stability study).

2.6 Data Analysis

The toxicity data generated from the acute toxicity tests (Experiments I and II) were transformed into clone-specific EC50s (median effect concentrations) using Probit analysis (IBM SPSS *v*24). Clonal differences in sensitivity to CPF and CPF-oxon between age classes (S1-S3 vs. T1-T3) were assessed using ANOVAs (IBM SPSS *v*24), where $\alpha = 0.05$. The constitutive AChE activity for each clone was expressed as the mean specific activity of the water-only control replicates. Statistical differences in constitutive activity were evaluated using an ANOVA. Target-site sensitivity for each clone was represented as an IC50—the concentration of CPF-oxon that resulted in 50% AChE

inhibition relative to control—which was calculated using Probit analysis. Differences in IC50 were similarly evaluated using an ANOVA.

3. RESULTS

3.1 Quality Control

The overall extraction efficiency of the stability study was 93 ± 16%. The samples that were extracted immediately (0.7 µg/L, 0.4 µg/L, and 0.1 µg/L) yielded the following extraction efficiencies: 104 ± 11%, 92 ± 20%, 84 ± 13%, respectively. Similarly, the two CPF-oxon concentrations were reasonably stable after 48 hours: 0.7 µg/L (81 ± 13% recovery), 0.4 µg/L (66 ± 16% recovery).

3.2 Metabolic Tolerance Mechanisms

Experiment I exposed the six *D. pulicaria* clones to CPF-oxon for 48 hours. The T1-T3 clone group was significantly more tolerant to the parent compound, chlorpyrifos, than the S1-S3 group ($F_1 = 27.5$, $p = 0.006$), with T1-T3 clones being on average 4.7-fold more tolerant (Simpson et al. 2015). Exposure to CPF-oxon reduced this disparity to a 1.7-fold difference in sensitivity (Figure 1); however, the T1-T3 clones still exhibited significantly higher EC50s ($F_1 = 19.4$, $p = 0.012$).

Experiment II exposed the clones to CPF-oxon and PBO for 48 hours. The effect of PBO on sensitivity was dose-dependent and independent of clonal sensitivity, such that the 200 µg/L treatment increased sensitivity by 18 ± 3.7% relative to the 100 µg/L treatment. Simultaneous exposure to CPF-oxon and PBO (200 µg/L) further reduced the variation between groups, resulting in a non-significant 1.2-fold difference in sensitivity ($F_1 = 1.7$, $p = 0.26$) (Figure 1).

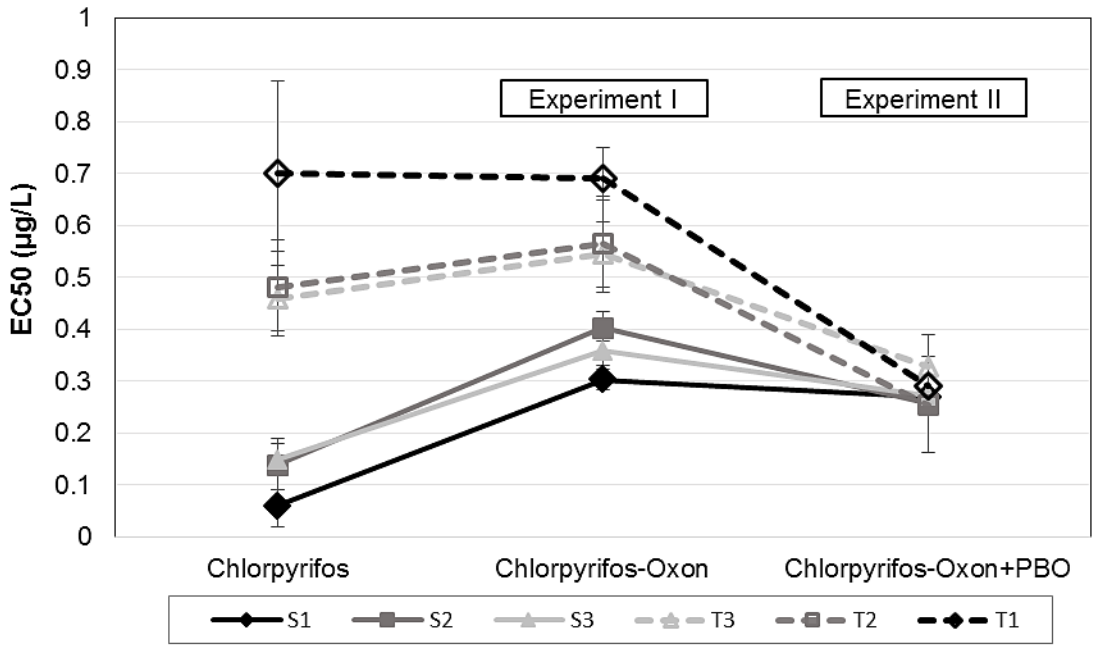

Figure 1. *Relative contribution of biotransformation mechanisms toward clonal variation in sensitivity.* The variation in sensitivity between sensitive (S1-S3; solid lines) and tolerant (T1-T3; dashed lines) clones decreases after factoring out key biotransformation pathways. Accounting for potential differences in bioactivation alone (Experiment I) reduced the variation between groups by a factor of three. The additional inhibition of CYP-driven detoxification mechanisms (Experiment II) accounted for virtually all of the inter-clonal variation. Error bars represent 95% confidence intervals.

3.3 Target-Site Tolerance Mechanisms

The *in vitro* AChE activity of the water-only control was significantly different from the solvent-only control ($F_1 = 4.6$, $p = 0.05$). Consequently, the solvent control activity for each clone was blank-subtracted from their respective CPF-oxon treatment activities to calculate IC50s, while the water-only control activities represented

constitutive AChE activity. Constitutive AChE activity did not significantly differ between tolerant and sensitive clones ($F_1 = 3.2$, $p = 0.15$) (Figure 2). Similarly, there was no difference in mean IC50 (i.e., target-site sensitivity) between tolerant and sensitive clones ($F_1 = 0.71$, $p = 0.45$) (Figure 3).

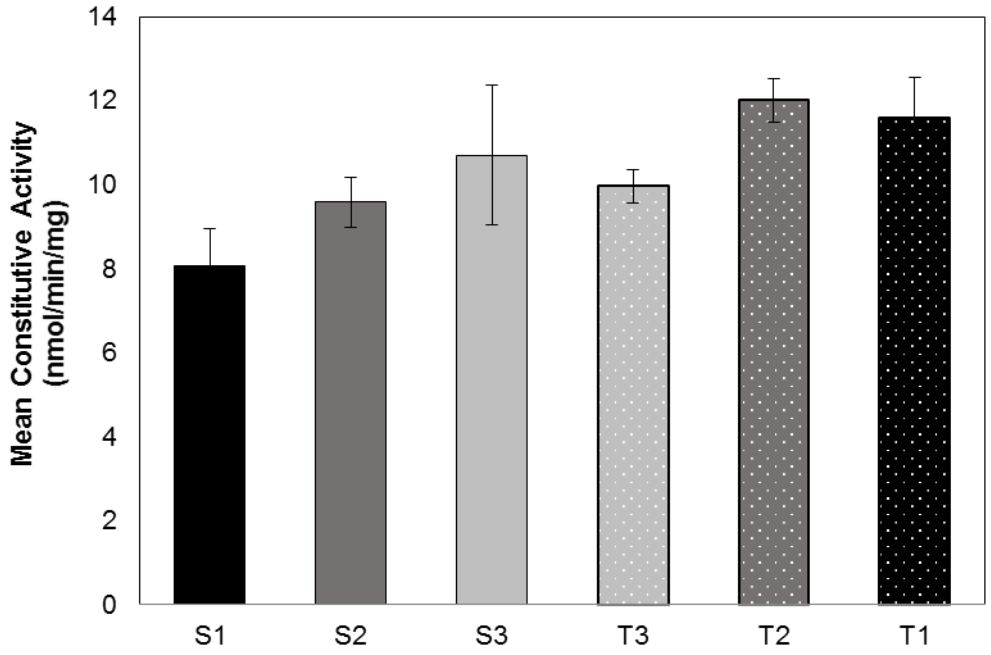

Figure 2. *Constitutive acetylcholinesterase activity of sensitive and tolerant clones.* *In vitro* acetylcholinesterase activity was quantified via Ellman method in the sensitive (S1-S3; solid bars) and tolerant (T1-T3; spotted bars) clones. Enzyme activity was standardized by protein content, and expressed as nmol of hydrolyzed substrate/minute/mg of protein. Mean activity did not differ between sensitive and tolerant clones ($p = 0.15$). Error bars represent standard deviation of the mean.

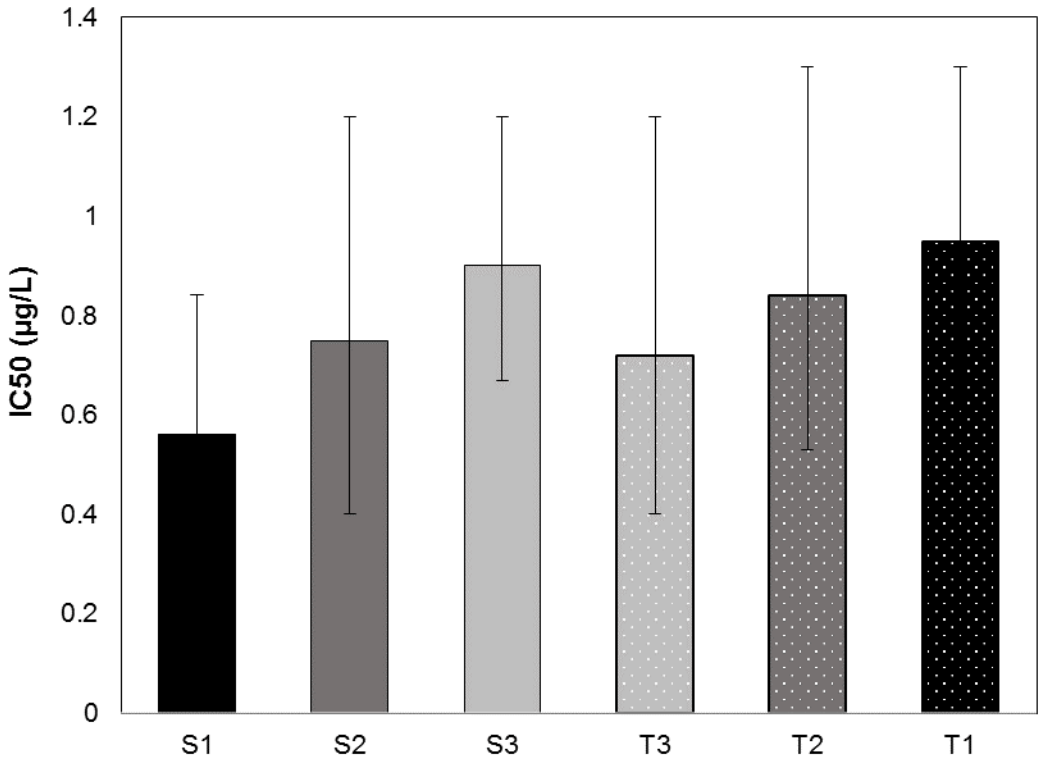

Figure 3. *Enzyme sensitivity to CPF-oxon in sensitive and tolerant clones*.

In vitro acetylcholinesterase activity was quantified via Ellman method in the sensitive (S1-S3; solid bars) and tolerant (T1-T3; spotted bars) clones following exposure to six nominal CPF-oxon treatments. Enzyme activity was standardized by protein content, then incorporated into dose-inhibition estimates (IC50s). No significant differences in enzyme sensitivity were detected between sensitive and tolerant clones ($p = 0.45$). Error bars represent 95% confidence intervals.

4. DISCUSSION

The objective of this study was to systematically evaluate the physiological mechanisms underlying temporal variation in chlorpyrifos sensitivity among representatives of a resurrected *D. pulicaria* population. Specifically, we examined the

most commonly described mechanisms of tolerance to organic toxicants, metabolic and target-site tolerance. We found strong evidence suggesting that metabolic tolerance mechanisms are largely responsible for the variation in chlorpyrifos sensitivity within this population.

4.1 Assessment of Metabolic Tolerance Mechanisms

Biotransformation is a crucial component of xenobiotic metabolism, as it is responsible for the conversion of toxic compounds into less toxic, readily eliminated metabolites (Belden and Lydy 2000). This process is usually initiated by Phase I enzymes, such as CYPs. As described in the Introduction, the biotransformation of chlorpyrifos is less straightforward than most other pesticides. Phase I reactions—carried out by various CYPs—can produce both non-toxic metabolites (predominantly 3,5,6-trichloro-2-pyridinol) and a highly potent oxygen analog (CPF-oxon) (Giesy et al. 1999). Here, we determined the relative contribution of Phase I bioactivation and subsequent CPF-oxon catabolism to the variation in sensitivity between clones. Our findings suggest that this variation is almost entirely explained by differences in both pathways, evidenced by the results of Experiment II. Exposing the clones to CPF-oxon and PBO accounted for inherent differences in both bioactivation and detoxification potential. Strikingly, factoring out these physiological mechanisms resulted in drastically different phenotypes; in the most extreme case, this reduced the disparity between S1 and T1 from a 10-fold difference to an insignificant 1.1-fold difference in EC50. These data strongly implicate biotransformation as the primary mechanism of tolerance to chlorpyrifos.

While these toxicity tests shed light on several aspects of biotransformation, some details regarding these processes remain unclear. For example, Experiment I was

designed to assess the importance of bioactivation by exposing the clones to the oxon form. However, this design also factored out the conversion of chlorpyrifos to non-toxic derivatives. Our toxicity test approach did not enable us to examine the bioactivation and detoxification of chlorpyrifos independently, as the addition of PBO would have inhibited both pathways simultaneously (Ankley and Collyard 1995). Furthermore, Experiments I and II provided indirect assessments of CYP activity, rather than direct quantification of chlorpyrifos biotransformation. Such approaches are regularly performed for more conventional test subjects (Abu-Qare and Abou-Donia 2001, Tang et al. 2001); however, numerous logistical concerns hamper *in vitro* and *in vivo* assays in small crustaceans (Gottardi et al. 2015). Nevertheless, our approach clearly demonstrated the contribution of metabolic tolerance mechanisms in this *D. pulicaria* population.

4.2 Assessment of Target-Site Tolerance Mechanisms

Chlorpyrifos elicits toxicity by irreversibly binding and phosphorylating AChE (Čolović et al. 2013). Our assessment of target-site tolerance examined the possibility of higher constitutive activity and reduced AChE sensitivity in tolerant clones via *in vitro* micro-plate assays. We found no significant differences in either parameter between sensitive and tolerant clones. Interestingly, the AChE activities of our *D. pulicaria* clones were noticeably lower than the activities reported in similar studies (Barata et al. 2001). The disparity might be explained by interspecific variation in this trait (*D. pulicaria* vs. *D. magna*), or perhaps by differences in biomass.

4.3 Conclusions

Based on our broad assessment of the biochemical properties exhibited by our *Daphnia* population, we conclude that the variation in sensitivity is largely explained by

differences in biotransformation. Conversely, we found no evidence to implicate target-site tolerance mechanisms. It is important, however, to acknowledge that we did not evaluate all potential mechanisms. Indeed, other traits could hypothetically affect the toxicokinetic and toxicodynamic properties of organic toxicants. The severity of exposure might be reduced through changes in behavior (Zalucki and Furlong 2017) and uptake (Sun et al. 2017), although these phenomena have not been described in *Daphnia*. Within the genus, general esterases (e.g., carboxylesterase) have been shown to be sensitive to other cholinesterase-inhibiting compounds (Barata et al. 2004). Elevated levels of these enzymes can buffer against neurotoxicity by sequestering the toxic molecules, thus reducing their ability to bind to the primary target, AChE. We cannot rule out this mechanism within the context of our system; however, given the definitive results from the toxicity tests, it seems unlikely that target-site mechanisms play a significant role in dictating tolerance to chlorpyrifos in this scenario.

The current study was motivated by the discovery of pronounced intra-population variation in sensitivity to chlorpyrifos. We postulated three potential evolutionary scenarios: 1) direct selection in response to historical chlorpyrifos exposure, 2) cross-tolerance acquired via historical exposure to other organic contaminants, or 3) indirect selection in response to cultural eutrophication. The findings of the current study aid in evaluating the validity of these hypotheses. Hypothetically, the discovery of target-site tolerance mechanisms would have provided strong support for the first hypothesis: the evolution of such traits is often a consequence of direct selection driven by exposure to a specific class of pesticide with a distinct mode of action (Le Goff et al. 2005, Soderlund 2012). The origins of metabolic tolerance, however, are sometimes more ambiguous, as

the overexpression or induction of metabolic enzymes could conceivably be an adaptive response to a variety of natural or anthropogenic stressors. Given that we found no evidence for target-site tolerance, it is less likely that tolerance was acquired from a direct selection scenario involving chlorpyrifos. Thus, we speculate that metabolic tolerance in our population may have evolved in response to 1) the presence of toxic food sources (e.g., cyanobacteria), 2) exposure to anthropogenic organic contaminants other than cholinesterase-inhibiting insecticides, or 3) the myriad of physiological stressors associated with eutrophication (e.g., disrupted elemental homeostasis). Further insight concerning these hypotheses might be acquired by parsing out the differences in xenobiotic biotransformation in greater detail.

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CHAPTER IV

DEVELOPMENT OF A COMPREHENSIVE *IN VIVO* BIOTRANSFORMATION ASSAY FOR FRESHWATER INVERTEBRATES

ABSTRACT

Toxicity testing with small-bodied invertebrates often requires considerable biomass, which generates a need for biomass-efficient assessments of toxicant sensitivity. The development of *in vivo* assays addresses several of the logistical issues associated with invertebrate toxicology; however, few *in vivo* protocols have been developed for invertebrate models. The current study aims to validate an *in vivo* assay that quantifies biotransformation potential of toxins and toxicants in *Daphnia magna*. Organisms were exposed to three substrates that are common surrogates for measurement of biotransformation potential—*p*-nitroanisole (*p*-NA), 7-ethoxycoumarin (7-EC), and 1-naphthyl acetate (1-NpA)—for six hours. Subsequently, the metabolites of these substrates were extracted from the water, derivatizated (BSTFA; [N, O-Bis[trimethylsilyl] trifluoroacetamide]) to improve detection, and analyzed via gas chromatography/mass spectrometry. Two additional species were tested to validate the assay: *Physa acuta* and *Chironomus dilutus*. *Physa* snails exhibited detectable activity in the presence of all substrates. Only 1-NpA activity

was detected in *D. magna* and *C. dilutus*. This assay permits a comprehensive evaluation of xenobiotic metabolism for *Physa* snails, but raises questions regarding the suitability of conventional metabolic substrates for daphniids.

1. INTRODUCTION

The primary objective of ecotoxicology research is to characterize population- and community-level responses to environmental toxicants. Such investigations typically employ a tiered testing approach, where experimental complexity increases with each tier (Suter 2008). Within the lower tiers, the sensitivity of a species is assayed via acute toxicity tests, which are conducted to determine toxicity in a "worst case" exposure scenario (i.e., in a beaker) (Norton et al. 1992). In these assays biological/environmental relevance is sacrificed for experimental precision and control, allowing for the standardization of methods and reproducibility of results.

Invertebrate species within the phylum Arthropoda are commonly incorporated into lower-tier assessments of risk in aquatic ecosystems, as a substantial percentage of pesticides are engineered to target arthropod pests (Depledge and Fossi 1994, Roman et al. 2007). *Daphnia*—a genus of predominantly freshwater crustacean species—are among the most studied in ecotoxicology, largely due to their asexual life cycle and ease of culture (Shaw et al. 2008). Research with this taxonomic group has provided valuable insight into how toxicant exposure can alter processes across multiple levels of biological organization (Barata et al. 2002, Bendis and Relyea 2016, Orsini et al. 2016).

Conventional toxicity testing with small-bodied invertebrates, such as *Daphnia*, usually requires extensive laboratory culturing of the organisms to generate sufficient biomass to assess sensitivity to multiple toxicants. One solution to this issue is the development of *in vitro* techniques, which can identify alternative endpoints that potentially serve as proxies for organismal sensitivity. These approaches are becoming increasingly high-throughput, improving the utility of *in vitro* assays for large-scale risk assessments in public and private sectors (Judson et al. 2014). For vertebrate models, the use of *in vitro* techniques significantly reduces the number of organisms sacrificed in an experiment. A similar experiment with *Daphnia*, however, requires the homogenization of a whole individual, or even pools of individuals, which introduces complications and reduces the convenience of *in vitro* assays from a biomass standpoint.

While biomass is not usually a limiting factor when working with established laboratory cultures, it is a principal concern for assessments of toxicant sensitivity in wild populations. For example, if one is interested in formally evaluating the variation in sensitivity present within a *Daphnia* population, it is essential to 1) acquire and successfully establish genetically-distinct cultures, 2) culture sufficient biomass, and 3) conduct a lethal assay (e.g., toxicity test or *in vitro* assay). The labor and expense of conducting such research greatly reduces the number of genotypes or populations that can be tested.

An ideal alternative to the approach described above would be to identify reliable, non-lethal endpoints that can be observed *in vivo*. An *in vivo* design would be biomass-efficient and potentially non-destructive, which would enable repeated sampling and eliminate the need for establishing laboratory cultures. The selection of an adequate

endpoint is challenging, as it must be non-lethal, measurable *in vivo*, and strongly correlated with organismal sensitivity. Metrics of xenobiotic metabolism (i.e., biotransformation) show great promise as candidate endpoints, because the toxicity of an organic compound is highly dependent on the manner in which it is biotransformed (Livingstone 1998). Biotransformation processes recruit a myriad of metabolic enzymes, including cytochrome P-450s (CYPs) and esterases (Ranson et al. 2002). Both enzyme families are known to participate in the biotransformation of most common-use insecticides, such as organophosphates, carbamates, and pyrethroids.

Measurement of CYP and esterase activity is commonly accomplished by exposing organisms or homogenized tissue to non-toxic compounds that are known substrates of the enzymes. The production of the expected metabolites from these reactions is then quantified, which allows for a direct determination of enzyme activity. For example, the CYP-mediated *O*-dealkylation of 7-ethoxyresorufin, 7-methoxyresorufin (7-MR; MROD activity), and 7-ethoxycoumarin (7-EC; ECOD activity) is commonly quantified via spectrofluorometry, and has been associated with tolerance to organic contaminants (Lee and Scott 1989, González Audino et al. 2004, Clark and Di Giulio 2012). Similarly, the enhanced *in vitro* biotransformation of *p*-nitroanisole (*p*-NA; PNOD activity) has been correlated with resistance to pyrethroids (Thomas et al. 1996, Chen et al. 2005) and neonicotinoids (İşci and Ay 2017). Esterase activity is also known to be representative of organismal sensitivity: enhanced *in vitro* biotransformation of 1-naphthyl acetate (1-NpA) has been detected in several pest species, and associated with tolerance to cholinesterase-inhibiting insecticides (Wang et al. 2010, Buzzetti et al. 2016). Recently, efforts to quantify the catabolic activity of CYPs

and esterases *in vivo* have generated mixed results, particularly in crustaceans (Day and Scott 1990, Sturm and Hansen 1999, Gottardi et al. 2016). Consequently, it remains unclear whether the conventional metabolic substrates are suitable for such organisms, particularly *Daphnia*.

The objective of this study was to determine if an *in vivo* biotransformation assay can be used to measure biotransformation potential for daphniids. Thus, *D. magna* were exposed to two CYP substrates (7-EC, *p*-NA) and one esterase substrate (1-NpA) simultaneously, and the *in vivo* biotransformation of these compounds into their primary metabolites was quantified via gas chromatography (GC-MS). Two additional freshwater invertebrate species (*Physa acuta* and *Chironomus dilutus*) were also evaluated to validate the assay. These species belong to taxonomic groups that are known to exhibit detectable activity to at least one of the three substrates, and thus served as positive controls for the experiment (Gagnaire et al. 2010, Chen et al. 2015, Otero and Kristoff 2016).

2. MATERIALS & METHODS

2.1 Experimental Organisms

D. magna were graciously provided by ATC Group Services (Oklahoma City, OK), and cultured in low-nutrient COMBO media (Kilham et al. 1998). *Daphnia* were fed 3 mL of *Scenedesmus acutus* (3×10^7 cells/mL) daily. Adult *Physa* were collected from Sanborn Lake near Stillwater, OK (UTM: 36°15'36.6"N, 97°07'61.5"W), and housed in plastic bins with dechlorinated water until egg masses were deposited. The egg masses (containing F1 snails) were isolated into separate containers, and allowed to develop and hatch. F1 snails were randomly selected for the assay 12 days post-hatching. *C. dilutus*

were purchased from Aquatic Biosystems (Ft. Collins, CO). The chironomids arrived as third instar larvae, and were immediately used in the assay. All animal cultures were maintained in a temperature-controlled room (21˚C ± 1˚C) under a 16:8 light cycle.

2.2 Chemicals

7-EC was purchased from Alfa Aesar (99% purity). *p*-NA and 1-NpA were purchased from Sigma Aldrich (97% and 98% purity, respectively). 7-HC was purchased from Acros Organics (99% purity), while *p*-NP and 1-NpT were acquired from Fluka Analytical (both 99.9% purity). The derivatization agent, N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA), was acquired from Fluka Analytical. Stock solutions for the substrates were prepared as follows: 4.0 mM 7-EC in DMSO, 7.3 mM *p*-NA in methanol, and 6.6 mM 1-NpA in methanol. Similarly, stock solutions for the metabolic products were prepared as follows: 6.0 mM 7-HC in DMSO, 5.8 mM *p*-NP in methanol, and 5.4 mM 1-NpT in methanol. All organic solvents were reagent-grade.

2.3 Exposure Protocol

The assay media—composed of 6 µM concentrations of each substrate dissolved in COMBO media (final volume = 60 mL)—was prepared fresh before the experiment. The concentration of DMSO and methanol from the stock solutions did not exceed 0.15% of the test volume. Exposures were conducted in 5 mL of assay media contained within 20 mL scintillation vials. The experimental units contained either 10 adult *D. magna*, five chironomids, or four snails; these treatments were each replicated in triplicate. Additionally, duplicate blanks (assay media without organisms) were prepared to evaluate the potential for hydrolysis of the substrates. All organisms and blanks were

exposed to the assay media for six hours, after which extractions were conducted immediately.

2.4 Extraction of Analytes

Following the six-hour exposure period, a three-step liquid-liquid extraction was performed on each sample using diethyl ether. During each step of the extraction, a 3 mL volume of diethyl ether was added to the water samples, followed by 2 minutes of vigorous mixing by a vortex mixer (VWR). After each step, the organic layer (supernatant) was transferred to a 10 mL borosilicate conical vial, and evaporated to dryness under a gentle stream of nitrogen gas. In order to rinse the conical vials, 1 mL of fresh diethyl ether was added to each vial, then transferred to separate 2 mL amber GC vials. The solvent in the GC vials was once again evaporated to dryness under nitrogen gas, after which 0.1 mL of the derivatization agent (BSTFA) was added. Due to the chemical properties of the analytes, derivatization was required to improve the detection of these compounds via GC-MS. Derivatization was accomplished by incubating the samples with BSTFA for 60 minutes at 60˚C on a temperature-controlled shaker table (VWR). Following the incubation period, the samples were allowed to cool, after which 0.3 mL of hexane was added to bring the final volume of each sample up to 0.4 mL. The efficiency of this extraction protocol was assessed by performing the above steps on equal volumes of COMBO media spiked with a known concentration of each analyte.

2.5 Sample Analysis

The concentration of each analyte was quantified using gas chromatography-mass spectrometry (Agilent 5975c). The gas chromatography inlet was set to 70˚C, while the oven increased by 12˚C/minute until it reached 295˚C (total run-time = 26 minutes). Prior

to analysis of the experimental samples, a derivatized sample containing 5000 ng/mL of each analyte was used to identify ions for quantitation. Select ion monitoring successfully identified the analytes using the following ions: 7-HC (219, 234), 4-NP (196, 211), 1-NpT (185, 201, 216). High (6500-406 ng/mL) and low (320-5 ng/mL) calibration curves containing all three analytes were prepared and derivatized; quantitation based on these curves was conducted by external calibration. The metabolic activity of each substrate in the blanks and samples was expressed as the ng of analyte produced/individual/hour.

2.6 Data Analysis

The substrates were considered suitable metrics of metabolism in a species if *in vivo* activity was above the method detection limit (MDL) of the substrate. Substrate-specific MDLs were calculated from the standard deviation of control/blank activity:

MDL = (S.D. of control activity) X 10

The 10:1 signal-noise ratio serves as a conservative "safety factor" to ensure that detection is well above background (Konieczka and Namiesnik 2016).

3. RESULTS

3.1 Quality Control

The overall efficiency of the extraction method was 102 ± 25%. The extraction efficiency of each individual analyte were as follows: 7-HC (124 ± 15%), *p*-NP (92 ± 37%), and 1-NpT (91 ± 14%). The MDL for each substrate were as follows: 7-EC = 5.2 ng/hour, *p*-NA = 0.4 ng/hour, 1-NpA = 8 ng/hour.

3.2 In Vivo Biotransformation of Substrates

The success of this assay varied by species. *Physa* exhibited detectable activity to all substrates, with activities ranging from 1.7 to 6.8-fold above their respective MDLs (Figure 1A-C). *D. magna* and *C. dilutus* exhibited detectable activity for the esterase substrate only (1-NpA), with 3.2- and 6.5-fold higher activity relative to the MDL, respectively (Figure 1C).

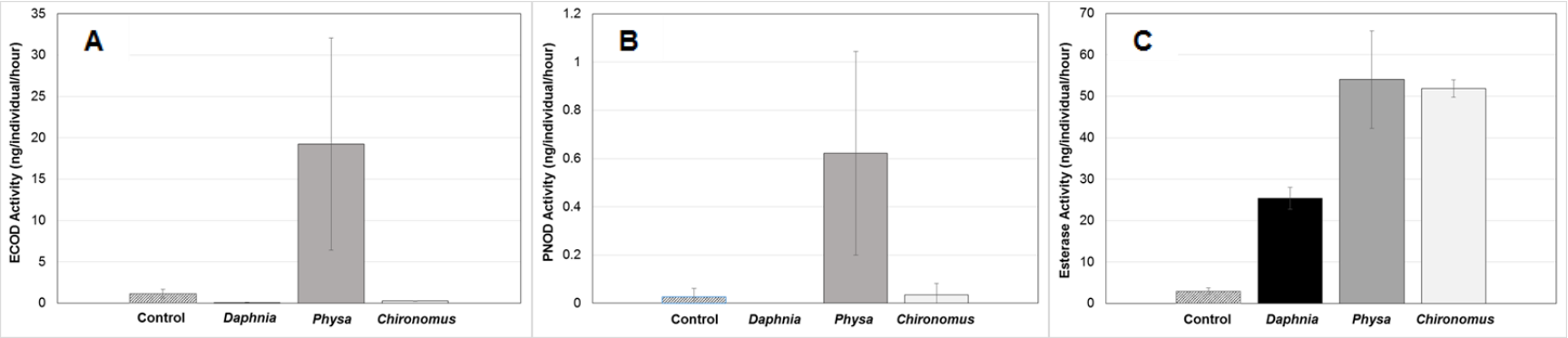

Figure 1. *Biotransformation of three metabolic substrates in freshwater invertebrates.*
Panel A: *In vivo* metabolism of 7-ethoxycoumarin into 7-hydroxycoumarin (i.e., ECOD activity) over six hours, expressed in ng of 7-HC produced/individual/hour. Only *Physa* exhibited activity above the method detection limit (MDL; 5.2 ng/hr). Panel B: *In vivo* metabolism of *p*-nitroanisole into *p*-nitrophenol (i.e., PNOD activity) over six hours, expressed in ng of *p*-NP produced/individual/hour. Only *Physa* exhibited activity above the MDL (0.4 ng/hr). Panel C: *In vivo* metabolism of 1-naphthyl acetate into 1-naphthol (i.e., esterase activity) over six hours, expressed in ng of 1-NpT produced/individual/hour. All species exhibited esterase activity above the MDL (8 ng/hr). Error bars represent standard deviation around the mean.

4. DISCUSSION

The objective of this study was to quantify *in vivo* biotransformation of various metabolic substrates in *Daphnia* simultaneously. We were able to successfully quantify the biotransformation of a predominantly esterase substrate (1-NpA) in *Daphnia*. Previous studies have used 1-NpA to quantify carboxylesterase activity *in vitro* in *D. magna* (Barata et al. 2004, Trac et al. 2016), but to our knowledge, this is the first successful *in vivo* assay using this substrate with *Daphnia*. Our ability to detect activity from at least one of the substrates is promising, because it demonstrates the validity of the assay, provided a suitable substrate is used. The legitimacy of the study design is further strengthened by the detection of activity in multiple arthropod taxa. Chironomids are frequently used in ecotoxicology research, and have been shown to exhibit measurable activity to numerous substrates *in vitro* (Forcella et al. 2007, Chen et al. 2015) and occasionally *in vivo* (Gottardi et al. 2016); however, our study is the first to detect 1-NpA activity in chironomids *in vivo*. Additionally, our assay detected activity to all three substrates in *Physa* snails. While an *in vivo* assay has recently been optimized for measuring ECOD activity in other freshwater snail species (Gagnaire et al. 2010), our study is the first to quantify PNOD and 1-NpA activity *in vivo* in snails. The ability to quantify the metabolism of three substrates simultaneously is extremely valuable for future studies, in that it permits a more comprehensive assessment of xenobiotic metabolism. Furthermore, this assay yielded detectable activity using little biomass (e.g., four snails); this low biomass requirement greatly reduces the logistical concerns associated with assessments of population-level sensitivity.

We were unable to detect activity to any of the CYP substrates (7-EC, *p*-NA) in *D. magna*. The inability to detect activity to the CYP substrates could be a consequence of insufficient biomass; it is possible that the production and subsequent excretion of the analytes was too low to detect, and that an increase in the number of organisms per experimental unit would have improved the detection of the chosen analytes. The most likely explanation, however, is that *Daphnia* simply do not exhibit detectable activity to conventional CYP substrates. Indeed, this is not the first study that failed to detect activity in *Daphnia*: other studies have reported negative results using the same substrates in *Daphnia* and other aquatic invertebrates (Livingstone 1991, Sturm and Hansen 1999), although these assays were performed *in vitro*. Only one study has successfully quantified *in vivo* ECOD activity in *D. magna* (Gottardi et al. 2016). It is important to note, however, that personal attempts to reproduce their findings have failed, despite strictly adhering to their published protocol (*unpublished data*). Further, it is known that *Daphnia* and other crustaceans possess CYPs, and that these enzymes do play a role in xenobiotic detoxification (Akkanen and Kukkonen 2003, Connon et al. 2003, Simpson et al. *unpublished* – **see Chapter III**). For these reasons, it seems highly likely that 7-EC and *p*-NA are merely poor substrates with which to measure CYP activity in *Daphnia*, and that quantification of biotransformation is hypothetically possible if the appropriate substrates are employed.

5. CONCLUSIONS

This study validated an *in vivo* biotransformation assay for three invertebrate species, although the suitability of the tested substrates was species-specific. Namely, we

found detectable ECOD, PNOD, and 1-NpA activity in *Physa* snails, while *Daphnia magna* and *Chironomus dilutus* only exhibited activity to 1-NpA. The success of this assay is promising for the future development and implementation of *in vivo* techniques in ecotoxicology; however, it also exemplifies the challenge of working with small-bodied invertebrates, particularly *Daphnia*. More research is needed to identify suitable substrates with which to measure CYP activity in *Daphnia.*

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CHAPTER V

THE IMPORTANCE OF EVOLUTIONARY HISTORY AND ENVIRONMENTAL CHANGE IN ECOTOXICOLOGY

The following chapter appears as submitted to *Frontiers in Ecology and the Environment*:

- Human-induced rapid environmental change (HIREC) exerts strong selective forces on contemporary populations.
- HIREC selects for phenotypes composed of favorable suites of physiological traits, most often involving aspects of metabolism.
- Natural populations exhibit variation in tolerance to anthropogenic contaminants, such as pesticides; this intrinsic variation is present even when the contaminant is novel.
- We posit that physiological shifts driven by rapid environmental change can influence naïve populations' susceptibility to novel toxicants.
- We emphasize the importance of considering evolutionary history when evaluating pesticide tolerance scenarios.

Predicting the threat of environmental contaminants, and evaluating how species respond to these stressors, is a central frontier in ecotoxicology. Decades of research have established that populations harbor significant variation in sensitivity to contaminants (e.g., pesticides), and population-level sensitivity can change over time (Oziolor and Matson 2015). Such temporal changes in sensitivity are often a function of *direct selection* imposed by high concentrations of a toxic contaminant that serves as a

persistent selective factor. The evolution of tolerance to pesticides has critical implications for society and human health. Tolerant pest populations can greatly reduce crop yields, and populations of resistant disease vectors (e.g., mosquitoes) can facilitate the transmission of devastating diseases. For these reasons, literature concerning the evolution of pesticide tolerance has historically focused on species that have direct implications for human health (i.e., target species). In these scenarios, *tolerance ratios* are usually steep, as these organisms are the intended targets of the toxicants. For example, field-collected populations of crop pests, such as the fall army worm (*Spodoptera frugiperda*), have been shown to exhibit extreme tolerance to pyrethroid, organophosphate, and carbamate insecticides; these populations were upwards of 216-fold, 271-fold, and 192-fold more tolerant, respectively, compared to a susceptible laboratory strain (Yu 1991). Tolerance of this magnitude is likely acquired via direct selection, a process that can drive rapid evolution when populations are exposed to frequent, high doses of the pesticide.

When considering exposure scenarios in non-target organisms, it is also frequently assumed that the differences in tolerance are a direct result of exposure to either the contaminant of interest, or perhaps other compounds with similar routes of toxicity and/or metabolism (i.e., *cross-tolerance*) (Coors et al. 2009, Bendis and Relyea 2014). This assumption is justified in some systems. For example, killifish (*Fundulus* spp.) have been observed inhabiting estuarine sites that are heavily contaminated with carcinogenic compounds such as polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) (Clark and Di Giulio 2012, Whitehead et al. 2012). In these established systems, it is highly likely that tolerance has evolved as a direct

consequence of chronic, intense contaminant exposure. However, when assessing the risk of pesticides to other non-target species, exposure is often unintentional and occurs via agricultural runoff or incidental contact from other non-point sources. Most current-use pesticides are 1) specifically designed to have shorter environmental half-lives than those of the persistent legacy compounds of the 20th century, 2) only applied to part of the landscape, and 3) have been regulated based on risk assessments to avoid severe contamination. Thus, non-target organisms are less likely to be frequently exposed to high concentrations of pesticides compared to target species. One notable exception involves populations of hyper-tolerant amphipods (*Hyalella* spp.) inhabiting pyrethroid-contaminated streams, exhibiting as high as a 550-fold difference in sensitivity compared to reference populations (Weston et al. 2013). Given that certain pyrethroids can have long half-lives in the sediment (relative to most current-use pesticides), it seems likely that the benthic life history of this species might have exacerbated exposure to the pesticide. Nevertheless, situations such as this are not necessarily reflective of most contemporary exposure scenarios involving non-target species. Whether milder exposure scenarios are sufficient to result in direct selection for tolerance remains to be explicitly addressed within ecotoxicology.

It is also possible that population-level susceptibility to pesticides may change due to rapid evolution in response to other agents of environmental change. Human-induced rapid environmental change (HIREC) can drastically alter the structure and function of ecosystems (Palumbi 2001). HIREC can impose selective pressure on contemporary populations through an assortment of stressors, including climate change, habitat fragmentation, and various forms of pollution (Sih et al. 2011). Populations can

rapidly respond to these threats via *acclimatization*, *adaptation*, or a combination of both (Somero 2010). Such environmental change along multiple axes often results in multifarious selection, acting on suites of interacting behavioral and physiological traits (Chapin et al. 1993, Egea-Serrano et al. 2014). For example, microevolutionary shifts in nutrient utilization and metabolism have been empirically demonstrated in a *Daphnia* population experiencing cultural eutrophication (Frisch et al. 2014). These physiological shifts were also shown to alter the population's susceptibility to a current-use pesticide, possibly implicating *indirect selection* in the acquisition of tolerance to the novel contaminant (Simpson et al. 2015; Simpson et al. *in prep* – see **Box 1**).

The nearly ubiquitous use of pesticides, coupled with the biotic and abiotic consequences of HIREC, poses significant challenges for contemporary populations. In order to address the complex nature of HIREC-pesticide interactions, it is vital to integrate the responses to these stressors at multiple levels of biological organization (Hooper et al. 2013). Indeed, current interdisciplinary assessments of these responses are increasingly adopting a multiple-stressors approach (see Kimberly and Salice 2015). Such experiments typically treat environmental change as an additional stressor to pesticide exposure, which is an effective way to provide information concerning the potential interactions of co-occurring challenges (Almeida et al. 2015). Although HIREC and pesticides are indeed co-occurring threats, these conditions are not necessarily novel for some populations. Climate change has rapidly progressed for over a century, and synthetic pesticides have been a staple of pest control since the 1940s; therefore, it is probable that evolutionary change in response to these stressors—encountered simultaneously or in isolation—has already occurred in some populations.

Recent studies have incorporated evolutionary history into predictive frameworks to infer the influence of HIREC on ecological interactions (Sih et al. 2011) and heavy metal sensitivity (Kimberly and Salice 2014); however, the importance of evolutionary history in predicting susceptibility to current-use pesticides remains to be verified. Indeed, the commonly shared physiological mechanisms that underlie organismal responses to both climate change and pesticide exposure indicate that adaptive shifts in response to one of these stressors might affect susceptibility to the other. We propose that an examination of the biochemical mechanisms underlying acclimatization and adaptation to HIREC and pesticides will yield evidence supporting alternative origins of pesticide tolerance, such as indirect selection. Further, we emphasize the importance of examining past and present environmental histories to better predict the evolutionary trajectories of threatened systems. Here, we illustrate this perspective by *i*) briefly reviewing mechanisms of tolerance to global climate change and current-use pesticides, and *ii*) examining the environmentally-driven shifts in physiology that could alter pesticide susceptibility.

Mechanisms of Tolerance to Pesticides

The most commonly described mechanisms of tolerance to pesticides are typically classified into two categories: metabolic and target-site tolerance (Hemingway et al. 2004). Individuals exhibiting metabolic tolerance frequently possess enhanced detoxification and clearance abilities, which are usually mediated by the amplification and/or overexpression of genes that control toxicant metabolism (Li et al. 2007). Biotransformation (i.e., metabolism) of foreign compounds is largely performed by cytochrome P450 enzymes (CYPs); consequently, mechanisms of tolerance in both target

and non-target species often involve altered CYPs (Scott 1999). For example, in disease-carrying mosquitoes (*Anopheles arabiensis*), the overexpression of a specific CYP was quantitatively linked to high levels of tolerance to multiple pyrethroid insecticides (Ibrahim et al. 2016). Further, molecular docking simulations indicated that this single CYP exhibited an enhanced ability to break down specific pyrethroids, thus reducing their toxicity. Conversely, selection might favor *decreased* CYP activity if the toxicant of concern is metabolically activated by these enzymes (i.e., toxicity of metabolite > parent compound). For instance, the general mechanism of PCB and PAH tolerance in the previously described killifish system involves a decrease in responsiveness of the aryl hydrocarbon receptor pathway, an essential regulator of xenobiotic metabolism (including CYPs) in vertebrates (Oziolor et al. 2014).

Target-site tolerance similarly results in a considerable reduction in sensitivity, often as a consequence of point mutations within the genes that encode the receptors or enzymes targeted by the pesticide (Hemingway et al. 2004). In extreme cases a mutation can alter enzyme or receptor structure in such a way that it effectively prevents the toxicant from exerting its toxic effect altogether; in these situations, tolerance ratios are expected to be high (Soderlund and Knipple 2003). High tolerance ratios, resulting from a target-site mutation, have been measured in populations of the plant bug (*Apolygus lucorum*) in China (Wu et al. 2015). These populations possessed a point mutation in the *ace-1* gene, which is responsible for the production of acetylcholinesterase. Acetylcholinesterase is the target of many neurotoxic pesticides (e.g., organophosphates and carbamates); this mutation alters the amino acid composition of acetylcholinesterase, resulting in an enzyme that is less sensitive to the toxic effects of these pesticides.

Mechanisms of Tolerance to Global Climate Change

Climate change is one of the best studied stressors associated with HIREC. Several studies examine the potential for evolution in this context. Schoville et al. (2012) investigated differences in thermal tolerance between copepods (*Tigriopus californicus*) inhabiting intertidal habitats along the coast of California. Using next generation RNA sequencing (RNAseq), they demonstrated that the northern and southern populations of copepods exhibited differential gene expression. Specifically, the southern population exhibited greater up-regulation of genes that produce heat-shock proteins and ubiquitin; these proteins are instrumental in minimizing the cellular damage that is associated with thermal stress. In addition to heat-shock proteins, various metabolic enzymes have been implicated as a thermal tolerance mechanism. Rosic et al. (2010) examined the effect of thermal stress on CYPs in a coral-symbiont dinoflagellate species (*Symbiodinium*) using quantitative PCR. Following exposure to a moderately elevated temperature (+6˚C above average temperature), the dinoflagellates exhibited a two- to four-fold increase in expression of CYP genes, demonstrating that thermal stress can induce greater metabolic activity. However, CYP gene expression drastically decreased after exposure to more severe heat stress (+9˚C above average temperature). Thus, they concluded that thermal stress can potentially induce an organism's metabolism, although this effect is only expected to occur at temperatures at or below the upper critical thermal limit.

Shifts in the expression of metabolic/maintenance enzymes have been empirically established in other taxonomic groups. McCairns et al. (2016) utilized RNAseq to evaluate transcriptional variation in captive Australian rainbow fish (*Melanotaenia duboulayi*) raised at different temperatures in a common-garden design. They

demonstrated that the fish which were raised at temperatures near their upper critical thermal limit significantly up-regulated multiple genes involved with metabolism and oxidative stress. These genes encode the production proteins that might be adaptive in warm environments, such as heat-shock proteins, CYPs, and other metabolic enzymes. Furthermore, McCairns et al. (2016) discovered that a significant fraction of the overall transcriptional variance observed was explained by additive genetic effects; in other words, the transcriptional plasticity in these potentially adaptive candidate genes has an underlying genetic basis. Consequently, if wild populations of rainbowfish were to experience selection from climate change, it is conceivable that these acclimatory responses could become heritable adaptations. Such evolutionary shifts in physiology could allow persistence in unpredictable environments, or alter responses to future environmental stressors.

Integrating Evolutionary Responses to Environmental Stressors

Based on the many (but non-exhaustive) examples described above, physiological responses to selection imposed by HIREC and pesticides commonly involve some aspect of metabolism. Considering the diversity of biochemical functions that are classified under metabolism, this is not surprising. The molecular participants (e.g., enzymes and cofactors) responsible for carrying out these essential metabolic processes are often highly conserved across taxonomic groups (Peregrín-Alvarez et al. 2009). Included among these conserved metabolic components is the CYP superfamily, which encompasses a broad group of enzymes that metabolize endogenous and exogenous molecules. These enzymes possess broad substrate specificities and are often the first line of defense against toxic chemicals. In other words, CYPs serve as a metabolic Swiss

Army knife: they can effectively break down toxic by-products of other metabolic processes, as well as detoxify a variety of xenobiotic compounds encountered in the environment.

It is well understood that pesticide exposure can result in direct selection for metabolic tolerance, usually involving CYPs. We posit that selection on a trait which utilizes a suite of CYPs could *indirectly* result in selection for other traits, especially if this collection of CYPs is capable of performing other metabolic functions. Evolutionary theory refers to this phenomenon as indirect selection. It is evident that HIREC has driven evolutionary shifts in contemporary populations, but how do these adaptive responses influence susceptibility to pesticides? Is there potential for environmentally-induced evolution to indirectly select for populations that are less (or more) tolerant to a novel stressor (**Figure 1)**? Delving into the molecular properties of metabolism reveals a plausible link between thermal adaptation and pesticide susceptibility.

Climate science has thoroughly demonstrated that temperature can strongly influence metabolism. It is generally understood that increases in temperature can accelerate a number of chemical processes, including diffusion, respiration, and enzyme activity (Karasov and Martínez del Rio 2007). If these shifts are adaptive in such a stressful environment, then evolution is expected to occur. Many of these processes could play a direct role in pesticide exposure and metabolism: exposure in aquatic organisms often occurs via diffusion, and enhanced enzyme activity (esterases and CYPs) has been repeatedly described as an effective mechanism of tolerance to pesticides (Hemingway et al. 2004). In the context of xenobiotic biotransformation, enzyme activity is clearly important, but so are substrate-enzyme interactions. Temperature has been shown to

directly alter enzyme-substrate binding (**K_M**). As temperature approaches the upper critical thermal limit, enzymes are usually less effective at binding to substrates, signified by a high K_M (Somero 1995). However, enzymes from thermally-adapted species have been shown to exhibit lower K_M values (i.e., greater binding ability) at elevated temperatures compared to non-adapted species (Somero 1995). What does this mean for population-level susceptibility to pesticides? When considering CYPs and esterases, such a change in enzyme kinetics could alter the biotransformation capabilities of a population. Depending on the manner in which selection alters CYP activity, an increase in binding ability could 1) improve the survival of a population facing future exposure to a novel contaminant by enhancing detoxification abilities, or it could 2) increase susceptibility to metabolically-activated compounds (e.g., organophosphate insecticides). Regardless of the specific outcome, it is essential that the evolutionary history of a population is considered when attempting to explain a tolerance scenario or predict a population's response to future insults.

Future Directions

The application of this perspective allows research to advance in multiple areas. First, an awareness of the physiological characteristics that are mutually favored in the presence of natural and anthropogenic stressors might improve our ability to fully understand the responses of non-target populations in systems with multiple environmental challenges. Cursory acceptance of the prevailing assumption—that tolerance in a population was acquired via direct selection—might overlook other equally intriguing evolutionary scenarios (e.g., indirect selection). Indeed, these alternative scenarios are particularly relevant, given that HIREC can potentially act as a more

persistent selective force than most current-use pesticides. The generation of hypotheses that address how adaptation to other HIREC stressors might influence pesticide susceptibility is a critical first step for future investigations into this phenomenon (for eutrophication example, see **Figure 2**). Second, our perspective fosters a more comprehensive understanding of how rapid environmental change influences pesticide tolerance, which might improve our ability to perform pesticide risk assessments for labile environments. We support the recent movements to *i*) construct an interdisciplinary framework featuring climate- and toxicant-induced evolution (Hooper et al. 2013), and *ii*) adopt approaches that address the potential interaction of natural and synthetic stressors in these environments (Kimberly and Salice 2015). While more research is likely required before these concepts are effectively incorporated into regulatory decision-making, the future impact of this line of research will be strengthened by the alternative scenarios discussed in this perspective. Finally, this perspective has implications for unique approaches that seek to predict population-level responses to future challenges. Phylogenetic patterns of pesticide tolerance have been detected in non-target species, and are generally considered to be divergent in nature (Hammond et al. 2012). In other words, closely related species would likely exhibit similar levels of tolerance to a contaminant. However, if other environmental factors (e.g., climate change) are significantly changing susceptibility, then it is possible that local adaptation to these factors might weaken this phylogenetic signal. Thus, the environmental context, as well as the life history of a species, should be interpolated when evaluating patterns of susceptibility for risk assessment purposes.

Conclusions

This perspective highlights the multifarious nature of evolution in the face of modern environmental challenges. We focus on the physiological shifts that are frequently associated with tolerance to both pesticides and climate change, with the aim of encouraging future research to consider unconventional origins of pesticide tolerance. Namely, the potential for HIREC to shift pesticide susceptibility via indirect selection. Although this perspective singularly concentrates on evolutionary history with respect to thermal adaptation, an exhaustive empirical analysis of other HIREC-associated stressors might yield further insight. Indeed, the physiological modifications that underlie adaptive responses to stressors such as eutrophication might have important toxicological ramifications. This perspective should serve as a fruitful extension of the recent efforts to assimilate an evolutionary perspective into ecotoxicology.

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Panel 1: **Helpful Definitions**

Direct Selection – a scenario where evolutionary change occurs *directly* in response to a known selection pressure.

Tolerance Ratio – a metric that compares the sensitivity of a tolerant population relative to a susceptible one. A higher ratio indicates a more tolerant population.

Cross-tolerance – a scenario where the direct evolution of tolerance to a stressor also results in tolerance to a different stressor with a similar mode of action.

Acclimatization – an inducible, within-generation phenotypic response to changing environmental conditions that increases the probability of survival and reproduction; involves plastic traits, which may or may not be heritable.

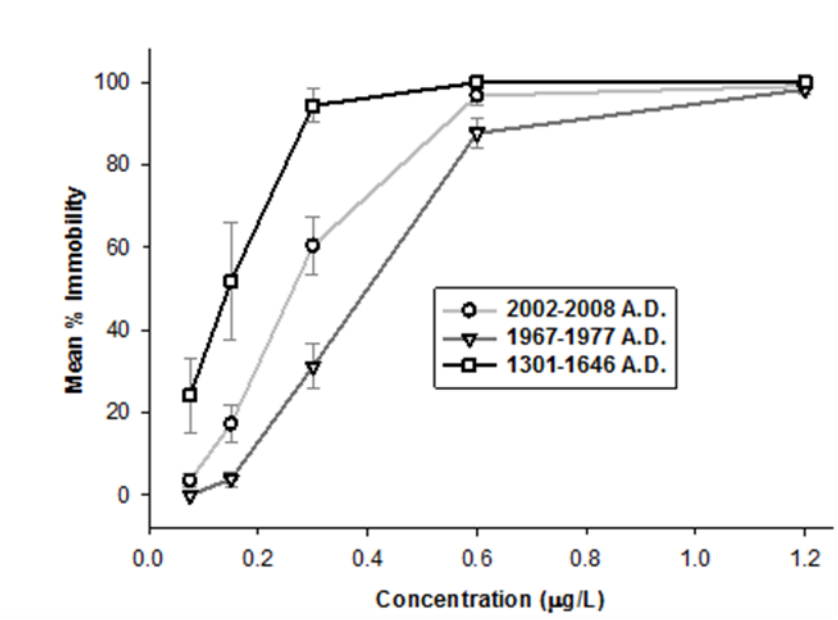
Adaptation – an across-generation genotypic response that increases the probability of survival and reproduction; involves heritable traits.

Indirect Selection – a scenario where direct selection imposed by one selection pressure (Stressor A) results in a phenotype that is also adaptive to another potential selection pressure (Stressor B), despite the absence of direct selection from Stressor B.

K_M – a constant in the Michaelis-Menten kinetic model describing the ligand-binding ability of an enzyme.

Box 1. Insights from Resurrection Ecology

Resurrection ecology utilizes the dormant egg banks of various species to reconstruct ancestral lineages. This technique enables one to link changes in population genetic structure to historical environmental conditions, which provides insight into the population's evolutionary history. Simpson et al. (2015) employed this approach to investigate microevolutionary shifts in pesticide sensitivity within a *Daphnia* population. Acute toxicity tests using chlorpyrifos revealed a temporal trend in sensitivity, where ancient individuals (1301-1646 A.D.) exhibiting greater sensitivity than the more contemporary individuals (1967-1977, 2002-2008 A.D.; see below). Subsequent biochemical assays strongly implicated temporal differences in metabolism as the primary mechanism of tolerance (Simpson et al. *in prep*). As there is no evidence to suggest direct selection for tolerance to chlorpyrifos, these metabolic shifts are potentially the result of evolution in response to an unrelated stressor. The first study conducted on this system demonstrated significant shifts in nutrient physiology concurrent with rapid cultural eutrophication over time, suggesting adaptation to this environmental stressor (Frisch et al. 2014). Thus, Simpson et al. (2015) hypothesized that selection imposed by eutrophication could have resulted in indirect selection for tolerance to a pesticide that was novel to the system.



Adapted from Simpson et al. (2015)

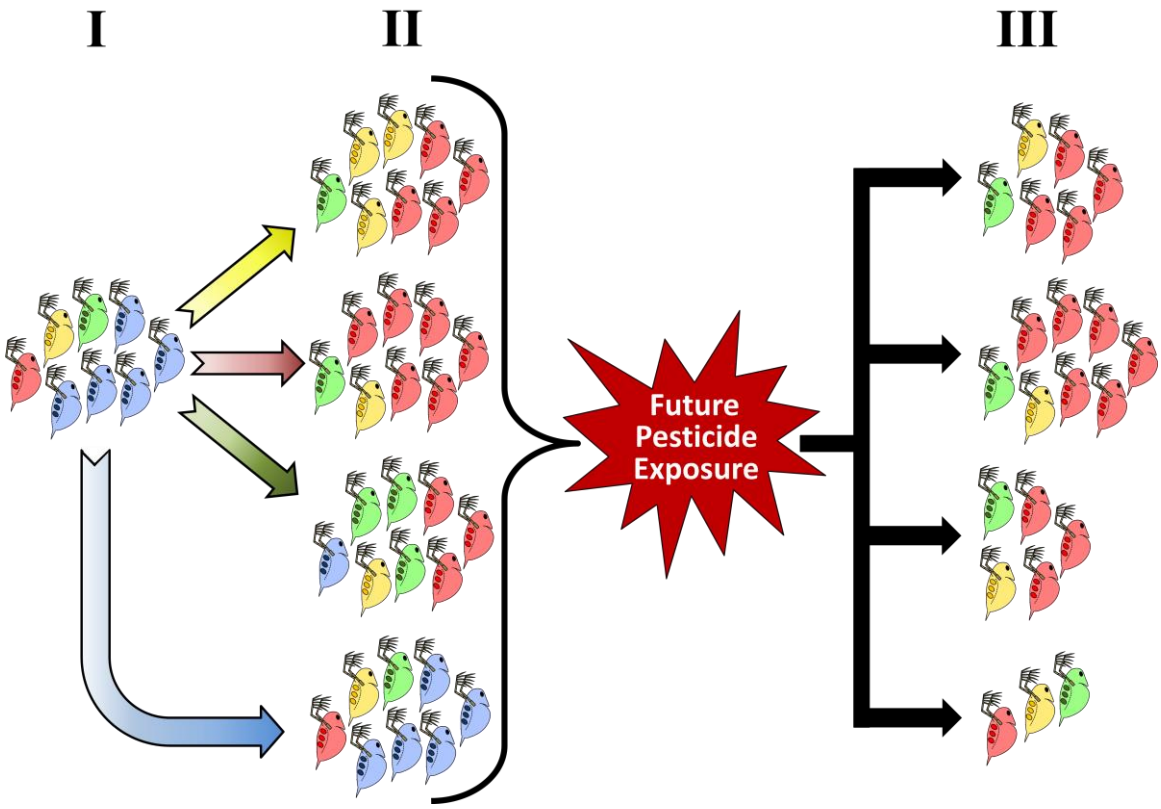


Figure 1. Evolutionary history can influence susceptibility to future anthropogenic stressors.

A naïve population (I) experiences four different evolutionary scenarios: solid arrows represent direct selection imposed by climate change (yellow), pesticide exposure (red), eutrophication (green), and the absence of selection (blue). After several generations under selection, the subsequent cohorts (II) simultaneously experience intense exposure to a pesticide. A comparison of the surviving phenotypes from each surviving cohort (III) theoretically illustrate differential susceptibility to a pesticide exposure event. Due to the presence of phenotypes that are co-favored by independent selection events (red, yellow, green), the cohorts derived from climate change and eutrophic conditions are less severely impacted by the pesticide exposure event (more surviving individuals). Populations that are tolerant to high temperatures, eutrophication, and a pesticide independently evolved metabolic profiles that are mutually adaptive in the presence of a novel pesticide, resulting in reduced susceptibility to future pesticide exposure.

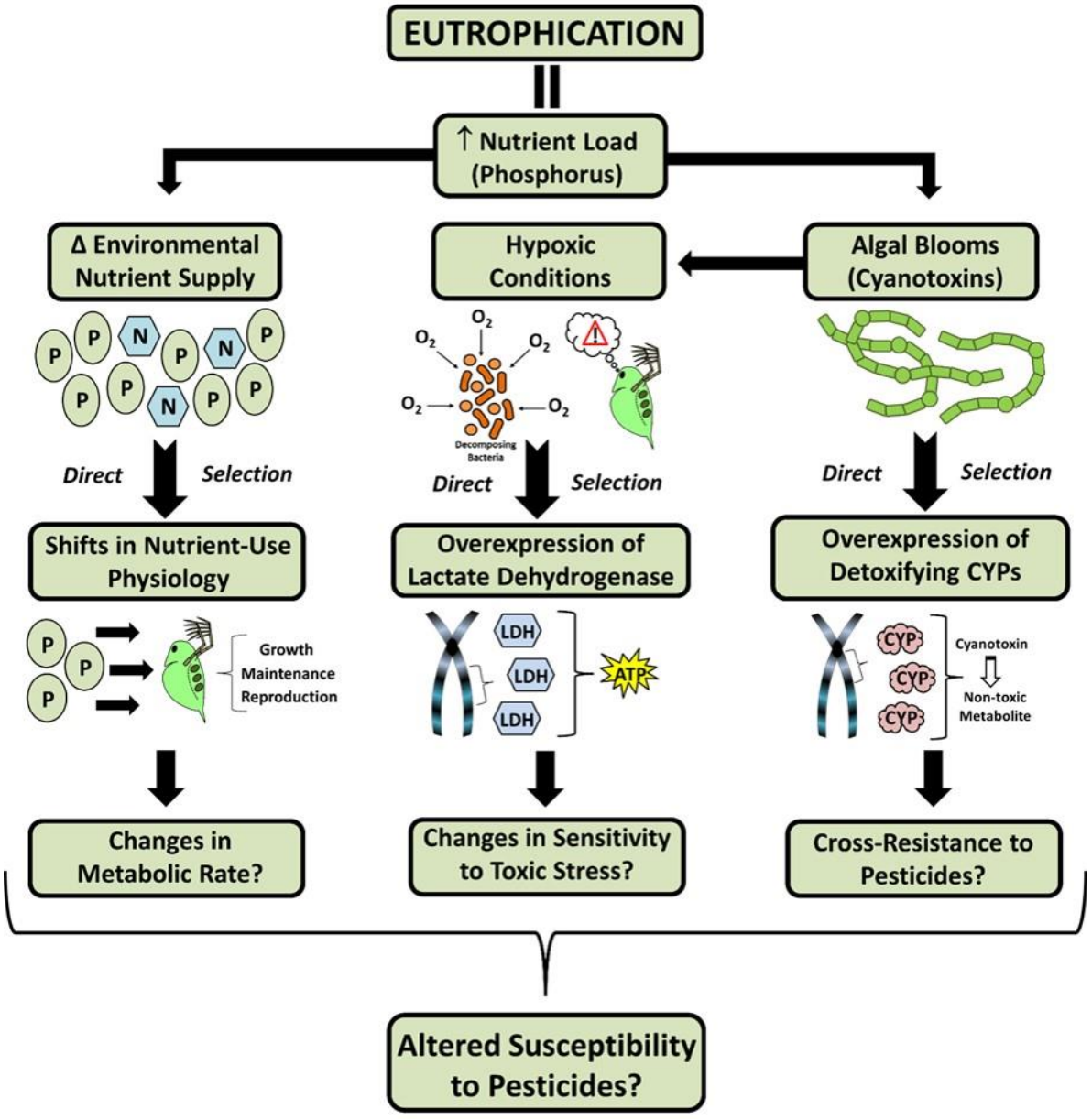


Figure 2. Formulating hypotheses: potential toxicological consequences of eutrophication.

Eutrophication is associated with a multitude of stressors in aquatic systems. *Left Column*: Shifts in elemental composition are likely to change a number of stoichiometric traits that may influence the metabolism of pesticides. *Center Column*: The presence of high concentrations of phosphorus accommodates high algal biomass. As masses of algal cells begin to die, populations of decomposers (bacteria) break down this organic matter, a process that depletes dissolved oxygen in the water body, creating hypoxic conditions. Organismal responses to hypoxia often involve an up-regulation of hemoglobin and

glycolytic proteins (Davies et al. 2011). One such protein, lactate dehydrogenase (LDH), enables the continued production of energy (ATP) in anaerobic conditions, which also facilitates cellular repair following exposure to toxicants. Expressing higher levels of LDH, due to adaptation/acclimatization to hypoxia, could alter pesticide susceptibility. *Right Column*: Eutrophic conditions are also favorable for cyanobacteria, which can produce compounds that are hazardous to zooplankton. Some zooplankton species have been shown to exhibit tolerance to these toxins (Sarnelle and Wilson 2005), which is likely expressed via metabolic shifts. Consequently, it is possible that this adaptation could confer *i*) cross-tolerance to pesticides that are detoxified via similar pathways, or *ii*) greater susceptibility to metabolically-activated pesticides.

VITA

Adam Martin Simpson

Candidate for the Degree of

Doctor of Philosophy

Thesis: INVESTIGATING THE EVOLUTIONARY ORIGINS AND MECHANISMS UNDERLYING XENOBIOTIC SENSITIVITY USING RESURRECTION ECOLOGY

Major Field: Integrative Biology

Biographical:

Education:

Completed the requirements for the Doctor of Philosophy in Integrative Biology at Oklahoma State University, Stillwater, Oklahoma in July, 2017.

Completed the requirements for the Bachelor of Science in Zoology at Oklahoma State University, Stillwater, OK in May, 2012.

Experience:

Graduate Research/Teaching Associate, Department of Integrative Biology, Oklahoma State University, Stillwater, OK. 2012-Present.

Professional Memberships:

Society of Environmental Toxicology and Chemistry
Southwestern Association of Naturalists
Society for Integrative and Comparative Biology