WETLAND DEFENSE: INSECTICIDE RESISTANCE IN NON-TARGET ORGANISMS AND ITS COMMUNITY-WIDE IMPLICATIONS

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Anthropogenic stressors are ubiquitous and have been implicated in species declines worldwide. Pesticides are one such stressor that can have profound effects on aquatic communities by directly affecting sensitive species and indirectly affecting other species via trophic cascades, which can alter ecosystem function. However, there is growing evidence that non-target species can evolve increased resistance to these chemicals. When such species are important drivers of the food web, such as zooplankton, then evolved resistance should help buffer communities from the effects of pesticides. Furthermore, given that some species can evolve cross-resistance to other pesticides, one would predict that cross-resistance could have pronounced effects on community stability.

The studies herein attempt to address these concerns through a series of experiments that build on each other in complexity. In the first study, we found that populations of two common, co-occurring zooplankton species collected from ponds near surrounding agriculture were more resistant to a commonly applied insecticide (chlorpyrifos) and this variation was correlated with surrounding agricultural land use. In the second study, we utilized this pre-existing variation in resistance to determine whether resistant populations of zooplankton could buffer an entire

aquatic community from the effects of pesticides. Chlorpyrifos caused direct mortality of zooplankton in communities containing sensitive populations and this led to a bloom of phytoplankton and subsequent declines in periphyton abundance and amphibian mass and survivorship. In the third study, we exposed communities to several concentrations of AChE-inhibiting or sodium channel-inhibiting insecticides. We discovered that communities containing resistant zooplankton were buffered from adverse effects at low-to-moderate concentrations of all AChE-inhibiting insecticides, but were not buffered against sodium channel-inhibiting insecticides. Conversely, communities with sensitive zooplankton experienced pronounced trophic cascades when exposed to all insecticides. The fourth study manipulated the diversity of zooplankton within the experimental communities. We discovered that populations of cladocerans and copepods living near agriculture were more resistant to chlorpyrifos, but rotifers did not show a clear pattern of variation in that could be associated with land use. Furthermore, unlike communities with cladocerans, communities containing only copepods and rotifers were unable to buffer the community from the effects of the pesticide.

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PREFACE

When I was younger, I used to thoroughly enjoy mathematics. In particular, I was fascinated by a sequence of numbers known as the Fibonacci sequence, which was named after the renowned Italian mathematician. The sequence, which is recursive in nature, begins with 0 and 1 (or 1 and 1) and each subsequent number in the sequence is the sum of the two preceding numbers. At first, this seems rather uninteresting – that is, until you attempt to visualize it graphically. If you take the first two numbers (1 and 1), made two unit squares corresponding to them and placed them side by side, and then added a new square consisting of the same length of the largest side of the original rectangle to those two original unit squares and continued this process over and over, you create an outward spiral of progressively larger rectangles. Finally, if you use quarter-circle arcs starting at the smallest square and connect them together, you find that the line that is created is a logarithmic spiral that continues outward towards infinity.

As time went by, my enthusiasm for math waned (as it does for most), and I became increasingly more interested in the sciences. Then, one fortuitous day in high school, the sequence reappeared in a short film during my AP biology class which completely reignited my interests. The film showed how the Fibonacci numbers were pervasive in nature: they could be found in the petals of a rose, to the conical shape of a pinecone, the distribution of seeds in a sunflower, the spiral shape of the nautilus shell and even the cochlea of the human inner ear. With all of the complexity and diversity of life that exists, how could it be that this pattern shows

up time and time again? Not often can we pinpoint an exact moment in our lives as a truly life-altering experience, but I can certainly say that this was the exact moment that I knew that I was destined to become a biologist.

When I recount my journey through graduate school, I cannot help but to relate it to the spiraling nature of the Fibonacci sequence as well. In my first year of study, the vast majority of my time the lab was spent pouring over was seemed to be an insurmountable amount of literature in order to narrow down potential areas of research. At times it seemed almost hopeless and there were several instances in which I considered leaving the program. However, once I found one broad, testable and important question, it opened the doors to new and exciting opportunities for future avenues of research. By the end of my time in graduate school, I almost felt as if I was left with more questions than answers. That is the beauty of science. From one question comes a handful more, and from those, even more still.

Though this has largely been a five year process of discovery, intellectual development and self-improvement, there have certainly been low points. Often, graduate students become overly frustrated because of failed experiments, seemingly harsh criticisms from colleagues, or the knowledge that a particular pet hypothesis was most likely untrue. However, it is in the company that we keep that we are able to summon the strength to persevere. As I reflect back on my time in graduate school, I now realize that all of this would not be possible without the input, assistance, guidance and friendship of many individuals. Although it would be nearly impossible to thank everyone who I have come into contact with throughout these past five years, I do want to take the time to reflect and thank those individuals who have left an indelible mark on my life and have helped shape me into the person I am today. Firstly, I have to thank my advisor, Rick Relyea, who was undoubtedly the best advisor I could have ever possibly imagined. When Rick

informed the lab group that he would be leaving the University of Pittsburgh to join the faculty at Rensselaer Polytechnic Institute for my final year of graduate study, we all assumed it was an elaborate hoax (he told us on April Fools' Day of all days). However, when we all came to the realization that this was not a joke, we were all faced with a rather difficult decision: to remain in Pittsburgh or to go with Rick to New York. In reality, this was not a difficult decision for me. In my mind, there is no possible way that I could have made it to where I am today without all of Rick's tireless efforts to improve my abilities as not only a scientist, but as a human being. Rick maintains one of the most intimidating schedules that one could ever imagine, but still prioritizes time for his students and treats them as if they were members of his own family. Words cannot describe how much that meant to me over the five years that I spent in his lab.

I would also like to thank the members of my dissertation committee. Nate Morehouse was always able to provide tremendous amounts of insight towards my experimental designs and his helpful criticisms have molded the experiments within this dissertation into a coherent and logical set of studies that hopefully advance our knowledge of the ecology of wetland communities. Susan Kalisz was able to use her academic dexterity, particularly in her knowledge of *Daphnia* biology, to provide helpful insight at meetings and was also critical in highlighting the importance of feasibility in my experimental designs. Mark Rebeiz was able to offer a unique perspective at meetings as a non-ecologist that aided in generating stimulating discussions among our committee members. I would also like to thank Larry Weider who was generous enough to allow me to visit his lab group in Oklahoma to see how a laboratory that specializes in *Daphnia* resurrection ecology functions. Larry has been an invaluable resource over these past few years and his expertise and knowledge on experimental design and the interpretation of data have helped to greatly improve my abilities as a new ecologist.

The work in this thesis is largely a collaborative effort and would not be possible without the assistance of a large number of undergraduates. Although there are far too many to name, there are a few students in particular who deserve particular praise. John Golden was the first undergraduate student that I worked with extensively. He through the growing pains of dealing with me as a novice graduate student advisor with a smile on his face was always able to make me laugh. Elisa McAfee spent hours running phytoplankton samples and counting zooplankton samples and generated some of the most beautiful data I have ever seen. Brian Mattes is one of the hardest working undergraduates to have come through our lab and his assistance on the two largest mesocosm studies that our lab has ever done are a testament to his diligence. I wish him (and the fate of the Relyea lab) the best of luck as he is now the current lab technician. Madeline Kelly, like Elisa, spent countless hours under a microscope and generated copious amounts of data for several of these studies and was extraordinarily patient with me when I continually inundated her with work. There are, of course, many other undergraduates that were involved with these studies and they have been listed in the Acknowledgement sections for each chapter. I wish all of them the best of luck in all of their respective future endeavors.

I would also like to take time to show my gratitude to the post-docs and graduate students that I have had the privilege to share this journey with. Rickey Cothran spent hours of time driving me between ponds to collect zooplankton and is one of the most dedicated and hard-working human beings I have ever met. John Hammond was always willing to spend time with me discussing how data should be analyzed and assisted me in calculating my first LC50 values. Jessica Hua was (and, for my money, still is) the undisputed queen of community mesocosm experiments. No matter what her workload consisted of, she came into lab every single day with a smile on her face and was a living example of what a successful graduate student must do in

order to achieve their goals. Will Brogan was the other "*Daphnia* guy" in the lab and was responsible for teaching me how to properly dose experiments, but also provided stimulating discussions on a daily basis, both in the realm of ecology or in general off-topic news stories (he might be addicted to NPR.) Devin Jones has always provided the perfect mix a strong work ethic and a lighthearted sense of humor to the lab and it made the last two years of my dissertation work much more enjoyable because of it. I already miss the ability to have a few craft beers with both Devin and Brian after a rough day at work. Other graduate students and post-docs that have left a major impact on my development include Maya Groner, Aaron Stoler, Heather Shaffery and Kate LeCroy. Their advice and friendship are things that I will carry with me forever, regardless of my future trajectory.

Finally, I want to thank my parents. I would be nothing without them (obviously.) I am entirely indebted to them for the remainder of my life as there is no possible way that I would ever be able to repay them for all they have provided for me. My work ethic comes from my father who is a self-employed businessman who literally worked his way to the top of his field. He is one of my heroes and has been the perfect example of what it means to be a caring and devoted father. My mother is the most compassionate, kind-hearted woman to ever grace this planet. She would literally drop everything to help out anyone at any time and has taught me countless invaluable lessons on what it means to be a considerate and loving human being. All of the good qualities that exist in me as a man are, at their root, derived from my interactions with my parents. I managed to pick up any of my negative qualities on my own.

To all of you, I owe you my eternal gratitude and much like the Fibonacci spiral that moves outwards to the unknown, I hope to continually grow and develop with your guidance, friendship and love as a both human being and scientist, regardless of where life takes me.

1.0 INTRODUCTION

One of the most central and on-going goals in the field of ecology is to be able to predict the consequences of anthropogenic disturbances on community structure and function. This has proven to be a rather difficult undertaking, as there is seemingly limitless amounts of complexity when attempting to understand the nature of interactions within any ecosystem (Simberloff 2004). A range of these stressors including (but not limited to) habitat fragmentation, deforestation, over-harvesting of natural resources and the production and release of chemical contaminants have directly led to what some are considering the 6th largest mass extinction of species in our planet's history; the 'Anthropocene extinction' (Barnosky et al. 2011). One such stressor that has received a great deal of attention both from an economic and an ecological point of view has been the use of synthetic pesticides. Undoubtedly, synthetic pesticides have increased our capacity to produce the food resources needed to meet the needs of a burgeoning human population, but the use of these chemicals has been shown to come at a significant cost (Pimentel 2005). From deleterious effects on human health, direct toxicity to both target and non-target organisms as well as the development of insecticide-resistant pest species, the effects of pesticides have been a major focus of ecological, entomological, and economic studies for decades (Pimentel et al. 1992). What has garnered markedly less attention, however, are the array of indirect effects that low, sublethal and, most importantly, environmentally-relevant concentrations of pesticides can have on community stability and ecological function.

Wetlands and vernal ponds are two such community types that are often indirectly inundated with an array of pesticides via aerial drift, run-off, and even inadvertent direct application (Davidson et al. 2012). Wetlands and ponds can contain diverse assemblages of organisms within a multi-trophic community that can be highly sensitive to anthropogenically-produced stressors (De Meester et al. 2005). They are often the preferred breeding habitat of amphibians, a unique group of vertebrates containing more than 7,000 species that are facing worldwide declines in both diversity and abundance (Blaustein et al. 2003, Stuart et al. 2004). Most importantly, ponds communities are model systems that are ideal for asking ecologically or evolution-based questions, in that they are relatively contained systems and easier to study and manipulate when compared to terrestrial systems (De Meester et al. 2005).

Studies within the past few years have shown that aquatic community function can be significantly impaired by extremely low concentrations of insecticides that are sublethal to most species within the community (Mills and Semlitsch 2004, Relyea and Diecks 2008). Insecticides often extirpate the most sensitive species within the community, which are typically the zooplankton. Zooplankton are essential for proper aquatic community function, as they maintain top-down control on their primary food resource, phytoplankton. When low concentrations of insecticides enter these communities, zooplankton immediately decline in abundance causing a sharp increase in phytoplankton content. This bloom in phytoplankton shades out competing species of algae within the community, particularly the attached periphytic algae along the substrate at the bottom of the pond. The decline in periphytic algae can then cause dramatic effects on grazers that consume periphyton such as amphibians (Relyea and Diecks 2008). Many of these pond communities are ephemeral and throughout the summer, the hydroperiod will continually get shorter, so amphibians that cannot attain the necessary energy and resources to

successfully metamorphose before the pond dries, will die. In these studies, which have utilized natural pond-drying regimes, low concentrations of insecticides (that are in some cases more than 100x lower than concentrations that have been shown to be directly toxic to amphibians) resulted in pronounced insecticide-induced trophic cascades that can result in delayed growth and development in tadpoles leading to amphibian mortality (Mills and Semlitsch 2004, Boone et al. 2004, Relyea and Diecks 2008).

This thesis aims to expand upon and explore how natural variation within zooplankton assemblages can potentially buffer aquatic communities from the detrimental effects of low and environmentally-relevant concentrations of commonly applied insecticides. In the first chapter, we explore whether or not there is natural variation in insecticide resistance among two different species of zooplankton (*Daphnia pulex* and *Simocephalus vetulus*) that were collected from ponds that varied in the amount of agricultural land use around them. Whereas insecticide resistance is a well-known phenomenon that has received a great deal of attention over the years due to the economic implications associated with it, insecticide resistance in non-target organisms, like zooplankton, is a remarkably understudied area that may potentially have dramatic effects within a community. We hypothesized that populations of zooplankton from ponds surrounded by a high (>30%) amount of agriculture would be more resistant to the commonly applied insecticide chlorpyrifos, than those from ponds with little to no (<5%) surrounding agriculture. Using standardized toxicological methodology (i.e. LC50 tests), we found that *D. pulex* and *S. vetulus* populations showed marked natural variation in resistance to chlorpyrifos, and that this resistance was, in general, highly correlated with surrounding land use, such that ponds with high amounts of surrounding agriculture had populations of zooplankton that were more resistant to the insecticide. To our knowledge, these are only the second and

third cladoceran species to have been shown to have developed naturally-occurring population-level variation in resistance to a pesticide, and the first study to show that this variation is correlated with surrounding agricultural land use. This paper is co-authored by Rick Relyea and is published in *Environmental Toxicology and Chemistry*.

In the second chapter of this thesis, we utilized the variation in resistance among naturally-occurring *D. pulex* populations to determine if these differences in sensitivity to chlorpyrifos had the potential to affect the entire aquatic community. We hypothesized that communities with sensitive populations of *D. pulex* would experience higher rates of mortality with the insecticide and that this would in turn, cause insecticide-induced trophic cascades through the food web across a range of chlorpyrifos concentrations. This trophic cascade would then have the potential to affect phytoplankton and periphyton abundance, as well as larval amphibian development. Conversely, communities with resistant populations of *D. pulex* would be buffered from the effects of the insecticide and the communities would not exhibit these pronounced cascades. To test this theory, we cultured four populations of *D. pulex* that we had previously demonstrated were either sensitive or resistant to chlorpyrifos. Using outdoor mesocosms that contained identical aquatic communities of phytoplankton, periphyton, and leopard frog tadpoles (*Lithobates pipiens*), we manipulated these four *D. pulex* populations and four chlorpyrifos concentrations. As we monitored the communities for nearly three months, we found that the insecticide caused significant direct mortality of *D. pulex* in communities containing sensitive populations and this led to a bloom of phytoplankton. In contrast, chlorpyrifos caused significantly less direct mortality in communities containing resistant *D. pulex* populations and the trophic cascade was prevented under low to moderate insecticide concentrations. Across all treatments, survivorship of leopard frogs was ~72% in communities

with resistant *D. pulex* but only 35% in communities with sensitive *D. pulex.* This is the first study using naturally-occurring population variation in insecticide resistance to show that the evolution of pesticide resistance in zooplankton can mitigate the effects of insecticide-induced trophic cascades and that this outcome can have far-reaching community effects. This paper is co-authored by Rick Relyea and is in review at *Oecologia*.

In the third chapter of this thesis, we built on previous knowledge from the studies performed in chapters 1 and 2 in order to determine if the resistant and sensitive populations of *D. pulex* were cross-resistant to multiple insecticides and, if so, whether or not this cross-resistance was related to the insecticide mode of action. Cross-resistance to insecticides, particularly those with similar modes of action, is a fairly common phenomenon that has been found in many pest species. However, due to a lack of economic incentive there is almost no evidence of cross-resistance in non-target organisms, nor are there any studies that attempt to discern whether or not there are any pertinent community-wide effects associated with this cross-resistance that may be generalizable to a wide array of insecticides. In this study, we hypothesized that *D. pulex* populations that were resistant to the acetylcholinesterase (AChE)-inhibiting insecticide chlorpyrifos, would also be resistant to insecticides that share the same mode of action, but not necessarily to those with a markedly different mode of action. To address this hypothesis, we conducted a mesocosm experiment comprised of 200 identical aquatic communities. We then added one of the four *D. pulex* populations that were either resistant or sensitive to chlorpyrifos (based on previous studies) and exposed the communities to either no insecticide or three different concentrations of AChE-inhibiting insecticides (chlorpyrifos, malathion or carbaryl) or sodium channel-inhibiting pyrethroid insecticides (permethrin or cypermethrin). We found that communities containing sensitive *D. pulex*

experienced phytoplankton blooms and dramatic community-wide effects at moderate to high concentrations of all five insecticides. However, communities containing resistant *D. pulex* were buffered from these effects at moderate concentrations of all AChE-inhibiting insecticides, but were not buffered against the pyrethroid insecticides. This suggests that resistance in zooplankton to a single insecticide can have widespread consequences for community stability and that the effects can potentially be extrapolated to a wide variety of pesticides that have similar modes of action. This paper is co-authored by Rick Relyea and will be submitted to *Ecological Applications*.

In the fourth and final chapter of this thesis, we continue to build on the narrative by adding additional complexity to our experimental aquatic communities. Our previous studies had only utilized variation within *D. pulex* populations, as cladocerans have been consistently cited as being responsible for maintaining top-down control on phytoplankton abundance as they are prolific consumers of phytoplankton (Tessier and Woodruff 2002, Korosi et al. 2012). Although cladocerans are typically the most sensitive group of zooplankton in terms of their response to anthropogenic disturbances, there are two other groups of zooplankton (copepods and rotifers), which also feed on and compete for phytoplankton that could potentially fill the same ecological role as cladocerans. To test this theory, we again utilized outdoor mesocosms and set up 152 identical aquatic communities. We then manipulated the identity of the zooplankton assemblages by adding an assemblage from either near or far from agriculture that was comprised of only cladocerans, a background assemblage of copepods and rotifers, or an entire assemblage of all three groups. These communities were then exposed to one of five chlorpyrifos concentrations. We discovered that populations of cladocerans and copepods living near agriculture were more resistant to chlorpyrifos. Rotifers, on the other hand, showed

population-level variation in resistance to chlorpyrifos, but it was not clearly associated with patterns of land use. Furthermore, the communities containing cladocerans collected from near agriculture were able to buffer the community from the cascading effects of chlorpyrifos, but communities composed of only copepods and rotifers from the same pond were not. This is one of the first empirical tests to show that pesticide-induced trophic cascades cannot be fully prevented by copepods and rotifers and that there is no real evidence of functional redundancy within zooplankton assemblages regarding the ability to buffer communities from insecticide-induced trophic cascades. Such information may be crucial in determining future effects of contaminants on aquatic communities, as it indicates that cladocerans are critical to the stability of the community and cannot be functionally replaced with either of the two more resistant major groups of macro-zooplankton. Rick Relyea is also a co-author on this paper which will be submitted to *Oikos*.

The four chapters within this thesis logically build on each other, as we have continually attempted to increase the ecological realism and level of complexity with each consecutive experiment. Our findings indicate, however, that there are still numerous questions left to be fully answered. In the concluding chapter, I discuss the implications of this research in terms of conserving vernal pond and wetland communities and furthering our understanding of the ecological theory surrounding the impacts of pesticides on aquatic communities.

2.0 LIVING ON THE EDGE: POPULATIONS OF TWO ZOOPLANKTON SPECIES LIVING CLOSER TO AGRICULTURAL FIELDS AND MORE RESISTANT TO A COMMON INSECTICIDE

2.1 INTRODUCTION

Anthropogenic alterations of numerous environments across the globe force organisms living in these habitats to adapt to novel conditions or face extirpation (Zalasiewicz et al. 2010). Numerous sources of anthropogenic change have been the focus of ecological research for decades including deforestation, urban encroachment, habitat destruction, and pollution (Urban 2004, Tylianakis et al. 2008). Pollution has been implicated in a wide range of detrimental outcomes including trophic cascades (Mills and Semlitsch 2004, Relyea and Diecks 2008, Clements and Rohr 2009), increasing organismal susceptibility to pathogens (Jansen et al. 2011*a*), and reducing biodiversity and altering community structure in terrestrial and aquatic ecosystems (Tilman et al. 2001, Relyea 2005). In addition to documenting ecological effects, there has also been an effort to understand the evolution of resistance to pesticides. However, this effort has been almost entirely focused on targeted pest species.

Evolved resistance to pesticides in targeted pest species has been observed for nearly a century and is well established in the literature (Georghiou 1990, Hoy 1998). Today, the

application of nearly every major class of agrochemicals has caused evolved resistance in one or more pest species (ffrench-Constant 2007). Indeed, over 540 target pest species have evolved resistance to one or more pesticides; in many of these cases we also know the mechanisms by which this resistance occurs (i.e. metabolic or altered target-site resistance; Georghiou 1990, Hoy 1998). Worldwide, about 3 billion kg of pesticides are applied each year; in the U.S. alone, approximately 500 million kg of pesticides are applied annually at a cost of nearly 10 billion dollars (Pimentel 2005). The evolution of resistance to agrochemicals has caused approximately 1.5 billion dollars in economic costs per year (Pimentel 2005); this has been a major motivation to determine how insecticide resistance evolves in pest species and can be done to prevent further resistance to evolve in the future (Laurence 2001, Tilman et al. 2002). Because the physiological mechanisms that confer resistance in targeted species are largely evolutionarily conserved, we might expect non-target organisms to be capable of evolving resistance as well.

Freshwater zooplankton are a group of non-target organisms that have received some investigation of evolved resistance to insecticides. Zooplankton are a critical component of aquatic ecosystems worldwide (Relyea and Diecks 2008) and recent studies have found that low and environmentally relevant concentrations of insecticides can cause trophic cascades through communities by killing most of the zooplankton, which are generally highly sensitive to insecticides (Boone et al. 2004, Mills and Semlitsch 2004, Relyea and Diecks 2008, Clements and Rohr 2009). Many zooplankton have generation times of only 5 to 7 days, which means that tens of thousands of generations of zooplankton have been produced since the advent of organic insecticides (Hairston Jr. et al. 1999). Most studies demonstrating evolved resistance in zooplankton have used laboratory experiments that employed relatively high pesticide concentrations (Hanazato 2001, Brausch and Smith 2009, Jansen et al. 2011*b*). Such studies

confirm that the evolution of increased resistance is possible, but we need to determine whether natural populations with different proximity to pesticide are more resistant to pesticides when they are located closer to areas of pesticide applications such as agricultural areas. Recent work has shown patterns of resistance in *Daphnia magna* that exhibited nearly significant correlations with greater agricultural land use and markedly lower genetic variation among clones hatched from dormant egg banks in ponds located closer to agriculture (Coors et al. 2009). These results suggest that inadvertent exposures of zooplankton to anthropogenic chemicals may impose strong bouts of selection for resistance. However, we need to determine the extent to which zooplankton populations positioned across the landscape can vary in their susceptibility to insecticides based on their proximity to agriculture.

To address this challenge, we tested whether two species of common freshwater zooplankton show variation in resistance to a common insecticide (chlorpyrifos) and whether this variation is associated with surrounding agricultural land use. We hypothesized that zooplankton populations collected from ponds near agricultural fields—which are assumed to experience more frequent exposures to pesticides—would be more resistant to commonly applied insecticides than populations collected from ponds far from agricultural fields.

2.1.1 Insecticide background

In the United States, chlorpyrifos is one of the most widely applied organophosphate insecticides in agriculture and is also one of the most common insecticides found in water bodies (Christensen et al. 2009). Chlorpyrifos has broad-spectrum abilities as an insecticide and miticide. As an inhibitor of acetylcholinesterase (AChE), it is used to control foliage and soil-

borne insect pests on a variety of crops. The chemical was first registered in 1965 by the Dow Chemical Company and was originally available for home and garden use under various trade names such as Dursban and Lorsban. By the end of 2001, Dow Chemical halted sales of the chemical for household use due to impending regulatory action by the Environmental Protection Agency (EPA). However, the insecticide is still extensively used in agriculture; approximately 4.54×10^6 kg of the active ingredient in chlorpyrifos are applied annually (Christensen et al. 2009). According to the United States Geological Survey's (USGS) National Water Quality Survey, chlorpyrifos is used extensively in northwestern Pennsylvania with primary applications on corn and soybeans (Stone 2013). Chlorpyrifos is considered to be highly toxic to most aquatic invertebrates.

2.2 METHODS

2.2.1 The *Simocephalus vetulus* experiment

We conducted a laboratory experiment at the University of Pittsburgh's Donald S. Wood Field Laboratory in Linesville, Pennsylvania. The experiment was a completely randomized design employing a factorial combination of 10 populations of the zooplankton species *Simocephalus vetulus* crossed with seven treatments: five nominal concentrations of chlorpyrifos (0.05, 0.25 μg/L, 0.5 μg/L, 1.0 μg/L, and 5.0 μg/L), a negative control of 0 μg/L chlorpyrifos, and a vehicle control (ethanol [EtOH]) because the chlorpyrifos is moderately insoluble in water. For the vehicle control, we added the same amount of ethanol as used in the 5.0 μg/L chlorpyrifos treatment (0.25 mL EtOH/L of water) to demonstrate that the amount of ethanol added to each

jar did not cause mortality. The chlorpyrifos concentrations were chosen based on published chlorpyrifos LC50 (lethal concentration causing 50% mortality of a tested population) values of *S. vetulus* (0.2 to 0.5 μg/L; Brock et al. 1992, Grist et al. 2006, van den Brink et al. 2007). In order to detect differences among the populations in their LC50 values, we set our concentrations from sublethal to 10 times higher than the highest LC50 values for the species. Furthermore, our lower three concentrations (0.05, 0.25. and 0.50 μg/L chlorpyrifos) were similar to those reported as environmentally relevant and found in natural wetland ecosystems (0.01-0.65 μg/L; Brock et al. 1992). The 70 treatment combinations (10 populations x 7 pesticide treatments) were replicated three times each for a total of 210 experimental units.

We made all pesticide concentrations in large batches using carbon-filtered, UV-irradiated well water and then distributed them into the appropriate containers. A sample of each solution was saved in pre-cleaned, glass amber jars and shipped to the University of Georgia's Chemical Analysis Laboratory for independent analysis of chlorpyrifos concentrations. These analyses determined that four of the nominal concentrations of chlorpyrifos (0.25, 0.50, 1.0 and 5.0 μg/L) produced actual concentrations of 0.27, 0.33, 0.82, and 3.10 μg/L (hereafter termed 0.25, 0.5, 1.0, and 5.0 μg/L for simplicity). Thus, on average, the actual concentrations were 80% of the nominal concentrations. The negative control (0 μg/L chlorpyrifos) had no detectable traces of chlorpyrifos. The lowest concentration (0.05 μg/L) was not tested because it was below the 0.1 μg/L detection limits of the University of Georgia's equipment.

We selected ponds and wetlands in northwestern Pennsylvania based on previous research that examined wood frog (*Lithobates sylvaticus*) population-level resistance to chlorpyrifos in relation to their distances from agricultural fields (Cothran et al. 2013). We collected *S. vetulus* from 10 wetlands using dipnets and zooplankton tows during 6 to 12 June

2011 (Table A.1). We cultured each population in the lab using five, 8-L containers of UV-filtered water per population. We initiated each of these lab populations with 20 juvenile (instars 3-4) *S. vetulus* and the zooplankton within each container were fed 2 mL of concentrated *Scenedesmus* spp algae that had been grown in high phosphorus COMBO medium every two days. Each population was culled regularly and water in each of these containers was changed every seven days to prevent fouling. The *S. vetulus* populations were cultured in the lab for at least three generations to reduce variation due to environmental and maternal effects among the populations. To do this, we removed mothers that had released their young and placed them in separate bins, thereby creating populations separated by generation i.e. GO, F1, F2, F3 etc. Only F3 individuals and beyond were used to assess resistance.

For the LC50 test, we used 210, 200-mL glass jars that were filled with UV-filtered water containing either no chlorpyrifos (negative control), an ethanol control or one of the five chlorpyrifos concentrations. Once the jars were filled, we pipetted 10 juvenile *S. vetulus* from a given population onto a mesh net and then added the animals to a jar to prevent additional water from entering the jar and diluting the insecticide concentration. After adding the animals, we recorded the number of individuals that survived after 2, 6, 12, 24, and 48 hours. Mortality was negligible during the first 12 hours of the experiment (except in the 5.0 μg/L treatment); therefore, we only analyzed the 24 and 48 hour results. An individual was considered "alive" if it was moving or moved after being gently sprayed with water from a pipette after any of three attempts.

2.2.2 The *Daphnia pulex* experiment

The following year, we conducted a second laboratory experiment using a second species of zooplankton (*Daphnia pulex*). The experiment was a randomized design employing a factorial combination of four populations of *D. pulex* crossed with the same five chlorpyrifos treatments used in the *S. velutus* experiment plus the negative and vehicle controls (EtOH). The same chlorpyrifos concentrations were used because they are also within the range of chlorpyrifos LC50 values of *D. pulex* (0.1 to 0.8 μg/L; Brock et al. 1992, van der Hoeven and Gerritsen 1997). Again, the lower three concentrations (0.05, 0.25. and 0.50 μg/L chlorpyrifos) are environmentally-relevant based on previous research (Christensen et al. 2009). The 28 treatment combinations (4 populations x 7 treatments (0 [negative control], 0.05, 0.25, 0.5, 1.0, 5.0 μg/L chlorpyrifos, and EtOH control) were replicated 4 times for a total of 112 experimental units.

As in the first experiment, we made all pesticide concentrations in large batches using carbon-filtered, UV-irradiated well water and then distributed them into the appropriate jars. A sample of each solution was saved in pre-cleaned, glass amber jars and shipped to the University of Georgia's Chemical Analysis Laboratory for independent analysis of chlorpyrifos concentration. These analyses determined that the four testable concentrations of chlorpyrifos (0.25, 0.5, 1.0, 5.0 μg/L) produced actual concentrations of 0.28, 0.39, 0.91, and 3.92 μg/L (hereafter termed 0.25, 0.5, 1.0, and 5.0 μg/L for simplicity). Thus, on average, the actual concentrations were 90% of the nominal concentrations. As in the previous experiment, the negative control did not contain any detectable chlorpyrifos and is hereafter termed 0 μg/L.

We selected the four *D. pulex* populations based on the results of the *S. vetulus* experiment. We sampled *D. pulex* from two ponds that were close to agriculture (Mallard and

Love) and contained a more resistant *S. vetulus* population as well as one pond that was close to agriculture (Hopscotch) and contained a less resistant *S. vetulus* population. Only one of the ponds that were far from agriculture and contained *S. vetulus* also contained *D. pulex*. Therefore, we had to sample an additional pond (Minnow) containing *D. pulex* that was relatively far from agriculture. We hypothesized that populations of *D. pulex* would show a similar pattern of resistance across the landscape as *S. vetulus*. The ponds were sampled with dipnets and zooplankton tows on May 2 2012. We cultured each population in the lab using five, 8-L containers of UV-filtered water per population. Again, each population was initiated with 20 juvenile (instars 4-6) *D. pulex* females. Zooplankton within each container were fed 2 mL of concentrated *Scenedesmus* spp algae that had been grown in high phosphorus COMBO medium every two days. Populations were culled and water in each of these containers was changed every seven days to prevent fouling. As in the previous experiment, the *D. pulex* were grown in the lab for at least three generations to limit variation due to environmental and maternal effects.

For the LC50 test, we used 112, 200-mL glass jars that were filled with UV-filtered water containing 1 of the 7 treatments. Assignment of individuals to jars and assessment of survival over the course of the experiment followed the same methods utilized in the *S. vetulus*. Again, although survivorship data was monitored 2, 6 and 12 hours into the experiment, mortality was negligible during this time period across most treatments, so the data were not analyzed for these early time points.

2.2.3 Statistical analysis

For both experiments, we used the mortality data from each pesticide concentration to estimate $LC50_{24h}$ and $LC50_{48h}$ values with 84% confidence intervals (CI) using standard probit analyses. Simulation tests have shown that when 84% confidence intervals do not overlap between two LC50 estimates, this method approximates an $\alpha = 0.05$ (Payton et al. 2003). We then used Abbott's formula to account for any mortality within our negative and ethanol vehicle controls (Rosenheim and Hoy 1989).

To determine the amount of agriculture surrounding each wetland, which served as a proxy of historic pesticide use, we took aerial images of each of the wetlands using Google Earth Pro. We visually estimated the center of the pond and drew three concentric circles with radii of 200, 300, and 500 m. We chose 500 m as the upper boundary of our land use estimates because recent studies have found that agricultural fields outside of this boundary have negligible effects on aquatic systems (Declerck et al. 2006). To verify that the land in each image was used for agriculture, we used USGS crop use overlay maps (Stone 2013) to determine if the land was being actively used and what crops were produced there.

To quantify the amount of agriculture within each of these circles, we used Photoshop software to crop out all areas containing agriculture. We then quantified the proportion of pixels that contained agriculture relative to the total number of pixels contained in the circle around the wetland to find the total percentage of agriculture (Table 2.1). We then used Pearson product-moment correlation analysis to assess whether population $LC50_{24h}$ and $LC50_{48h}$ estimates were associated with agricultural land use. In all cases, we set the significance level at $\alpha < 0.05$.

Table 2.1. The percentage of agriculture surrounding the ponds where populations of *S. vetulus* and *D. pulex* were collected. Percentage of agriculture was calculated across 3 different spatial scales (200, 300 and 500 m from the center of each pond.) An asterisk '*' denotes ponds used in both experiments whereas two asterisks '**' denotes a pond only used in the *D. pulex* experiment.

Pond Name	200-m radius	300-m radius	500-m radius
Blackjack	0%	2%	7%
Graveyard	16%	21%	25%
Hopscotch*	0%	0%	0%
Log	0%	2%	10%
Love*	30%	32%	35%
Minnow**	2%	10%	6%
Mallard*	35%	29%	20%
Road	0%	1%	5%
Staub	20%	23%	29%
Trailer Park	10%	20%	16%
Turkey Track	0%	0%	2%

2.3 RESULTS

2.3.1 The *Simocephalus vetulus* experiment

Across all 10 populations, survival in the control treatments remained high after 48 hours (range: 93 to 100%) and the ethanol vehicle controls showed a similar pattern of high survival (range: 90 to 100%). This indicates that the ethanol had a negligible effect on mortality.

Across the 10 populations, the $LC50_{48h}$ values were 52 to 84% of the $LC50_{24h}$ values. The populations of *S. vetulus* exhibited differences in their $LC50_{24h}$ or $LC50_{48h}$ values. However, the general relationship between LC50 values for each of the 10 ponds remained fairly constant between both time points. Across populations, LC50 estimates exhibited a three-fold difference, ranging from 0.14 to 0.43 µg/L (Table 2.2). Taking into account the upper and lower bounds of the 84% CIs, the most resistant populations of *S. vetulus* (i.e. Love, Mallard, and Staub) were 2 to 3.5 times more resistant than the least resistant population (i.e. Log). Survivorship curves for each population after 48 hours are provided as supplemental data in Appendix B (Figure B.1).

We then examined whether LC50 values were associated with percent agriculture that existed around each wetland. At all three spatial scales (200, 300 and 500 m), we found significant, positive relationships (all $p < 0.02$) with R^2 values ranging from 0.53 to 0.62; populations collected from wetlands surrounded by a higher percentage of agricultural land possessed higher $LC50_{48h}$ values than populations collected from areas surrounded by little to no agricultural land (Figure 2.1). While we present the correlations only for the $LC50_{48h}$ values, the results were similar for both time points.

Table 2.2. The LC50 estimates after 24 and 48 hours for the 10 populations of *S. vetulus* along with the lower and upper boundaries of 84% confidence intervals (CI). The populations are ordered by ascending 48 hour LC50 values.

Population	24-hr LC50	84% CI lower boundary	84% CI upper boundary	48-hr LC50	84% CI lower boundary	84% CI upper boundary
Log	0.27	0.23	0.30	0.14	0.11	0.17
Hopscotch	0.34	0.29	0.38	0.22	0.18	0.26
Trailer Park	0.26	0.21	0.31	0.22	0.18	0.26
Road	0.28	0.24	0.31	0.23	0.19	0.26
Blackjack	0.33	0.28	0.38	0.26	0.22	0.30
Graveyard	0.37	0.32	0.42	0.26	0.22	0.30
Turkey Track	0.38	0.33	0.43	0.26	0.22	0.31
Mallard	0.42	0.38	0.48	0.35	0.31	0.40
Staub	0.51	0.44	0.59	0.39	0.34	0.46
Love	0.51	0.44	0.60	0.43	0.38	0.49

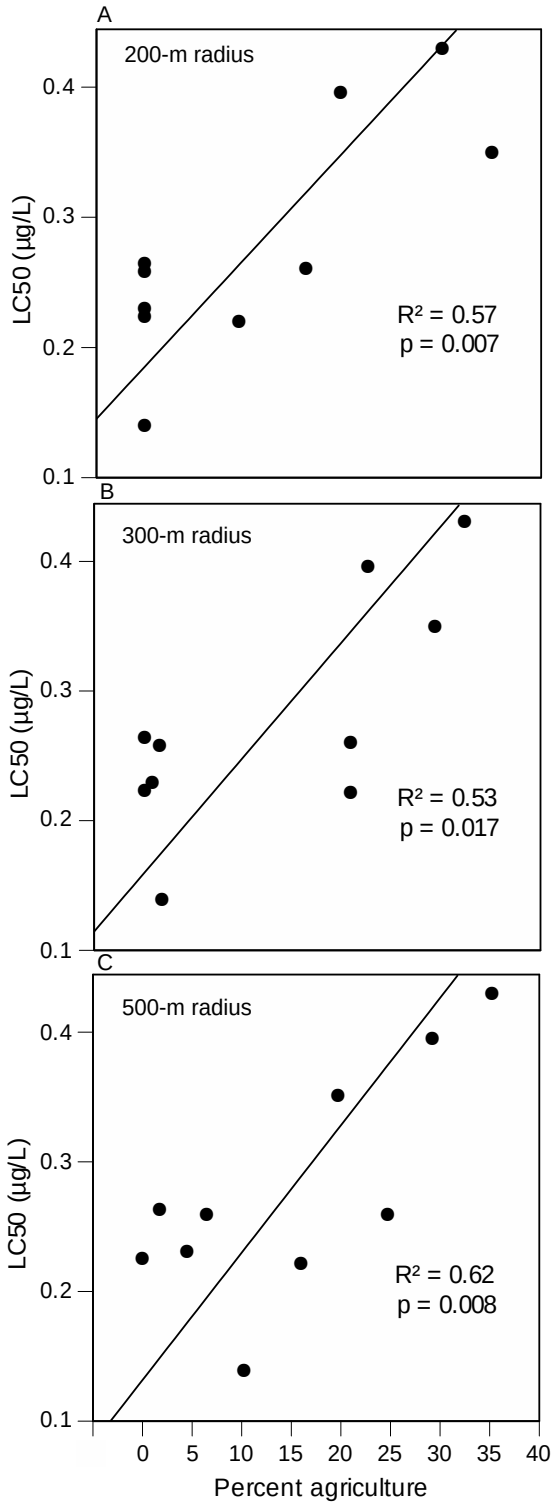

Figure 2.1. The relationship between percentage of agriculture in the area surrounding each population and the $LC50_{48\text{-hr}}$ value of each of the 10 *S. vetulus* populations. Data are presented for correlations conducted using radii of A) 200 m, B) 300 m, and C) 500 m

2.3.2 The *Daphnia pulex* experiment

Across the four populations of *D. pulex*, survival in the control treatments remained high after 48 hours (range: 97 to 100%) and the ethanol vehicle controls showed a similar pattern of high survival (range: 93 to 100%). Again, these data demonstrate that ethanol did not affect survival.

The chlorpyrifos $LC50_{48h}$ values differed among the four populations of *D. pulex.* Across the 4 populations, the $LC50_{48h}$ values were 62 to 84% of the $LC50_{24h}$ values. Based on the lack of overlap among the 84% CIs, there were two populations that were significantly more resistant and two that were more sensitive. The two most resistant populations were not different from each other and the two least resistant populations were not different from each other. Across the four populations, LC50 estimates exhibited up to a nearly 3-fold difference, ranging from 0.18 to 0.53 μg/L (Table 2.3). Survivorship curves for each population after 48 hours are provided as supplemental data in Appendix B (Figure B.2).

We then examined whether LC50 values were associated with percent agriculture that existed around each wetland. At the smallest spatial scale (200 m) we found a significant (p = 0.029) relationship with a high R^2 value of 0.94, which indicates that populations collected from wetlands surrounded by more agricultural land possessed higher $LC50_{48h}$ values than populations collected from wetlands surrounded by less agricultural land (Figure 2.2). At larger spatial scales, the relationship remained positive, but the relationships were no longer significant (p > 0.09) and the R^2 values declined (0.54 to 0.82). Again, we present the correlations for $LC50_{48h}$ values, but results and data trends were similar for $LC50_{24h}$ values.

Table 2.3. The LC50 estimates after 24 and 48 hours for the 4 populations of *D. pulex* along with the lower and upper boundaries of 84% confidence intervals (CI). The populations are ordered by ascending 48 hour LC50 values.

Population	24-hr LC50	84% CI lower boundary	84% CI upper boundary	48-hr LC50	84% CI lower boundary	84% CI upper boundary
Hopscotch	0.29	0.22	0.36	0.18	0.12	0.24
Minnow	0.33	0.28	0.38	0.24	0.19	0.29
Love	0.59	0.53	0.65	0.41	0.35	0.47
Mallard	0.63	0.57	0.69	0.53	0.47	0.61

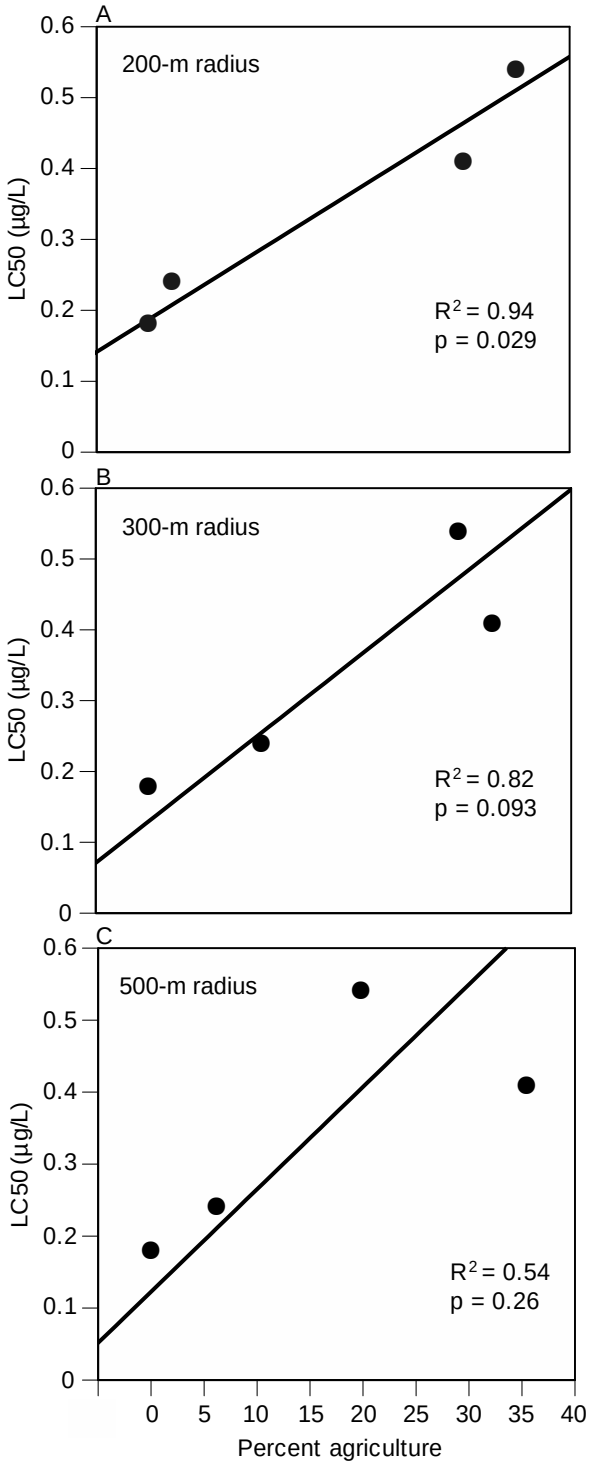

Figure 2.2. The relationship between percentage of agriculture in the area surrounding each population and the $LC50_{48\text{-hr}}$ value of each of the four *D. pulex* populations. Data are presented for correlations conducted using radii of A) 200 m, B) 300 m, and C) 500 m.

2.4 DISCUSSION

Using two species of common zooplankton (*S. vetulus* and *D. pulex*) we found that populations collected from ponds containing a higher percentage of agricultural land were more resistant to the insecticide chlorpyrifos than populations collected from ponds containing a low percentage of agricultural land. Moreover, this pattern was consistent across 3 different spatial scales (i.e. 200, 300, and 500 m) for both species. These two species, which are from different genera, differ a great deal in their behaviors, patterns of foraging, activity levels and many other traits (Tollrian 1995, Van Doorslaer et al 2007) yet we still see the same general pattern of variation in resistance across a geographic landscape. Given that the physiological mechanisms that confer resistance in targeted species are evolutionarily conserved, other species of zooplankton and even non-related taxa that co-occur with these populations may too possess genetic variation for resistance to pesticides. This pattern of spatial variation in resistance to insecticide exposure in these two species of zooplankton is consistent with the evolution of resistance to chlorpyrifos.

Resistance to agrochemicals, like chlorpyrifos, is a widespread phenomenon in pest species (Georghiou 1990, Hoy 1998, ffrench-Constant 2007). However, many non-target species are also inadvertently subjected to these and other related insecticides, which means that the evolution of increased resistance in populations living closer to agriculture may be a widespread phenomenon that has received little investigation. Our results are consistent with the findings of Coors et al. (2009) who found a nearly significant correlation between the resistance of *D. magna* exposed to the insecticide carbaryl and the percentage of land used for cereal and corn crops. Our results are also consistent with the work of Cothran et al. (2013) who found that wood frogs living closer to agriculture exhibited higher resistance to chlorpyrifos.

Chlorpyrifos is one of the most commonly applied insecticides in the United States. In northwestern PA, where we performed our studies, chlorpyrifos is regularly applied to crops to control pests of corn, winter wheat, and soybeans. Most importantly, chlorpyrifos is an organophosphate insecticide that shares its mode of action with numerous other insecticides. Therefore, the evolution of resistance in the two species of zooplankton may have occurred due to selection by chlorpyrifos or due to selection by other insecticides that inhibit AChE (e.g., carbaryl, malathion) and provide cross-resistance to chlorpyrifos. Cross-resistance occurs commonly among pest species (ffrench-Constant 2007) and recent research has shown it can also occur in non-target species such as wood frog tadpoles (Hua et al. 2013). As is often the case, we have no information on the concentrations of the pesticides in the water for the past several decades at each of the population locations, so it is difficult to determine whether chlorpyrifos specifically or other AChE-inhibiting pesticides selected for the high resistance of the zooplankton populations living near agricultural fields. We did not test the water when we collected the animals because most modern pesticides degrade relatively rapidly in the water column due to UV radiation, bacterial breakdown, sorption onto aquatic plants/detritus/soil and other various means (Brock et al. 1992, van den Brink et al. 2007, Clements and Rohr 2009).

Interestingly, previous studies that have examined variation in *Daphnia* resistance to other chemical stressors have shown similar differences in the amount of variation between populations or clonal lines. In the present study, LC50 values among populations of both species of zooplankton differed between 2 to 4-fold. Past studies have shown that different populations of *D. magna* collected from ephippial egg banks in the field show similar amounts of variation ($\leq$ 6 fold) when exposed to an array of other chemicals including cadmium, ethyl parathion (Barata et al. 2000), λ-cyhalothrin (Barata et al. 2002) and fenitrothion (Damásio et al. 2007).

Furthermore, studies have found that resistance in field populations is highly influenced by the genetic composition of the population whereas testing laboratory-reared, genetically distinct clonal lines has very limited usefulness in predicting the effects of chemicals in natural settings (Barata et al. 2000). With the recent publication of the *D. pulex* genome (Brede et al. 2009, Colburne et al. 2011), we can potentially unravel the mechanisms behind insecticide resistance in *D. pulex* to determine if resistance that naturally evolves in field populations is, in any way, analogous to resistance that is selected for in the laboratory.

Given the key ecological role that zooplankton play in aquatic food webs including being substantial grazers of phytoplankton, major cyclers of nutrients, and an important prey source for many species of aquatic predators (Mills and Semlitsch 2004, Relyea and Diecks 2008, Clements and Rohr 2009), studies on the impacts of pesticides on naturally-occurring zooplankton assemblages are of critical importance. For instance, the existence of population-level variation in pesticide resistance may have community-wide consequences. Past studies have shown that very low concentrations of insecticide can decimate zooplankton populations and this initiates a trophic cascade throughout the food web (Boone et al. 2004, Mills and Semlitsch 2004, Relyea and Diecks 2008). When the zooplankton die off, the phytoplankton that is normally consumed by zooplankton can dramatically increase in abundance, thereby producing a phytoplankton bloom. This bloom in phytoplankton shades out the periphytic algae that is attach to substrates in the benthos, which ultimately causes reduced growth, development, and survival of tadpoles that consume periphytic algae (Relyea and Diecks 2008). Based on this research, one can hypothesize that the occurrence of insecticide resistance in zooplankton populations may be able to buffer aquatic ecosystems from the cascading effects initiated by agrochemicals. This is an important hypothesis that needs to be tested in future studies.

2.4.1 Conclusions

Our results are one of the first examples indicating that zooplankton populations near agriculture are more resistant to pesticides than populations found far from agriculture, which is consistent with the evolution of pesticide resistance. While pesticides can have a multitude of effects on non-target organisms including direct lethal and deleterious sublethal effects via life history tradeoffs (i.e. reduced fecundity, increased susceptibility to parasites, etc.), we have also shown that zooplankton can evolve resistance to these chemicals. Such costs have been confirmed in lab selection studies using clonal zooplankton lines (i.e. Coors and De Meester 2008, Jansen et al. 2011*a*), but future studies should examine this question among populations that naturally differ in resistance. Finally, given the key role of zooplankton in aquatic food webs, future studies should examine whether the occurrence of resistant zooplankton populations can alter the trophic cascades that commonly occur when these systems are contaminated by insecticides, affect the rate at which nutrients are cycled throughout contaminated water sources, or potentially alter interactions among other taxa within the community.

2.5 ACKNOWLEDGEMENTS

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3.0 WETLAND DEFENSE: NATURALLY-OCCURRING PESTICIDE RESISTANCE IN ZOOPLANKTON POPULATIONS PROTECTS THE STABILITY OF AQUATIC COMMUNITIES

3.1 INTRODUCTION

One of the most challenging tasks facing ecotoxicologists is to understand how anthropogenic chemicals can potentially affect the multitudes of non-target organisms that exist within communities (Boone et al. 2004, Relyea et al. 2005, Rohr et al. 2006, Relyea and Diecks 2008, Clements and Rohr 2009). To achieve this goal, the traditional approach has been to use highly controlled, short-term laboratory experiments to test the direct toxicity of contaminants on a number of model organisms (Moore et al. 1998). These single species laboratory tests can determine the concentrations of a particular chemical that cause 50% mortality of the experimental population i.e. LC50 (Stephan 1977) and which concentrations have no observable effects on the organisms i.e. NOEC (Chen et al. 2013). Such tests are important for assessing relative sensitivity among taxa and among contaminants, but they isolate organisms from their natural environmental context. Because there are myriad interactions within ecological communities that can be affected by contaminants, it is difficult to extrapolate the effects of a contaminant within communities based upon single-species laboratory tests (Liebold et al. 1997,

Brock et al. 2000 *a/b*, Boone et al. 2004, Mills and Semlitsch 2004, Relyea and Hoverman 2006, Rohr et al. 2006).

Aquatic ecosystems are particularly vulnerable to contamination by pesticides due to accidental drift via wind, through groundwater, or even direct application such as insecticide applications over water to remove disease vectors such as mosquitoes (Relyea 2005, Gilliom 2007, Downing et al. 2008). Furthermore, as our human population expands, agricultural production is projected to increase dramatically as well, along with new insecticides to combat the issue of insecticide resistance in pest species (Laurence et al. 2001, Tilman et al. 2001). Insecticide exposure is relatively common in natural aquatic communities (Gilliom 2007, Stone et al. 2014), but our understanding of how these insecticides affect organisms within these communities is derived from the aforementioned short-term laboratory tests which cannot highlight the indirect effects of pesticides on food webs (Boone et al. 2004, Mills and Semlitsch 2004, Relyea and Hoverman 2006, Relyea and Diecks 2008). We need to know more about the impacts of low, environmentally-relevant concentrations of pesticides on natural community assemblages by incorporating more realistic scenarios into ecotoxicological studies.

Insecticides at high concentrations are often directly lethal to a wide array of taxa found in aquatic communities such as zooplankton, macroinvertebrates, amphibians and fish (Boone et al. 2004, Mills and Semlitsch 2004, Rohr et al. 2006, Relyea and Diecks 2008). Environmentally-relevant concentrations of these insecticides, however, are typically quite low due to the relatively rapid breakdown of insecticides in water due to hydrolysis, bacterial action, UV-degradation and even sorption by aquatic plants (EPA ECOTOX database). Low concentrations of pesticides can have adverse effects throughout the food web by directly affecting sensitive species that interact with other species in the food web (de deNoyelles et al.

1994, Fleeger et al. 2003, Boone et al. 2004, Mills and Semlitsch 2004, Relyea and Hoverman 2006, Relyea and Diecks 2008). As highlighted in the above studies, low concentrations of commonly applied insecticides can cause marked declines of zooplankton. This decline in zooplankton can lead to a phytoplankton bloom that decreases light transmission down through the water column. As a result, periphytic algal content declines, which results in the decreased growth and survival of periphyton grazers such as tadpoles. These important community-level studies indicate that although many taxa that are directly affected by low pesticide concentrations, they can be susceptible to substantial indirect effects via altered food web interactions.

The above studies highlight the fact that pesticide-induced trophic cascades are initiated by the effect of insecticides that directly kill the zooplankton. However, an important issue that needs to be addressed is whether population variation in zooplankton resistance to insecticides can potentially prevent these trophic cascades. Such variation in zooplankton resistance may have important community-wide effects. Recent studies have discovered that various species of zooplankton show naturally occurring population variation in resistance to commonly applied insecticides and that this variation is associated with land use (Coors et al. 2009, Jansen et al. 2011*b*, Bendis and Relyea 2014). Due to this variation, it is conceivable that aquatic communities historically located near agriculture and containing zooplankton that have evolved higher insecticide tolerance may be buffered from the effects of a pesticide-induced trophic cascade. While this possibility has major implications for the persistence of communities and their proper functioning close to and far from agriculture, no studies have examined the role that population variation in zooplankton pesticide resistance has on aquatic communities.

We explored the community-wide impacts of insecticide resistance in zooplankton using

a mesocosm experiment, where we created otherwise identical aquatic communities, but with different populations of *Daphnia pulex* (*D. pulex*). We then exposed the communities to a range of low, environmentally-relevant concentrations of a commonly applied insecticide (i.e. chlorpyrifos). We hypothesized that communities with sensitive populations of *D. pulex* (collected far from agricultural fields) would experience higher rates of mortality with the insecticide and that this would in turn cause insecticide-induced trophic cascades through the food web across a range of insecticide concentrations, affecting phytoplankton, periphyton, and larval amphibians. Conversely, communities with resistant populations of *D. pulex* (collected close to agricultural fields) would be buffered from the effects of the insecticide and the communities would not exhibit an insecticide-induced trophic cascade.

3.2 METHODS

We conducted a mesocosm experiment at the University of Pittsburgh's Donald S. Wood Field Laboratory at the Pymatuning Laboratory of Ecology. Using mesocosms allowed us to replicate aquatic communities, while simultaneously subjecting these communities to a range of pesticide applications (Relyea and Diecks 2008). The experimental design was a full factorial combination of four distinct *Daphnia pulex* populations (two resistant populations from ponds with surrounding agriculture, two sensitive populations from ponds with little or no surrounding agriculture) and four nominal concentrations of chlorpyrifos (0, 0.25, 0.50 and 1.0 μg/L). The differences in sensitivity among *D. pulex* populations were determined via prior short-term LC50 pilot experiments in the lab (Bendis and Relyea 2014). These 16 treatment combinations were replicated 3 times for a total of 48 experimental units. A timeline of the experiment can be found

in Appendix F (Figure F.1)

These experimental units were 800-L cattle tanks that were filled with approximately 550 L of well water from 30 March to 2 April 2012. On 6 April we added 200 g of dry leaf litter (primarily *Quercus* spp.) to each mesocosm to provide both nutrients and additional surface area for periphyton growth. On 7 April we added 15 g of rabbit chow to provide an additional nutrient spike. On 20-21 April, we took pond water samples from each of the four ponds where our *D. pulex* populations were collected and we visually screened each for invertebrate predators. After removing predators, we ran the water through a series of cylinder sieves (1 mm, 250 µm, 80 µm) and then treated the pond water with carbonated water to remove any zooplankton. Once each sample of water had been processed, we combined the pond water samples and added equal aliquots to each experiment unit to provide a natural source of periphyton and phytoplankton. On 27 April, we added four unglazed ceramic tiles (15 cm x 15 cm) along the north side of each tank to provide a standardized methodology of sampling periphyton abundance.

We then isolated *D. pulex* from additional pond water samples taken from each of our four focal populations and placed each population in separate plastic containers (each population was replicated four times). Each container was filled with carbon-filtered, UV-irradiated well water and the *Daphnia* populations were fed 2 mL of lab cultured *Scenedesmus* spp. algae once every 2 days. As female *Daphnia* released their offspring, these older females were removed from the experimental populations. On May 3, after the *D. pulex* populations had produced at least three generations of offspring in the lab, we added 50 juvenile females from one of the four *D. pulex* populations to the corresponding experimental units.

Leopard frog tadpoles (*Lithobates pipiens)* were raised from egg masses that we collected from a single pond in northwestern Pennsylvania (Mallard Pond). We collected 10 egg masses

on March 20 and reared the hatched tadpoles in 200-L pools containing well water. Once hatched, the tadpoles were fed rabbit chow ad libitum. On 7 May, after the algal and bacterial assemblages had developed for 16 d, we added 30 leopard frog tadpoles to each mesocosm. We selected the tadpoles for our experiment by mixing all 10 egg masses and then selected individuals of a similar size (initial mass ± SE: 45 mg ± 6 mg). Survival of the leopard frog tadpoles after a 24-hour handling test was 100%.

We allowed the tadpoles to acclimate to experimental conditions for 4 d before applying the insecticide treatments. On 11 May (day 1), we exposed the mesocosms to one of four chlorpyrifos concentrations (0, 0.25, 0.50, and 1.0 μg/L); these concentrations were based on our data examining the sensitivity of the four *D. pulex* populations to chlorpyrifos in the laboratory (Bendis and Relyea 2014). All details related to the application of the pesticides and the resulting concentrations in the mesocosms can be found in Appendix C.

3.2.1 Abiotic response variables

During the course of the experiment, we measured pH, temperature, dissolved oxygen, and the decay rate of light with increased water depth. All details of these measurements can be found in Appendix C.

3.2.2 Biotic response variables

We also quantified several biotic response variables during the experiment. We sampled *Daphnia* abundance seven times during the experiment (typically 3 to 4 days before and after

each pesticide applications; Figure F.1). We also measured phytoplankton and periphyton four times during the experiment. All methodological details can be found in Appendix C.

The first leopard frog metamorphs emerged on day 38; every day thereafter we conducted checks for metamorphs from all tanks. We visually scanned each tank and removed all individuals when both hindlimbs and forelimbs emerged and their tail was almost completely resorbed. Once metamorphs were removed from the mesocosms, they were kept in 1-L plastic containers in the laboratory containing a layer of moist sphagnum moss. Each metamorph was checked daily and when the tail was completely resorbed, we euthanized the metamorph using a 2% solution of MS-222. All metamorphs were then preserved in a solution of 10% formalin.

On day 76 (July 27) we began a tank drying protocol to simulate the natural drying cycle that occurs in wetlands in our region during the late summer. It is important to draw down the water gradually because amphibians can sense the drying of a pond by sensing a reduced volume and respond to speeding up their development (Denver et al. 1998). To simulate the gradual drying of a pond, we removed 20 L of water each day for a period of two weeks; on day 89 we terminated our experiment when the water depth was ~10 cm (~145 L) in each mesocosm. On that day, we drained the remainder of water from the tanks and sorted through the leaf litter and detritus to recover all amphibians that had not metamorphosed. If an amphibian had at least one emerged forelimb (i.e. Gosner stage 46; Gosner 1960), we allowed that individual to complete metamorphosis in the lab. All other amphibians that did not have any forelimbs were humanely euthanized using MS-222 and preserved in a 10% formalin solution. These latter individuals were categorized as not surviving to metamorphose. For the leopard frogs that metamorphosed, we recorded time to metamorphosis (from the start of the experiment), mass at metamorphosis, survivorship to metamorphosis (% of animals ≥ Gosner stage 46 at the end of the experiment)

and overall survivorship (% of all leopard frogs [i.e metamorphs + tadpoles] that were alive at the end of the experiment). For leopard frogs that did not metamorphose, we recorded the mass of the tadpoles and developmental stage (Gosner 1960).

3.2.3 Statistical analysis

All details of the statistical analysis can be found in Appendix C. In brief, our preliminary analyses found no differences between the two sensitive populations and no differences between the two resistant populations, so we pooled the four populations into one "sensitive" and one "resistant" category. We tested for effects on the abiotic and biotic variables using analyses of variance including repeated measures analyses for responses that were repeatedly measured over time using SPSS statistical software (IBM, Version 22).

3.3 RESULTS

3.3.1 Abiotic variables

A detailed analysis of all abiotic response variables can be found in Appendix D. In brief, pH and DO experienced an increase with higher concentrations of chlorpyrifos, and this difference grew larger over time. Temperature experienced small, but significant changes in response to the treatments. Light attenuation increased with higher concentrations of chlorpyrifos on all three of the sample dates.

3.3.2 Zooplankton

The rm-ANOVA of *D. pulex* abundance indicated that there were significant effects of insecticide concentration, *D. pulex* sensitivity, time, and several interactions (Table E.4). We then ran individual univariate ANOVAs for each time point (Table E.5A). On the first sampling date (day 14), there was an effect of insecticide concentration and *D. pulex* sensitivity, but no significant interaction (Figure 3.1). Treatments containing no insecticide had more *D. pulex* than treatments containing any of the three insecticide concentrations ($p < 0.001$). However, the effect of *D. pulex* sensitivity was clearly driven by the three treatments that contained chlorpyrifos.

On the second sampling date (day 21), which immediately preceded the second pesticide application, we observed a similar pattern, but now the difference in *Daphnia* sensitivity with increasing chlorpyrifos was large enough to cause a significant concentration-by-sensitivity interaction (Table E.5A). Communities containing sensitive *D. pulex* populations had a lower

abundance of *D. pulex* when exposed to the two highest concentrations (0.5 and 1.0 μg/L, both $p < 0.001$) compared to when the insecticide was absent. However, communities containing resistant zooplankton only experienced a decline in *D. pulex* when exposed to the highest concentration (1.0 μg/L; $p = 0.023$). Communities exposed to the lowest pesticide concentration (0.25 μg/L) did not differ from the control regardless of the sensitivity of the *D. pulex* population within the community ($p > 0.05$).

On all subsequent sample dates (days 28, 42, 50, 62, and 83), we continued to observe effects of concentration, *D. pulex* sensitivity, and their interaction (Table E.5A). Throughout all 7 samples, there was a consistent decline in the abundance of sensitive *D. pulex* populations whenever they were exposed to ≥ 0.50 ppb chlorpyrifos ($p \leq 0.008$ in all cases). In communities exposed to 1.0 μg/L chlorpyrifos, nearly every community containing sensitive *D. pulex* experienced complete extirpation of the *D. pulex* within the community after the first application.

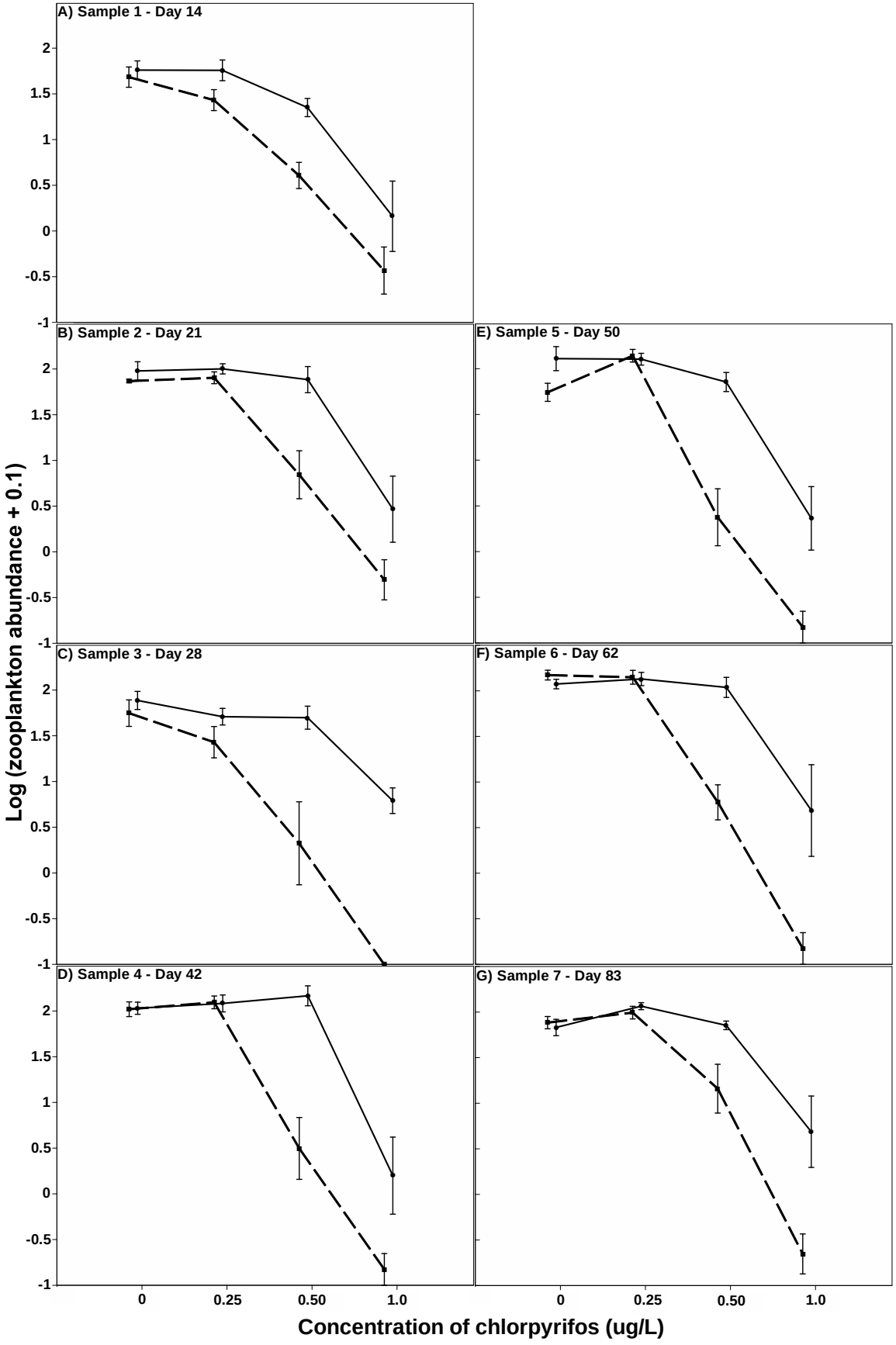
A) Sample 1 - Day 14
B) Sample 2 - Day 21
C) Sample 3 - Day 28
D) Sample 4 - Day 42
E) Sample 5 - Day 50
F) Sample 6 - Day 62
G) Sample 7 - Day 83
Log (zooplankton abundance + 0.1)
2
1.5
1
0.5
0
-0.5
-1
0
0.25
0.50
1.0
Concentration of chlorpyrifos (ug/L)

Figure 3.1. *D. pulex* abundance across seven sampling dates in experimental communities that were exposed to a range of chlorpyrifos concentrations. The solid line indicates communities with resistant *D. pulex* whereas the dashed line indicates communities with sensitive *D. pulex*.

3.3.3 Phytoplankton

The rm-ANOVA of the phytoplankton data revealed significant effects of insecticide concentration, the sensitivity of the *D. pulex* populations, time, an insecticide concentration-by-time interaction, and a marginal concentration-by-*Daphnia* sensitivity interaction (Table E.4). To further understand these effects, we analyzed phytoplankton at each sample date (Table E.5B; Figure 3.2).

On the first sampling date (day 20), there was a significant effect of insecticide concentration as well as *D. pulex* sensitivity, but no significant interaction term. Compared to control treatments, communities subjected to the highest two insecticide applications (0.50 and 1.0 μg/L) had more phytoplankton ($p \leq 0.010$). Across all concentrations, the increase in phytoplankton with sensitive *Daphnia* populations was relatively small. On the subsequent sample dates (days 40, 61, 81), we saw two distinct patterns emerge. On all sample dates, increases in chlorpyrifos caused increases in phytoplankton. Across the concentrations, we repeatedly observed no difference in phytoplankton between sensitivity categories at the lowest two concentrations of chlorpyrifos (all $p > 0.052$), a large increase of phytoplankton with sensitive *Daphnia* populations at 0.5 μg/L of chlorpyrifos when compared to resistant *Daphnia* populations (all $p < 0.024$), and no difference in phytoplankton between sensitivity categories at the highest concentration of chlorpyrifos (all $p > 0.431$).

3.3.4 Periphyton

The rm-ANOVA of the periphyton data revealed significant effects of insecticide concentration, the sensitivity of the *D. pulex* populations, and time (Table E.4; Figure 3.2). Periphyton abundance increased over time but decreased with higher concentrations of chlorpyrifos. Across all sample dates and chlorpyrifos concentrations, periphyton abundance was higher when communities contained resistant *Daphnia* populations than when they contained sensitive *Daphnia* populations.

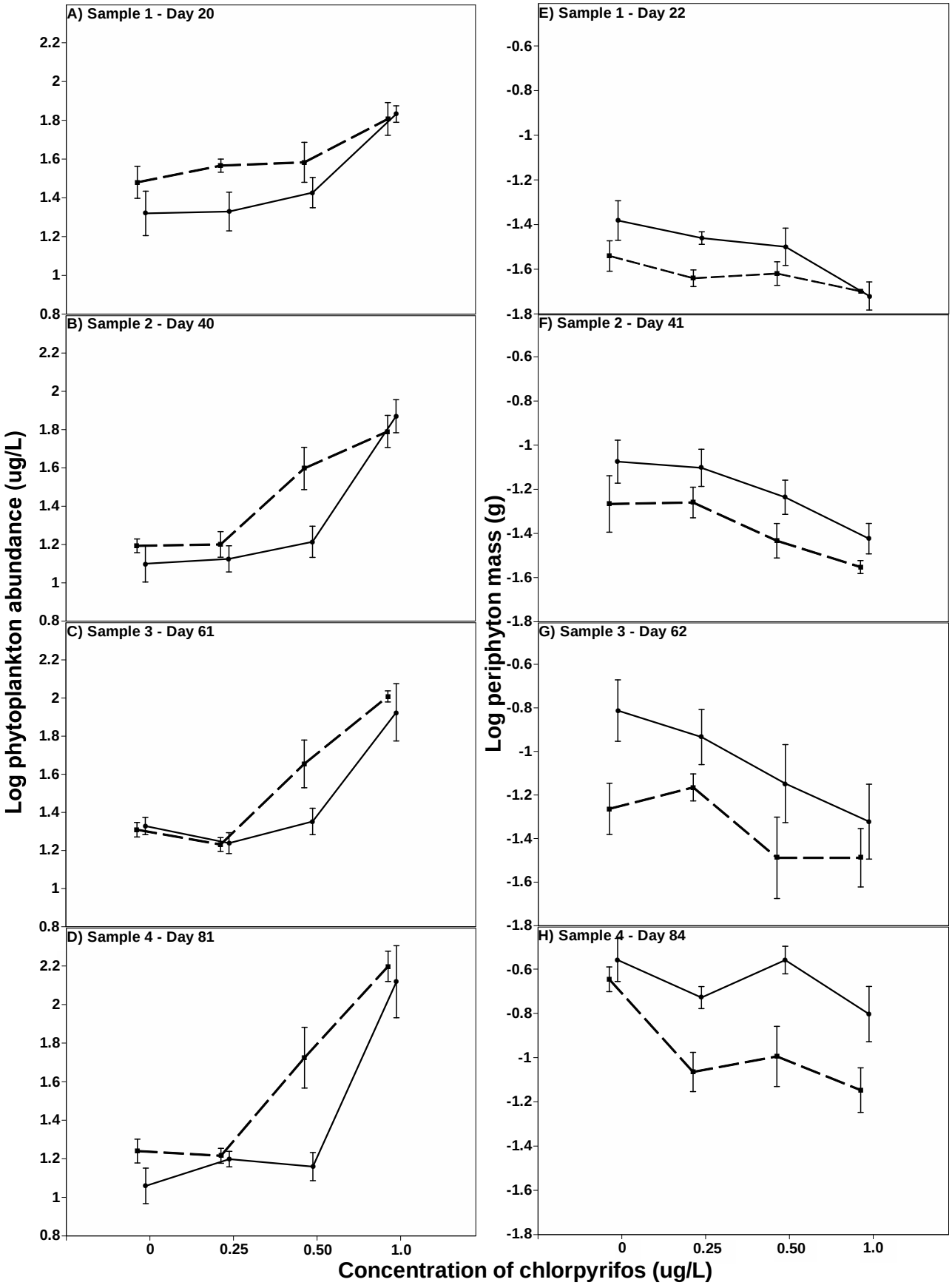
A) Sample 1 - Day 20
B) Sample 2 - Day 40
C) Sample 3 - Day 61
D) Sample 4 - Day 81
E) Sample 1 - Day 22
F) Sample 2 - Day 41
G) Sample 3 - Day 62
H) Sample 4 - Day 84
Log phytoplankton abundance (ug/L)
Log periphyton mass (g)
Concentration of chlorpyrifos (ug/L)
0
0.25
0.50
1.0

Figure 3.2. Phytoplankton and periphyton abundance across four sampling dates in experimental communities that were exposed to a range of chlorpyrifos concentrations. The solid line indicates communities with resistant *D. pulex* whereas the dashed line indicates communities with sensitive *D. pulex.*

3.3.5 Leopard frogs

When we analyzed the three life-history variables of the leopard frogs (overall survivorship, time to metamorphosis, and mass at metamorphosis), we found multivariate effects of concentration, *Daphnia* sensitivity, and their interaction (Table E.6A). We then ran separate univariate analyses of the life history traits of the leopard frogs: survivorship, time to metamorphosis and mass at metamorphosis.

To better understand how the treatments affected amphibian survival, we considered two different measures: 1) overall survival of all metamorphs and tadpoles at the end of the experiment or 2) just the survivorship to metamorphosis. The ANOVA of overall survivorship indicated that there was an effect of *D. pulex* sensitivity ($F_{1,40} = 8.229$, $p = 0.007$) but no effect of insecticide concentration ($F_{3,40} = 1.570$, $p = 0.741$) nor was there a significant interaction ($F_{3,40} = 0.846$, $p = 0.477$, Table E.6B; Figure 3.3B). In general, overall survivorship of leopard frogs was significantly higher in communities with resistant zooplankton, but this difference was only significant under the two highest concentrations of chlorpyrifos (both $p < 0.032$).

When we analyzed survival to metamorphosis (Figure 3.3A), we found significant effects of insecticide concentration ($F_{3,40} = 24.162$, $p = 0.001$) and *D. pulex* sensitivity ($F_{1,40} = 49.713$, $p < 0.001$) but no significant interaction ($F_{3,40} = 2.403$, $p = 0.082$). Because the interaction term

was nearly significant and the data suggested a strong difference in survival to metamorphosis at low versus high pesticide concentrations, we conducted mean comparisons at each concentration. Leopard frogs from communities with resistant *D. pulex* had increased survival to metamorphosis across all four treatment levels (all $p < 0.001$). At the lowest two concentrations of chlorpyrifos, 78-80% of the leopard frogs metamorphosed from communities containing resistant *D.* pulex, whereas only 55-59% metamorphosed from communities containing sensitive *D. pulex*. At the highest two concentrations of chlorpyrifos, 61-72% of the leopard frogs metamorphosed from communities containing resistant *D. pulex*, whereas only 17-23% metamorphosed from communities containing sensitive *D. pulex*.

For time to metamorphosis, the ANOVA indicated that there were no significant main effects of concentration or sensitivity of the *D. pulex* population, but there was a chlorpyrifos concentration-by-sensitivity interaction (Table E.6B; Figure 3.3C). In the control and 0.25 μg/L treatments, leopard frogs took 8.5 to 10.8 d longer to metamorphose if they were from communities with sensitive *D. pulex.* In the 0.50 μg/L treatment, time to metamorphosis did not differ between communities with either a resistant or sensitive population of *Daphnia* ($p = 0.718$). In the 1.0 μg/L treatment, the relationship was reversed and leopard frogs took ~9 d longer to metamorphose if they were from communities with resistant *D. pulex* ($p < 0.001$).

For mass at metamorphosis, there was an effect of *D. pulex* sensitivity, but no effect of chlorpyrifos concentration or their interaction (Table E.6B). Across all chlorpyrifos treatments, leopard frogs from communities with resistant *D. pulex* metamorphosed at a larger size than leopard frogs from communities with sensitive *D. pulex* (all $p \leq 0.05$; Figure 3.3D). In summary, increasing insecticide concentrations had an effect on survivorship to metamorphosis, but the majority of the effects on leopard frog life history were driven by the genetics of the *D. pulex*

populations within the communities.

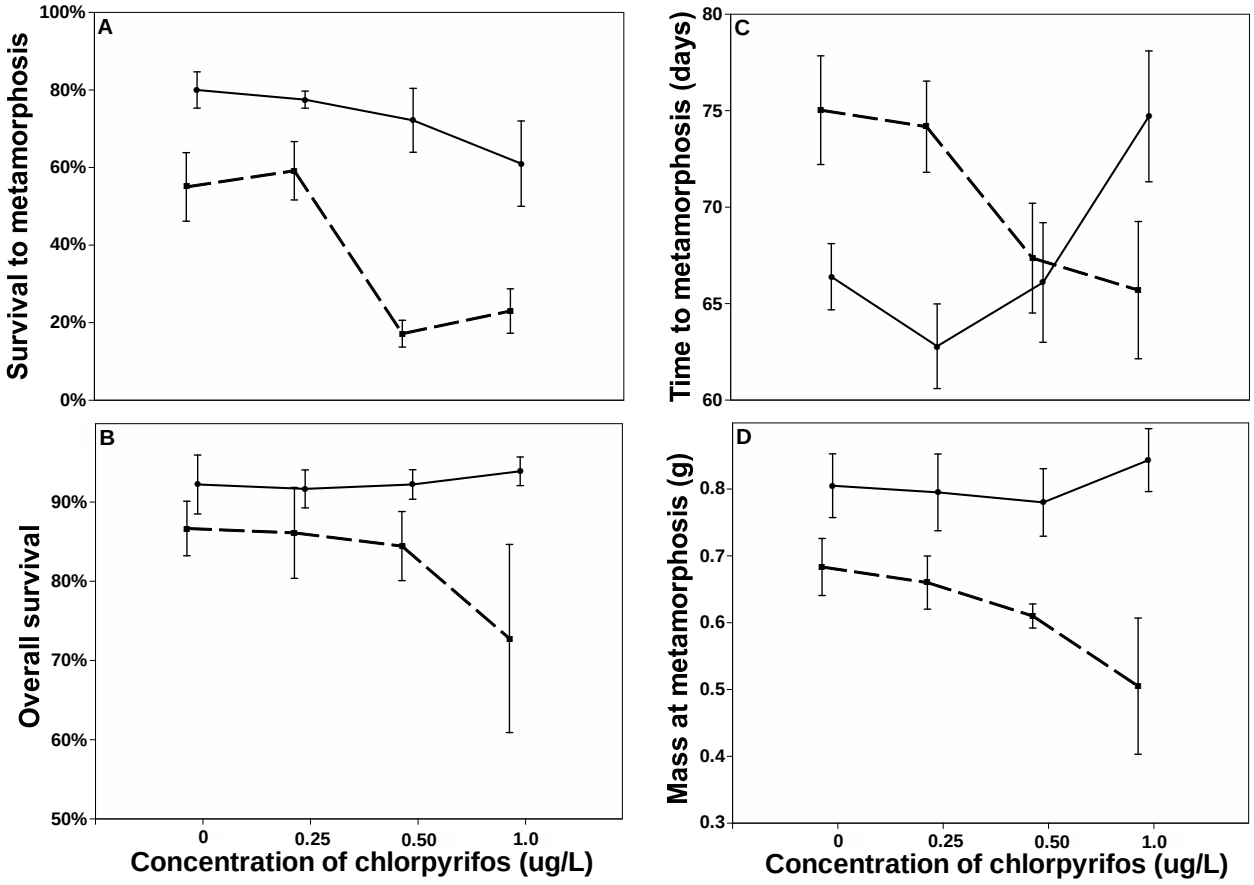

Figure 3.3. Respones of leopard frog tadpoles exposed to combinations of different chlorpyrifos concentrations and *Daphnia* populations that were either resistant or tolerant to the pesticide: A) survivorship to metamorphosis and B) overall survivorship of all metamorphs and tadpoles, C) time to metamorphosis, and D) mass at metamorphosis. The solid line indicates communities with resistant *D. pulex* whereas the dashed line indicates communities with sensitive *D. pulex*.

3.4 DISCUSSION

The results of this particular study indicate that extremely low and environmentally relevant concentrations of a historically common insecticide, chlorpyrifos, can trigger a series of cascading events, which can ultimately result in reduced growth and survivorship of amphibians. Our study is the one of the first to show that differences in population genetics of the zooplankton within the community had a marked impact on the trajectories of these communities after insecticides were added. Communities with resistant populations of *D. pulex* were buffered from the detrimental effects of the trophic cascade to a much greater extent than communities with sensitive populations of *D. pulex*. To our knowledge, this is the first study that suggests that differences in naturally occurring population-level variation in resistance to insecticides in zooplankton can determine whether or not a pond community is dramatically affected by, or almost entirely buffered from, the effects of an insecticide-induced trophic cascade. Furthermore, our chlorpyrifos concentrations spanned the range from having no observable effects on *D. pulex* abundance to completely extirpating all *D. pulex* in some experimental communities. However, abundance varied greatly between the tolerant populations that were collected near agricultural areas versus sensitive populations that were collected far from agricultural areas. This is consistent with our previous laboratory studies that demonstrated differences in sensitivity among the four populations (Bendis and Relyea 2014). Moreover, the concentrations utilized in this experiment (all $\leq$ 1.0 μg/L) are substantially lower (10-500x) than previously utilized concentrations of other related chemicals with similar modes of action. Interestingly, even at such extremely low concentrations, the community-wide effects of these insecticides were dramatic.

Our highest pesticide concentration (1.0 μg/L) was lethal to almost all of the *D. pulex*

within the experimental communities. In communities with sensitive *D. pulex*, the zooplankton were entirely eliminated from the community after the first application and never recovered. In communities with resistant *D. pulex*, most of the mesocosms experienced a similar phenomenon where all *D. pulex* were extirpated after the first application. This is not surprising as most zooplankton are generally highly susceptible to a wide array of insecticides (Boone and James 2003, Boone et al. 2004, Mills and Semlitsch 2004, Relyea and Diecks 2008). However, in several of the communities where *D. pulex* were virtually eliminated, cladoceran populations rebounded to some degree as resistant genotypes repopulated the mesocosms.

Our third highest concentration (0.5 μg/L) caused the greatest differences in *D. pulex* abundance between communities with different *D. pulex* sensitivities. This is consistent with our previous laboratory LC50 work on these populations (Bendis and Relyea 2014) as well as several other studies that have examined chlorpyrifos toxicity in cladocerans (van der Hoeven and Gerritsen 1997, Caceres et al. 2007, Palma et al. 2009). In the 0.5 μg/L treatment, both populations of resistant zooplankton had abundances that were not different from the control communities. However, in communities with populations of sensitive zooplankton, there were significantly fewer zooplankton present. It was this difference in survivorship of *D. pulex* populations between the different community types that drove the differences throughout the entire community.

Whereas the sensitive and resistant populations differed in their response to chlorpyrifos, the two resistant populations responded similarly to each other and the two sensitive populations responded similarly to each other, despite being collected at different locations and dispersal between the ponds is unlikely. The resting eggs (i.e. ephippia) of cladoceran zooplankton are typically dispersed through surface water, transfer via animals (i.e. humans, birds and

Notonecta), and even the wind (Havel and Medley 2006, van de Meutter et al. 2008). However, there is still much debate concerning how far these eggs typically disperse. One study found that, within 15 months, an average of four cladoceran species colonized newly created pools; this represented nearly 40% of the species richness within 3 km of the pools (Louette and De Meester 2005). However, another study that examined the range expansion of the invasive cladoceran *Daphnia lumholtzi* found that all 40 ponds sampled within the watersheds of 11 reservoirs known to have *D. lumholtzi* did not contain any of the species of interest (Dzialowski et al. 2000). The conclusion of this study (and several others) is that non-human dispersal mechanisms probably plays an insignificant role in the zooplankton dispersal (Jenkins and Underwood 1998). The two ponds from which we collected sensitive *D. pulex* are located > 55 km apart from each other, which would make it unlikely that cladocerans could have dispersed between the two ponds. Although the two ponds where we collected resistant *D. pulex* are closer to each other (~2.5 km apart), both ponds are located on private property and are not frequently visited by people.

Previous studies have shown that many species of *Daphnia*, and other species of non-target organisms, often exhibit marked life-history trade-offs associated with the maintenance of resistance to pesticides or parasites such as reduced growth rates and smaller clutch sizes which both would lead to slower overall population growth rates (Ebert 1995, van der Hoeven and Gerritsen 1997, Shirley and Sibley 1999, Little and Ebert 2001, Duffy and Sivars-Becker 2007, Coors and De Meester 2008, Little et al. 2008, Jansen et al. 2011, Auld et al. 2013). Interestingly, in our communities with 0 μg/L chlorpyrifos, there were no significant differences in the abundance of *Daphnia* during any of the seven time points. This suggests that higher tolerance in both of our *Daphnia* populations collected near agricultural areas did not experience a life-history trade-off that affected the growth rates of their populations.

Because *D. pulex* is a major consumer of phytoplankton, additions of chlorpyrifos that caused declines in *D. pulex* also caused phytoplankton blooms. Further evidence of a phytoplankton bloom was that the increase in DO and pH in communities where *D. pulex* were nearly eradicated at high chlorpyrifos concentrations. Such increases in pH and DO are due to increased photosynthetic activity from the phytoplankton bloom. Similar results have been found in other studies where insecticides indirectly caused phytoplankton blooms by eliminating zooplankton from aquatic communities (Sierzen et al. 1998, Boone et al. 2004, Mills and Semlitsch 2004, Relyea and Diecks 2008, Hua and Relyea 2012).

We also found that light decay rates were higher in communities containing sensitive *D. pulex* and high concentrations of chlorpyrifos, which reflects the fact that phytoplankton blooms reduce the amount of light that can pass through the water column. Communities with 0 or 0.25 μg/L chlorpyrifos never experienced a full phytoplankton bloom. At the next higher chlorpyrifos concentration (0.50 μg/L), we observed the largest difference in phytoplankton abundance between communities with resistant and sensitive *D.* The highest concentration (1.0 μg/L) caused a phytoplankton bloom in all communities, regardless of *D. pulex* sensitivity because although the *D. pulex* populations differed in sensitivity, there is a limit to their tolerance. In short, our results show that incorporating natural genetic variation among *Daphnia* populations can have dramatic effects on the phytoplankton.

Because phytoplankton and periphyton compete for resources including light, the phytoplankton blooms that occurred at the highest two insecticide concentrations led to a decline in periphyton abundance by the end of the experiment. Similar phytoplankton blooms and subsequent declines in periphyton abundance have been found in several other studies examining the effects of insecticide-induced trophic cascades in experimental mesocosms (Mills and

Semlitsch 2004, Relyea and Diecks 2008, Relyea and Hoverman 2008). However, as the experiment progressed there was also a divergence in the abundance of periphyton between communities with resistant and sensitive *D. pulex*. By the final sample of periphyton, this difference was most noticeable at two highest chlorpyrifos concentrations; in short, the concentration where we saw the greatest difference in zooplankton abundance among populations was also where we saw the largest increase in phytoplankton abundance and the largest decrease in periphyton.

While the decline in periphyton with sensitive *D. pulex* populations was most pronounced under the higher chlorpyrifos concentrations, we also observed that periphyton was less abundant with sensitive *D. pulex* populations even when no chlorpyrifos was present during the first and third samples. Because we could not detect any differences in the abundance of the *D. pulex* populations in the absence of chlorpyrifos, it is unclear why periphyton was less abundant with sensitive *D. pulex* populations. It may be that the *D. pulex* populations differ in the species of phytoplankton that they consume or the amount of nutrients that they recycle, which in turn may affect light transmission and alter periphyton abundance. Clearly, further research should be undertaken to determine how population-level differences in resistant and sensitive *D. pulex* can potentially alter the abundance of periphyton in aquatic communities.

The trophic cascade initiated by the direct lethal effects of chlorpyrifos on the zooplankton also affected the leopard frogs in the communities. As we simulated pond drying at the end of the experiment, we found that the insecticide-induced trophic cascade indirectly caused a decline in tadpole survivorship to metamorphosis. As in previous studies, the proximate cause for the leopard frog mortality was pond drying, but the ultimate cause was the alteration of the food web by the repeated insecticide applications.

When we examined overall survivorship, we found that leopard frogs exposed to the two highest chlorpyrifos concentrations only survived better if they were from communities with resistant *D. pulex*. More importantly, we were able to rule out pesticide exposure being linked to leopard frog mortality because overall survivorship across all four treatments was above 90% in communities with either resistant population of *D. pulex*. Furthermore, the concentrations utilized in this experiment are entirely sublethal to leopard frogs (Gaizick et al. 2001, Relyea 2009). Therefore, the patterns in mortality and effects on time and mass at metamorphosis were the result of the populations of *D. pulex* that were present.

While there were no interactive effects of chlorpyrifos applications and differences in *D. pulex* sensitivity on overall survivorship, there were significant and striking effects on survivorship to metamorphosis. When we analyzed the data for survival to metamorphosis, we found that leopard frogs emerging from communities with resistant *D. pulex* had a higher chance of surviving to metamorphosis across all treatments, including the no-pesticide control. The likely reason for this pattern was the higher abundance of periphyton in communities with resistant *D. pulex*. Concordant with our other data, the largest declines in survivorship to metamorphosis was between communities with resistant versus sensitive *D. pulex* were at the two highest concentrations of the insecticide (0.5 and 1.0 μg/L) where leopard frogs were approximately 4.5 and 2.7 times more likely to successfully metamorphose from a community with resistant *D. pulex* relative to communities with sensitive *D. pulex*.

Several other studies have found that insecticide applications that are entirely sublethal to amphibians can have marked negative effects on survivorship to metamorphosis through a variety of direct and indirect effects on the aquatic food web (Mills and Semlitsch 2004, Boone et al. 2005, Relyea and Diecks 2008). Where our results differ, however, is that the effects of the

insecticide-induced trophic cascade are depend on the genetics of the zooplankton populations. To our knowledge, this is the first study to demonstrate that the population genetics of zooplankton in a community can cause significant effects on amphibian survivorship through the stabilization of an aquatic food web after the addition of an insecticide.

Leopard frog time to metamorphosis was also affected by the trophic cascade initiated by chlorpyrifos. When exposed to either 0 or 0.25 μg/L of the insecticide, leopard frogs emerging from communities with sensitive *D. pulex* took 8.5 to 10 days longer to metamorphose than leopard frogs emerging from communities with resistant zooplankton. Interestingly, leopard frogs in communities with 0.50 μg/L of the insecticide, where we saw the largest differences in zooplankton and phytoplankton abundance, metamorphosed at similar times regardless of whether the community contained resistant or sensitive *D. pulex*. In communities with 1.0 μg/L of the insecticide, however, the relationship was reversed: leopard frogs emerging from communities with sensitive *D. pulex* emerged an average of 9 days earlier than leopard frogs emerging from communities with resistant *D. pulex*.

One reason for this pattern may be the time lag associated with the phytoplankton bloom and the growth of periphyton. Although phytoplankton blooms were maintained throughout the experiment (particularly in the 0.5 μg/L treatment), the associated decline in periphyton abundance only began to differ more intensely towards the end of the experiment because the trophic cascade takes time to develop. As the phytoplankton bloom developed in communities with sensitive *D. pulex*, the periphyton within these communities were outcompeted by phytoplankton for access to resources (e.g., light) and subsequently declined in abundance which would have an effect on tadpole time to metamorphosis, particularly at the higher two insecticide concentrations. On the other hand, in communities with resistant *D. pulex*, the phytoplankton

bloom was prevented and the competing periphyton had the resources necessary to continually grow throughout the majority of the experiment. This led to higher abundances of periphyton which, in turn, gave the leopard frogs in these communities adequate access to the resources needed to metamorphose. Since periphyton abundance was high in these communities and the environment was relatively benign (i.e. free of predators) leopard frogs from communities with resistant *D. pulex* may have delayed metamorphosis. In communities with sensitive *D. pulex*, competition may have driven leopard frogs to complete metamorphosis earlier at a smaller, non-optimal size.

In terms of mass at metamorphosis, leopard frog metamorphs emerging from communities with resistant *D. pulex* always metamorphosed at a larger size when compared to those emerging from communities with sensitive *D. pulex*, even when the insecticide was absent. This difference in mass is consistent with the higher abundance periphyton in communities with resistant *D. pulex* relative to communities with sensitive *D. pulex* during two of the four sample dates. This increased abundance in periphyton in communities containing resistant *D. pulex* allowed leopard frogs to grow to a larger size compared to leopard frogs living in communities with sensitive *D. pulex*, which is a phenomenon that, to our knowledge, has not been previously observed.

At the highest concentration, however, all communities with sensitive *D. pulex* experienced large declines in abundance elimination and the phytoplankton bloom periphyton decreased the amount of resources (e.g., light) that were available to the periphyton. In communities with resistant *D. pulex*, cladoceran populations were negatively affected by the insecticide as well. However, some of the resistant *D. pulex* populations rebounded and dampened the magnitude of the phytoplankton bloom. This would lead to the pattern where

leopard frogs from communities with resistant *D. pulex* metamorphosed faster in the highest chlorpyrifos treatment (1.0 μg/L).

3.4.1 Conclusions

In this study, we have demonstrated that low and environmentally-relevant concentrations of a commonly applied insecticide had direct lethal effects on zooplankton and this led to numerous indirect effects throughout the aquatic community food web. Amazingly, the entire food web was affected by simply altering the population of *D. pulex* that was present in the community including abiotic conditions (pH, DO, and light transmission), phytoplankton abundance, periphyton abundance, and amphibian survival, mass at metamorphosis, and time to metamorphosis. To further our understanding of the effects of insecticides on aquatic communities, future studies should search for the potential for cross-resistance among zooplankton to insecticides of both similar and differing modes of action. This is essential as some populations may be resistant to a wide array of pesticides, especially those pesticides that share a similar mode of action. Furthermore, future studies should utilize more diverse zooplankton assemblages to determine whether or not the effects of these pesticide-induced perturbations are driven primarily by the loss of *Daphnia* or are there other species of zooplankton that are generally more resistant to insecticides that can fill the same functional role. To better understand how future perturbations will impact aquatic communities across an agricultural gradient, and to protect globally threatened species such as amphibians, we must incorporate as much ecological realism into our community studies as possible.

Only by doing so will we be able to determine how incidental exposure to anthropogenic chemicals will effect natural pond communities for generations to come.

3.5 ACKNOWLEDGEMENTS

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4.0 LIFE DOWN ON THE FARM: COMMUNITY-WIDE EFFECTS OF PESTICIDE CROSS-RESISTANCE IN ZOOPLANKTON POPULATIONS NEAR AND FAR FROM AGRICULTURE

4.1 INTRODUCTION

Synthetic pesticides are ubiquitous across the globe and have been largely responsible for increasing agricultural yields since their introduction in the late 1930s. It has been estimated that every $1 spent on pesticide production and use, has led to $4 in crops being saved from their target pest species (Pimentel et al. 1992). Although overall pesticide use in the United States has been slowly declining over recent years, due to the phasing out of specific classes of pesticides (i.e. organophosphates), worldwide production of these chemicals has increased dramatically to the point where nearly 2.4 billion kg of pesticides are applied every year (Grube et al. 2011). These chemicals are designed to affect the physiology of target pest species by either deterring, incapacitating, or killing them. However, widespread pesticide use can also cause direct, indirect, and sublethal effects on non-target organisms, as well as lead to the evolution of pesticide resistance. Evolved resistance in targeted pest species has received a lot of attention by researchers across the globe (Georghiou and Taylor 1977, Hoy 1998, Weill et al. 2003) and it is estimated that pesticide resistance causes more than $1.5 billion in crop losses each year

(Pimentel 2005). What is far less understood, however, is whether evolved pesticide resistance also occurs in non-target species that are inadvertently exposed to these chemicals (Brausch and Smith 2009*a*/*b*, Jansen et al. 2011).

Although there is less of an economic incentive to study evolved resistance in non-target species, such resistance may have important ecological and conservation implications (Hua et al. 2013). For instance, pesticides can markedly decrease population-level genetic variation and this can be disadvantageous in terms of responding to future environmental change (Georghiou 1990, Carriere et al. 1994). Pesticides can have a range of sublethal effects on non-target organisms (i.e. life history trade-offs) that can have direct effects on the survivorship and fecundity of the directly impacted species and can also have an array of indirect effects throughout the food web. On the other hand, populations of non-target species that have evolved resistance and play key roles in communities can buffer aquatic communities from the negative effects of a pesticide-induced trophic cascade (Bendis and Relyea, *in review*).

Today there are more than 1,055 active ingredients that are registered as pesticides in the United States (Goldman 2007). Due to this large number of chemicals, pesticides are often classified by their mode of action, which is the method by which chemicals affect target pest species. An interesting aspect of considering pesticide modes of action is that target species can commonly evolve cross-resistance to multiple chemicals of the same class or mode of action and sometimes even cross-resistance among pesticides with different modes of action, (Brengues et al. 2003, Brausch and Smith 2009*a*, Mitchell et al. 2012, Hua et al. 2013). It is reasonable to predict that cross-resistance may also be common in non-target species, but we know very little about the prevalence of cross-resistance among non-target species. Furthermore, we know

nothing about whether patterns of cross-resistance to pesticides that share a mode of action can have the same community-wide effects as pesticide that have different modes of action.

Pond communities are ideal for studying the community-wide effects of cross-resistance to insecticides because they are found across a wide range of distances from agricultural areas and are therefore subjected to a variety of pesticide types and application frequencies (De Meester et al. 2005). Habitats with higher proportions of surrounding agriculture and closer distances to agricultural areas have proven to be useful proxies of historic pesticide exposures that lead to populations evolving higher pesticide resistance (Coors et al. 2009, Cothran et al. 2013, Bendis and Relyea 2014). For example, recent studies have shown that wood frog populations (*Lithobates sylvaticus*) living closer to agricultural areas have higher resistance to the insecticide carbaryl (Cothran et al. 2013). A subsequent study found that these populations that were not only resistant to this one acetylcholine esterase (AChE)-inhibiting insecticide, but they also exhibited cross resistance to two additional AChE-inhibiting insecticides, thereby indicating that cross-resistance may be common among non-target species (Hua et al. 2013). Recent studies have also shown that zooplankton populations in the genus *Daphnia* can vary in their resistance to commonly applied insecticides such as carbaryl (*D. magna,* Coors et al. 2009) and chlorpyrifos (*D. pulex*, Bendis and Relyea 2014) and that these patterns of resistance are also related to agricultural land use surrounding the ponds. Such evolved resistance is important because zooplankton are one of the most sensitive taxonomic groups to insecticides, and they play a key role in the function of pond communities as consumers, as prey, and as cyclers of nutrients (Hanazato 1998, 2001). When zooplankton are exposed to an insecticide, they can experience large declines in abundance and their food resource (i.e. phytoplankton) typically experiences a dramatic increase in abundance. The increase in phytoplankton can, in turn, cause

a further trophic cascade that has numerous deleterious effects throughout the community (Barry and Logan 1998, Boone and James 2003, Fleeger et al. 2003, Boone et al. 2004, Mills and Semlitsch 2004, Relyea and Diecks 2008, Relyea 2009). However, we recently discovered that communities containing resistant populations of *D. pulex* can buffer the entire aquatic communities from the impacts of insecticides (Bendis and Relyea, *in review*). Although this is an important finding, we need to know if zooplankton can evolve cross-resistance to other pesticides with the same or different modes of action, and whether this allows the zooplankton to buffer communities from pesticide exposures.

We addressed this question using populations of *D. pulex* that vary in their resistance to the insecticide chlorpyrifos (Bendis and Relyea 2014). We created identical aquatic communities that varied only in the population of *D. pulex* that the community received (two "resistant" populations collected from ponds near agriculture, two "sensitive" populations collected from ponds far from agriculture). We hypothesized that communities containing populations of chlorpyrifos-resistant *D. pulex* would be buffered from the effects of low concentrations of not only chlorpyrifos, but also carbaryl and malathion, which have the same mode of action as chlorpyrifos (i.e. they inhibit AChE). In contrast, communities exposed to low concentrations of permethrin and cypermethrin, which have a different mode of action (i.e. Na^+ channel-inhibiting insecticides), should not exhibit any differences in resistance to these insecticides, regardless of the *D. pulex* population included and should therefore not be buffered.

4.2 METHODS

We conducted a mesocosm experiment at the University of Pittsburgh's Donald S. Wood Field Laboratory at the Pymatuning Laboratory of Ecology. Using mesocosms allowed us to replicate

aquatic communities, while simultaneously subjecting these communities to a range of pesticide applications (Relyea and Diecks 2008). The experimental design was a full factorial using four *D. pulex* populations: two resistant populations from ponds with surrounding agriculture (>30% agricultural land within a 300-m radius) and two sensitive populations from ponds with little or no surrounding agriculture (<5% agricultural land within a 300-m radius). These four populations were each exposed to 16 insecticide treatments ([0.25, 0.50 and 1.0 μg/L chlorpyrifos], [12.5, 25 and 50 μg/L carbaryl], [0.5, 1.0, and 2.0 μg/L malathion], [0.5, 1.0, and 2.0 μg/L permethrin], [0.5, 1.0 and 2.0 μg/L cypermethrin], and a negative control).

The insecticide concentrations were determined from our review of published data on recorded LC50s for *D. pulex* for each insecticide, a comparison of LC50 values from other LC50 pilots performed within our lab, and from a series of LC50 experiments that we performed prior to the setup of the mesocosm experiment (Bendis and Relyea 2014, Table I.1). For the LC50 pilot, we exposed 10 juvenile female *D. pulex* from either a resistant (Love pond) or sensitive (Minnow pond) to a range of concentrations of malathion, carbaryl, cypermethrin or permethrin for a period of 24 hours. Additionally, we utilized a negative control containing only UV-filtered water and an ethanol vehicle control to ensure that the highest concentration of ethanol used to dissolve the pesticides was not directly responsible for *D. pulex* mortality. We knew that these two populations significantly varied in their natural resistance to chlorpyrifos from previous studies (Bendis and Relyea 2014, Bendis and Relyea, *in review*). From this pilot, we found that the populations that were resistant to chlorpyrifos also showed signs of resistance to carbaryl and malathion, as their respective 84% confidence intervals (CI) did not overlap with those of the sensitive populations (simulation tests have shown that when 84% CIs do not overlap between two LC50 estimates, this method approximates an $\alpha = 0.05$, Payton et al. 2003).

Moreover, there was no evidence of cross-resistance to insecticides with different modes of action as the 84% CIs of the resistant and sensitive population LC50 values overlapped a great deal (Table I.1).

We used the LC50 data from our pilot lab experiments to find a range of suitable concentrations to use in the community experiment. Specifically, we set the middle concentrations to be similar to the LC50 of the resistant population utilized in our pilot studies. The lower concentrations were set to be sublethal and have no observable effect to the resistant population, whereas our higher concentration was set to be lethal to both resistant and sensitive populations (based on our pilot mortality studies). In setting these concentrations relative to each insecticide's LC50 value, our goal was to compare our responses of the community between D. pulex populations *within* a given insecticide; our goal was not to directly compare community responses to the different insecticides. The 64 treatment combinations were replicated three times and an additional eight experimental units (2 for each *D. pulex* population) were used as vehicle controls, since the insecticides were dissolved in ethanol. These vehicle controls allowed us to test the effects of ethanol on the community. In total, there were 200 individual mesocosm communities that were monitored throughout the remainder of the experiment.

4.2.1 Mesocosm set up

These experimental units were 75-L garbage cans (58.4 cm x 49.5 cm - Rubbermaid BRUTE^{TM}) that were filled with approximately 65-L of well water from 21 May to 22 May 2013. Each mesocosm was covered by a 60% shade-cloth lid to prevent any movement of animals. On 6 April, we added 200 g of dry leaf litter (primarily *Quercus* spp.) to each mesocosm to provide

both nutrients and additional surface area for periphyton growth. On 23 May, we added 1.5 g of rabbit chow and 20 g of leaf litter (*Quercus* spp.) to provide an initial nutrient spike. On this day, we also added four unglazed ceramic tiles (7.5 cm x 15 cm) along the north side of each mesocosm to provide a standardized measure of periphyton abundance. On 24 May, we took pond water samples from each of the four ponds where our *D. pulex* populations were collected (Love, Mallard, Minnow and Hopscotch), as well as one additional pond (Trailer Park); we visually screened the zooplankton samples for invertebrate predators. After removing predators, we ran the water through a series of sieves (1 mm, 250 µm, 64 µm) four times to remove all zooplankton and then treated the pond water with carbonated water to remove any smaller zooplankton, such as rotifers or copepod nauplii that may have made it through the sieves. Once each sample of water had been processed, we combined the pond water samples and added equal aliquots to each experimental unit to provide a natural source of periphyton and phytoplankton.

4.2.2 *Daphnia* population collection and rearing

On 6 May, we collected and isolated *D. pulex* (hereafter referred to as "*Daphnia*" for simplicity) from pond water samples taken from each of our four focal populations and placed each population in separate 12.5-L plastic containers (each population was held in eight replicate containers). Each container was filled with carbon-filtered, UV-irradiated well water and the *Daphnia* populations were fed lab-cultured algae (*Scenedesmus* spp.) *ad libitum*. As female *Daphnia* released their offspring, these females were removed from the experimental populations. On 25 May, after the *Daphnia* populations had produced at least 2-3 generations of

offspring in the lab, we added 100 juvenile females from one of the four *Daphnia* populations to the corresponding mesocosms.

4.2.3 Leopard frog collection and rearing

Leopard frog tadpoles (*Lithobates pipiens)* were raised from egg masses that we collected from a single pond in northwestern Pennsylvania (Mallard Pond; Lat 41.691669, Long-80.501070). We collected 12 egg masses on 17 April and reared the hatched tadpoles in 200-L pools containing well water. Once hatched, the tadpoles were fed rabbit chow *ad libitum*. On 5 June, after the algal and bacterial assemblages had developed for 13 days, we added five leopard frog tadpoles to each mesocosm. We chose the tadpoles for our experiment by mixing tadpoles from all 10 egg masses, and then selected individuals of a similar size (initial mass ± SE: 76.5 mg ± 7.1 mg). We used tadpoles that had experienced some growth since hatching to ensure that the individuals were healthy, and to simulate a scenario in which animals have lived a portion of their life under pesticide-free conditions and then are subjected to sublethal concentrations of pesticides. Survival of the leopard frog tadpoles 24 hours after being handled was 100%.

4.2.4 Pesticide additions

We allowed the tadpoles to acclimate to experimental conditions for 6 days before applying the insecticide treatments. On 11 June (defined as day 1 of the experiment), we exposed each mesocosms to one of the 16 pesticide treatments. We began by creating a stock solution for each insecticide by dissolving the respective chemical in ethanol (EtOH), as many of these chemicals

are moderately insoluble in water (for details, see Appendix G). For control mesocosms with 0 µg/L chlorpyrifos, we added 626 µL of carbon-filtered, UV-irradiated well water. For the eight mesocosms assigned the ethanol treatment, we added 626 µL of EtOH to verify that the largest amount of EtOH added to experimental communities (i.e. the amount included in the highest pesticide concentrations) did not affect the community. After the pesticide treatment was applied to a given mesocosm, we stirred and agitated the water in the mesocosm to equalize disturbance and to ensure that the pesticide was mixed throughout the water column.

To verify the actual concentrations of the insecticides used in our experimental communities, we collected an aliquot of water from each of the mesocosms assigned to a particular concentration within 1.5 hours of applying the insecticides and pooled the samples into pre-cleaned, 500-mL amber jars containing 2 mL of methylene chloride (CH_2Cl_2) to stabilize the insecticides. We sent these samples to an independent laboratory for chemical analysis using high-performance liquid chromatography (Center for Environmental Services and Engineering, University of Connecticut, Connecticut, USA). For the carbaryl, chlorpyrifos and malathion (the AChE-inhibiting insecticides), the actual average concentrations were within 93, 94 and 72% of the nominal concentrations, respectively. For permethrin and cypermethrin (the two pyrethroid insecticides), the actual average concentrations were significantly lower and only within 28 and 5% of the nominal concentrations, respectively (Appendix G). This, however, is not surprising as half-life for pyrethroid insecticides can be extremely low (~0.5 days) as the insecticide is rapidly broken down via both hydrolysis and UV-degradation (Lutnicka et al. 1999, Bennett et al. 2005). None of the five insecticides were detected in our non-insecticide controls.

Three weeks after applying the insecticides (day 23), we re-applied the insecticide concentrations and re-tested our nominal concentrations for the second application. For the

carbaryl, chlorpyrifos and malathion, the actual average concentrations were within 76, 57 and 65% of the nominal concentrations, respectively. For permethrin and cypermethrin, the actual average concentrations were 38 and 45% of the nominal concentrations, respectively (Appendix G). Again, there were no pesticides detected in our control samples.

4.2.5 Abiotic response variables

During the course of the experiment, we measured several abiotic response variables to help us understand the effects of the various insecticides on the communities (Appendix H). On days 4, 26 and 48, we measured pH, temperature, and dissolved oxygen (DO, Figure 4.1). Days 4 and 26 immediately followed our two pesticide applications and the day 48 was immediately before initiating the water drawdown on day 50. Temperature, pH and DO content readings were taken with a calibrated digital water meter (YSI, Yellow Springs, OH, USA). On days 8, 30 and 50 we took light measurements primarily because these days followed pesticide applications, were close to the days in which we sampled other abiotic factors and were clear, cloudless days, which are ideal for taking light measurements. We measured light radiation from the middle of each mesocosms at depths of 10 and 30 cm and calculated the decay rate of light with increased water depth (k) using the equation

$$k = [\ln(L_{10}/L_{30})]/d$$

where L_{10} is the intensity of sunlight from a depth of 10 cm, L_{30} is the intensity of sunlight from a depth of 30 cm, and d is the difference in depth between the two measurements of intensity (Relyea and Diecks 2008). Light attenuation was measured with an underwater light meter (LI-COR. Lincoln, Nebraska, USA).

4.2.6 Biotic response variables

We quantified several biotic response variables during the experiment. We sampled *Daphnia* abundance at four time points during the experiment (Figure 4.1). We sampled the *Daphnia* by submerging a 0.2-L plastic sampling tube in the middle of the water column at six different locations within each mesocosms (north, south and middle quadrants of each mesocosm both towards the surface and near the benthos). All six samples within each mesocosm were pooled and the sample was filtered through a 64-µm Nitex cloth screen and poured into a Whirlpak bag containing 30% ethanol to preserve the samples for subsequent enumeration. For *Daphnia* enumeration, we poured the ethanol from the Whirlpaks containing our zooplankton samples onto a Petri dish with a preset grid. We counted all *Daphnia* individuals in each grid and summed the total to get a count for each sample. We also identified and enumerated any zooplankton that were not *Daphnia*.

We measured phytoplankton twice during the experiment (days 22 and 51, Figure 4.1). Phytoplankton was sampled just prior to the second pesticide application and at the end of the experiment to determine how much phytoplankton was in each mesocosm. To measure phytoplankton, we sampled 0.5 L of water from the center of each mesocosm. The water samples were poured through a vacuum-filtration system and through GF/C Whatman glass microfiber filters (Whatman Industries Inc., Florham Park, New Jersey, USA). After each sample had been vacuum-filtered, each sample was wrapped in aluminum foil and stored in a freezer at -18 °C. To assess phytoplankton abundance, we used the concentration of chlorophyll *a* as our proxy which was quantified using a fluorometer (Turner Designs TD-700, Sunnyvale, CA, USA) and the protocols developed by Arar and Collins (1997).

Periphyton abundance was also quantified twice during the course of the experiment (days 26 and 53, Figure 4.1) by removing one of the four clay tiles from each mesocosm. Periphyton was always sampled within two days of our phytoplankton samples. Once a tile was removed, it was vigorously scrubbed with a toothbrush to remove all of the periphyton on the face of the tile and subsequently rinsed with carbon-filtered, UV-irradiated well water. The slurry containing water and periphyton was then vacuum-filtered onto a Whatman GF/C filter that had been previously dried for 24 hours at 80°C and weighed. After the periphyton sample was vacuum-filtered, the filters were again dried at 80°C for an additional 24 hours and weighed. The amount of periphyton biomass was measured as the mass of the filter paper containing the dried periphyton subtracted by the original mass of the dry, unused filter.

The first leopard frog metamorphs emerged on day 33; every day thereafter we conducted checks for metamorphs from all mesocosms. We visually scanned each mesocosm and removed all individuals when both hindlimbs and forelimbs emerged and their tail was almost completely resorbed. Once metamorphs were removed from the mesocosms, they were kept in 1-L plastic containers in the laboratory containing a layer of moist sphagnum moss. Each metamorph was checked daily and when the tail was completely resorbed, we euthanized the metamorph using a 2% solution of MS-222. All metamorphs were then preserved in a solution of 10% formalin.

On day 50 (31 July) we began a week-long drying protocol to simulate the natural drying that occurs in wetlands in our region during the late summer. It is important to conduct water drawdowns gradually because amphibians can sense the drying of a pond by detecting a reduced volume and respond by speeding up their development (Denver et al. 1998). To simulate the gradual drying of a pond, we removed 5 L of water each day for 1 week; on day 57 we terminated our experiment when approximately 30 L of water remained in each mesocosm. On

that day we drained the remainder of water from the mesocosms and sorted through the litter and detritus to recover all amphibians that had not metamorphosed yet. If we collected amphibians that had at least one forelimb, we allowed that individual to complete metamorphosis (Gosner stage 46, Gosner 1960) in the lab. All other amphibians that did not have any forelimbs were euthanized using MS-222 and preserved in a 10% formalin solution. Most animals (94.4%) did not metamorphose by the end of the experiment. For those individuals that metamorphosed, we recorded time to metamorphosis, mass at metamorphosis, and survival to metamorphosis. For those individuals that did not metamorphose, we recorded the mass of the tadpoles and developmental (Gosner) stage.

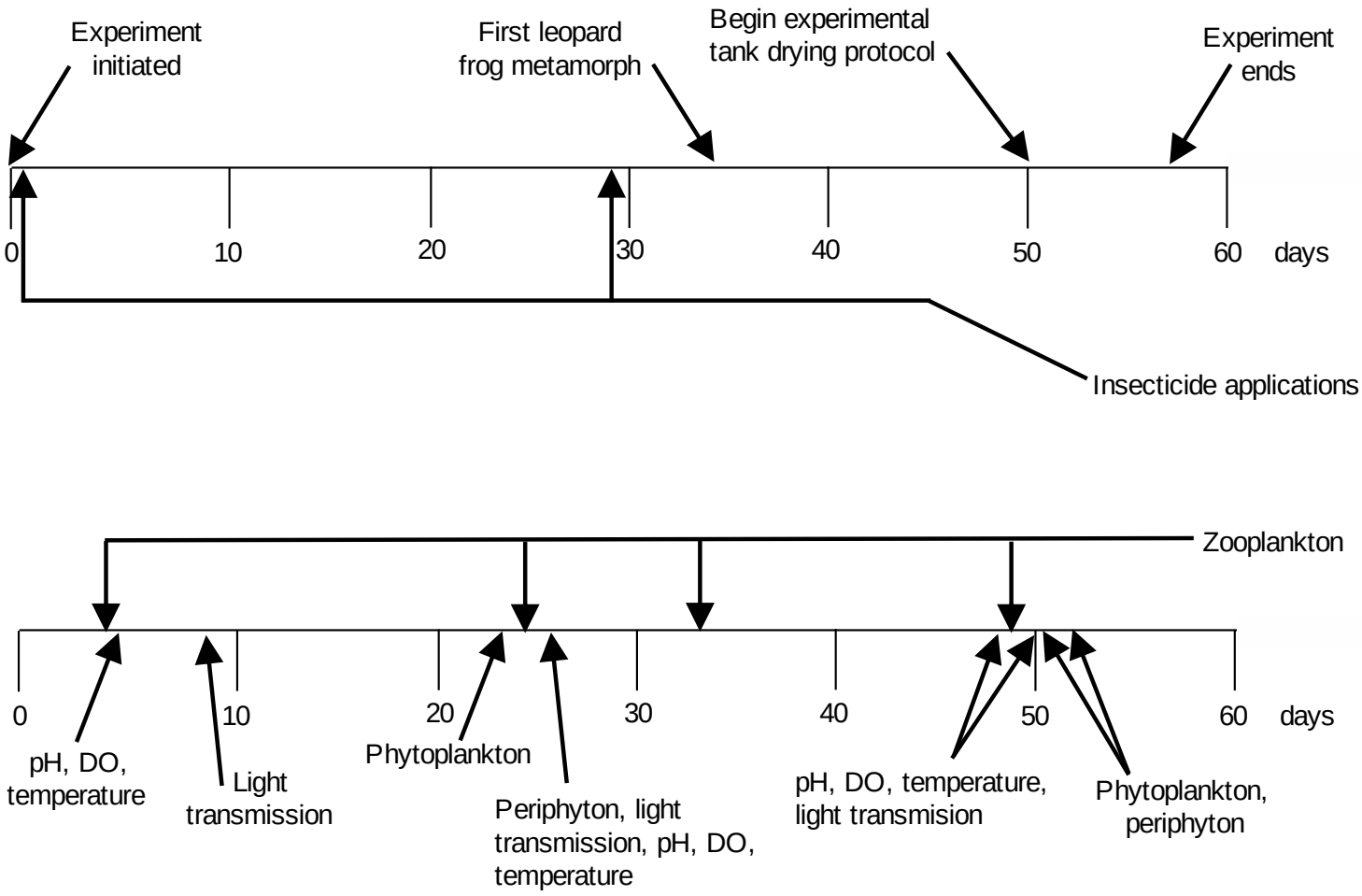

Figure 4.1. Experimental timeline to illustrate when the insecticides were added and when biotic and abiotic variables were measured. In this figure, "DO" stands for dissolved oxygen and "light transmission" indicates when light attenuation was measured to quantify light decay rates.

4.2.7 Statistical analysis

We conducted initial ANOVA analyses to determine whether we could group the two resistant *Daphnia* populations and the two sensitive *Daphnia* populations. Of the 21 response variables, including those measured over time, 19 showed no significant differences between the two resistant or between the two sensitive populations after a Bonferroni adjustment. As a result, and because the populations did not differ in the previous two studies that we had performed, we decided to pool the two resistant and two sensitive populations in all subsequent analyses.

We transformed any response variables that did not meet the assumption of homogenous variances. Data for temperature, pH and DO content were analyzed simultaneously on three separate occasions allowing us to conduct a repeated-measures MANOVA on our abiotic data. When we found significant multivariate effects, we then performed univariate repeated-measures ANOVAs (rm-ANOVA) on those variables that were measured multiple times throughout the experiment to determine how each variable was affected by the treatments. Whenever significant effects were found, particularly treatment-by-time interactions, we conducted independent ANOVAs for each variable at each time point in order to discern the factor(s) driving the significance of the multivariate tests. Finally, if we found significant results within the ANOVA test (particularly a significant effect of sensitivity), we ran mean comparisons between communities with resistant and sensitive *Daphnia* for each insecticide to see if there were population-level differences at each concentration. Light attenuation was measured only twice due to inclement weather, and therefore was not included in the overall MANOVA for abiotic variables. Light attenuation was analyzed independently using rm-ANOVA.

For the remaining biotic response variables, we first ran a MANOVA (as well as subsequent rm-ANOVAs and univariate ANOVAs) to test for the effects of pesticide type, concentration and sensitivity of the *Daphnia* population on the final measurements of periphyton, phytoplankton, and *Daphnia* abundance. The leopard frog response variables (survivorship, tadpole mass and tadpole developmental stage) were analyzed using a separate MANOVA (as well as subsequent univariate ANOVAs). For this experiment, survivorship was defined as survival to the end of the experiment as either a tadpole or a metamorph. Since so few individuals actually metamorphosed before the end of the experiment, we did not include the mass and developmental (Gosner) stage for the 5.6% of individuals that metamorphosed.

4.3 RESULTS

4.3.1 Abiotic variables

A detailed analysis of all abiotic response variables can be found in Appendix H. Because we found significant multivariate effects when we ran a MANOVA on the final sample of the abiotic variables, we ran subsequent rm-ANOVAs for each variable (Table I.2). Given that every abiotic variable exhibited treatment-by-time interactions, we then ran individual ANOVAs for each time point. Temperature, which fluctuated throughout the experiment, experienced small changes in response to the treatments but was unaffected by the sensitivity of the *Daphnia* within the community (Table I.3A; Figure J.1). For pH, the insecticides and *Daphnia* sensitivity both had effects during the experiment, but they only interacted on the final sample date, when adding the AChE-inhibiting pesticides to communities with sensitive *Daphnia* caused an increase in pH,

whereas adding AChE-inhibiting pesticides to communities with resistant *Daphnia* caused a decrease in pH (Table I.3B; Figure J.2). We observed a similar pattern of ANOVA results across the samples dates for DO (Table I.3C; Figure J.3). Light attenuation generally increased with higher concentrations of the five insecticides and this increase grew in magnitude over time (Table I.3D; Figure J.4).

4.3.2 Zooplankton

The rm-ANOVA of *Daphnia* abundance indicated that there were significant effects of insecticide treatment, *Daphnia* sensitivity, time, and several interactions (Table I.4). All sample dates exhibited effects of the insecticides and *Daphnia* sensitivity and three of the four sample dates exhibited insecticide-by-sensitivity interactions (Table I.5A). We then compared whether sensitive and resistant populations of *Daphnia* differed in abundance when exposed to each of the insecticides.

On the first sample (day 3; Figure 4.2), we found that there was a difference in control communities; there were significantly more *Daphnia* in communities with resistant *Daphnia* ($p = 0.004$). Furthermore, communities containing resistant *Daphnia* had more zooplankton than communities containing sensitive zooplankton if they were exposed to the two lowest concentrations of chlorpyrifos (C1 and C2; C3 was marginally non-significant, $p = 0.057$), the two lowest concentrations of carbaryl (C1 and C2), and the two highest concentrations of malathion (C2 and C3; all $p \leq 0.004$). *Daphnia* sensitivity had no effect on the abundance of *Daphnia* when exposed to either pyrethroid insecticide (all $p > 0.323$).

On the second sample (day 24; Figure 4.2), which preceded the second pesticide

application, we saw fewer differences as the pesticides continued to break down. Unlike the first sample, in the absence of pesticides there was no significant difference in *Daphnia* abundance between communities with resistant or sensitive *Daphnia* ($p = 0.623$). Communities containing resistant *Daphnia* populations had higher abundances when exposed to the middle concentration of carbaryl (C2; $p = 0.045$) and the highest concentrations of malathion and chlorpyrifos compared to communities containing sensitive *Daphnia* (C3; both $p \leq 0.023$). There were no effects of *Daphnia* sensitivity when communities were exposed to either pyrethroid insecticide (all $p > 0.478$).

On the final two samples (days 33 and 48; Figure 4.2), which followed the second application of the insecticides, again, in the absence of pesticides, there were no differences in *Daphnia* abundance between communities with resistant or sensitive *Daphnia* ($p = 0.504$ and 0.854 for samples 3 and 4, respectively). There were also higher *Daphnia* abundance in communities containing resistant *Daphnia* that were exposed to the three carbaryl and chlorpyrifos concentrations (C1, C2, C3) across both dates (all $p \leq 0.026$), compared to communities containing sensitive *Daphnia*. The same pattern was observed with the second highest malathion concentration on day 33 (C2; $p = 0.10$) and the highest malathion concentration on day 48 (C3; $p < 0.001$). For cypermethrin, there were no differences between the two community types at any concentration (all $p > 0.098$). Surprisingly, with permethrin there were lower *Daphnia* abundances in communities containing resistant *Daphnia* than communities containing sensitive *Daphnia* at the middle concentration on day 33 (C2; $p = 0.038$). In contrast, there were more *Daphnia* in communities containing resistant *Daphnia* at the highest concentration of permethrin on day 48 (C3; $p = 0.027$).

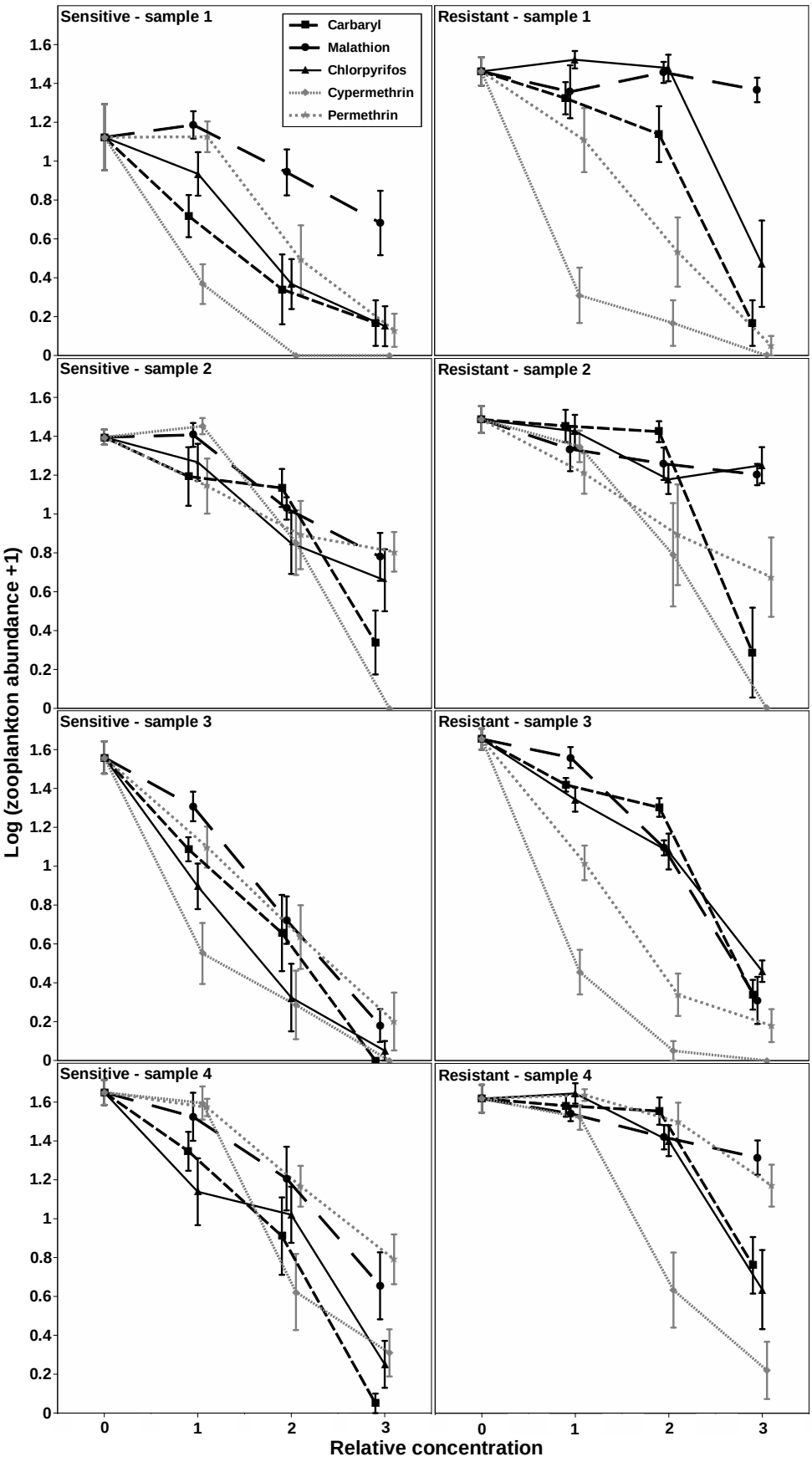
Sensitive - sample 1
Resistant - sample 1
Sensitive - sample 2
Resistant - sample 2
Sensitive - sample 3
Resistant - sample 3
Sensitive - sample 4
Resistant - sample 4
Carbaryl
Malathion
Chlorpyrifos
Cypermethrin
Permethrin
Log (zooplankton abundance +1)
Relative concentration
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
0
1
2
3

Figure 4.2. Differences in zooplankton abundance within experimental communities across the four sampling dates. For all figures, communities containing *Daphnia* populations that were previously shown to be sensitive to chlorpyrifos are on the left, populations that were resistant to chlorpyrifos are on the right.

4.3.3 Phytoplankton

The rm-ANOVA of the phytoplankton data revealed significant effects of insecticide treatment, *Daphnia* sensitivity, time, and multiple interactions with time (Table I.4). For both samples, there were significant effects of insecticide treatment, *Daphnia* sensitivity, and their interaction (Table I.5B).

On the first sample (day 22; Figure 4.3), communities with resistant *Daphnia* had less phytoplankton than communities with sensitive *Daphnia*, when exposed to the lowest two concentrations of chlorpyrifos (C1 and C2; both $p \leq 0.042$). The same pattern was observed in communities exposed to the lowest concentration of malathion, but this was marginally non-significant ($p = 0.063$). *Daphnia* sensitivity had no effect on the abundance of phytoplankton when exposed to carbaryl or either pyrethroid insecticide (all $p > 0.061$).

On the second sample (day 51; Figure 4.3), communities with resistant *Daphnia* had less phytoplankton when exposed to the middle concentration (C2) of all three AChE-inhibiting insecticides as well as the highest concentrations of carbaryl and malathion (C3), when compared to communities with sensitive *Daphnia* (all $p \leq 0.002$). Consistent with the surprising observation that permethrin exposures caused fewer *Daphnia* to be present in resistant communities, communities with resistant *Daphnia* had more phytoplankton than communities

with sensitive *Daphnia* when exposed to the highest two concentrations of permethrin (C2 and C3; both $p \leq 0.048$). Conversely, there was more phytoplankton in communities with sensitive *Daphnia* when exposed to the highest concentration of cypermethrin (C3; $p = 0.003$).

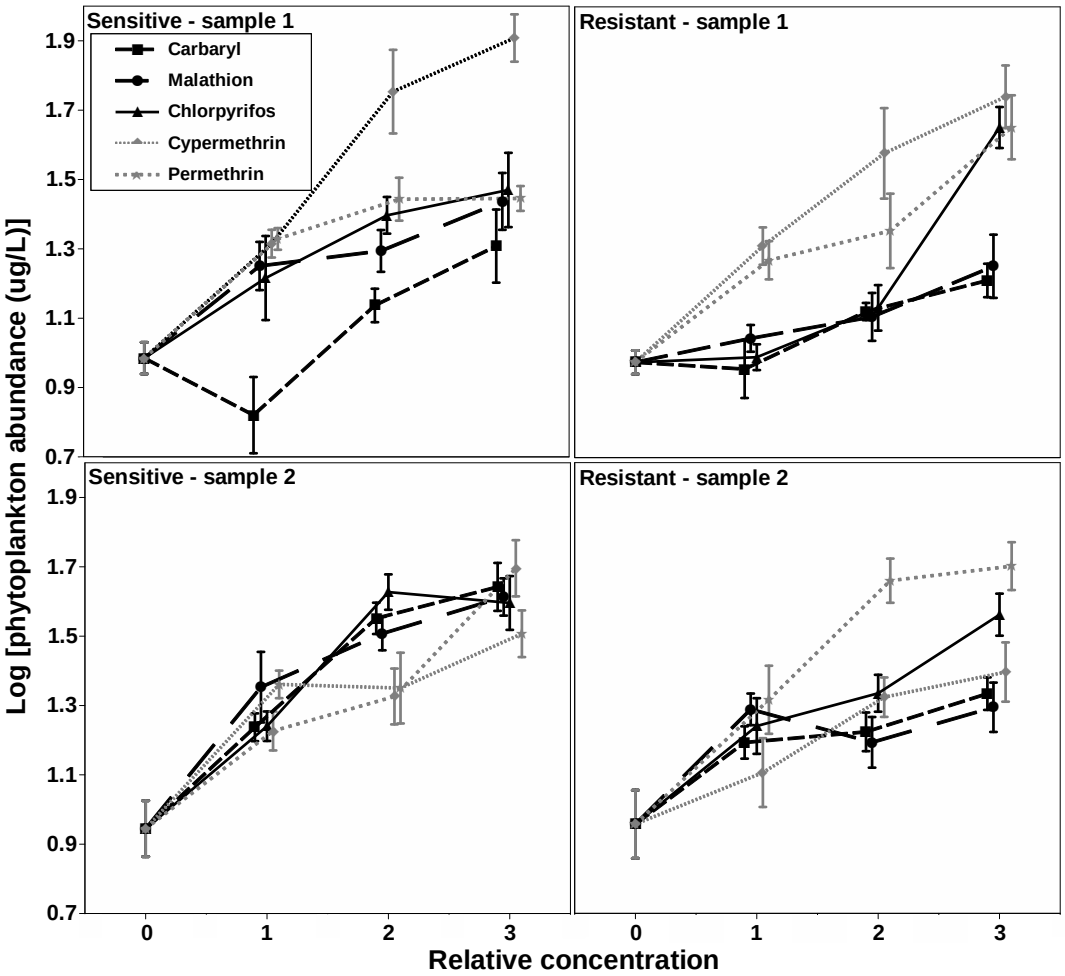

Figure 4.3. Differences in chlorophyll *a* abundance (in μg/L) within experimental communities across the two sampling dates of phytoplankton abundance.

4.3.4 Periphyton

The rm-ANOVA of the periphyton data revealed significant effects of insecticide treatment, the sensitivity of the *Daphnia* populations, and time as well as a significant insecticide treatment-by-*Daphnia* sensitivity interaction and a time-by-insecticide treatment interaction (Table I.4). On both sample dates, there were significant effects of insecticide treatment and *Daphnia* sensitivity, but there was only a significant interaction term for the second sample (day 53; Table I.5C).

On the first sample (day 26; Figure 4.4), there was more periphyton in communities with resistant *Daphnia* compared to communities with sensitive *Daphnia*, when exposed to the middle concentration of chlorpyrifos and malathion (C2; both $p \leq 0.003$) as well as the highest concentration of carbaryl (C3; $p = 0.010$). *Daphnia* sensitivity had no effect on the abundance of periphyton when exposed to either of the pyrethroid insecticides (all $p > 0.076$).

On the second sample (day 53; Figure 4.4), there was more periphyton in communities with resistant *Daphnia* than communities with sensitive *Daphnia* when exposed to the lowest two concentrations of chlorpyrifos (C1, C2), the middle concentration of malathion (C2) and the highest concentration of (C3) carbaryl (all $p \leq 0.045$). Consistent with the fact that the highest permethrin treatment caused fewer zooplankton and more phytoplankton in communities containing resistant *Daphnia*, communities with resistant *Daphnia* had less periphyton when exposed to the highest concentration of permethrin compared to communities with sensitive *Daphnia* ($p = 0.001$).

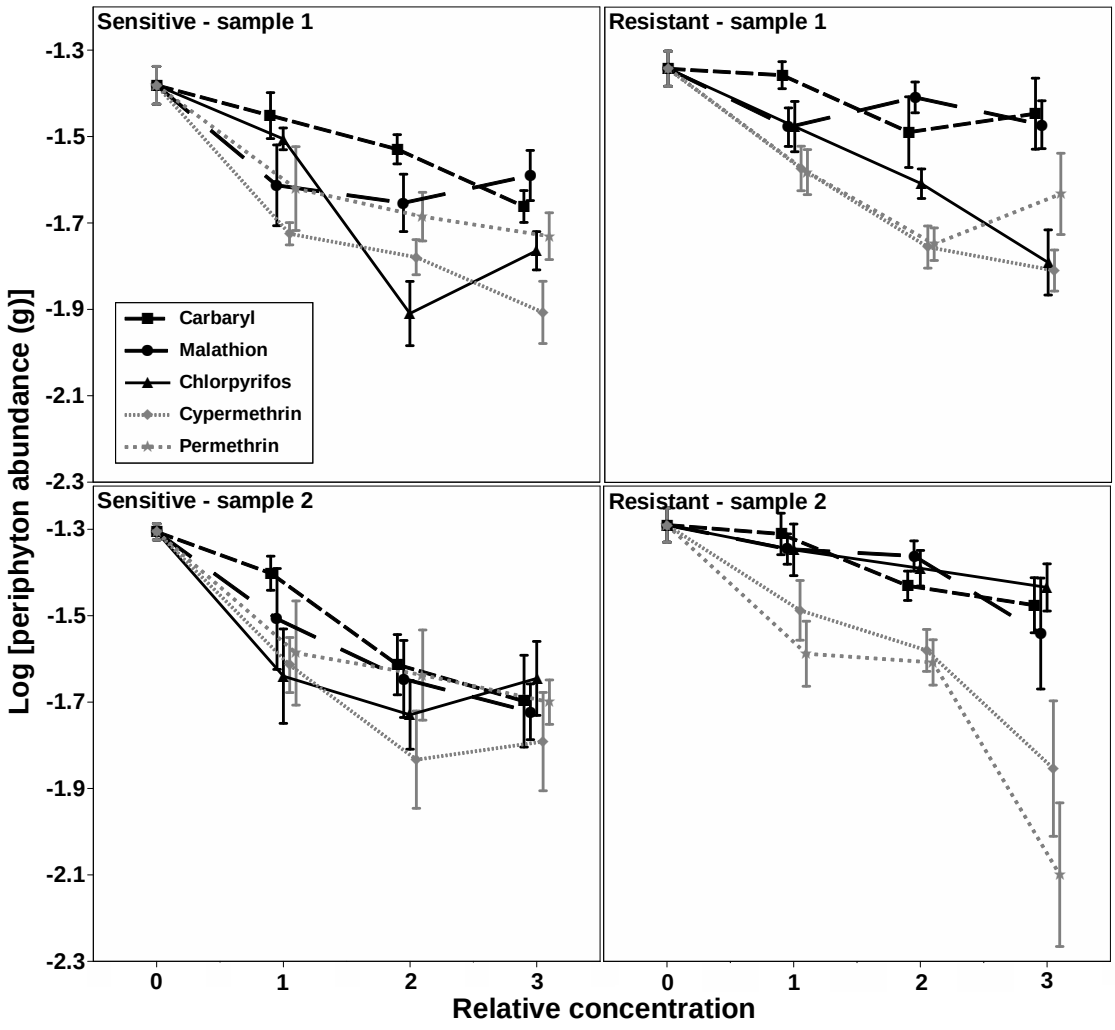

Figure 4.4. Differences in periphyton abundance (in g) within experimental communities across the two sampling dates of periphyton abundance.

4.3.5 Leopard frogs

We analyzed leopard frog survivorship, average tadpole mass and average developmental (Gosner) stage using a MANOVA. We found multivariate effects of insecticide treatment and *Daphnia* sensitivity (both $p < 0.001$), but no significant interaction (Table I.6). We then ran separate univariate analyses of the aforementioned leopard frog variables.

We began by analyzing survivorship (Figure 4.5). The ANOVA of survivorship indicated that there was an effect of insecticide treatment ($p < 0.001$), a marginally non-significant effect of *Daphnia* sensitivity ($p = 0.072$), and a non-significant interaction ($p = 0.861$, Table I.7A). Averaged across all pesticide treatments, overall survivorship of leopard frogs was slightly higher (3.7%) in communities with resistant *Daphnia*. Exposure to chlorpyrifos did not affect mortality beyond that of the control at any of the three concentrations in either community type (all $p > 0.255$). Malathion exposure did not result in lower survivorship in either community type, when compared to controls (all $p > 0.198$). Carbaryl exposure did not result in decreases in survivorship beyond those seen in controls at any concentration in either community type, but a comparison of communities with resistant versus sensitive *Daphnia* showed that there was lower survivorship of amphibians in communities with sensitive *Daphnia* exposed to the highest concentration relative to communities with resistant *Daphnia* ($p = 0.011$). Finally, in communities exposed to the two pyrethroid insecticides, in both community types there were declines in survivorship when exposed to the two highest concentrations of each of the pyrethroid insecticides compared to controls (all $p < 0.001$).

For tadpole mass, there was an effect of *Daphnia* sensitivity, insecticide treatment, and their interaction (all $p \leq 0.037$; Table I.7B; Figure 4.5). In control treatments, there was a marginal difference in average tadpole mass when comparing communities with sensitive versus resistant *Daphnia* ($p = 0.076$). Exposure to the three AChE-inhibiting insecticides led to larger leopard frog tadpoles in communities with resistant *Daphnia* when subjected to the lowest two concentrations of carbaryl (C1 and C2), the highest two concentrations of malathion (C2 and C3) and all three concentrations of chlorpyrifos (all $p \leq 0.035$). There were no differences in tadpole mass when we compared communities containing sensitive versus resistant *Daphnia* that were

exposed to either of the pyrethroids (all $p < 0.103$).

For Gosner stage, the ANOVA indicated significant main effects of insecticide treatment and *Daphnia* sensitivity as well as a significant interaction term (all $p \leq 0.002$; Table I.7C; Figure 4.5). The general trend was that leopard frogs in communities containing resistant *Daphnia* developed more quickly when compared to communities with sensitive *Daphnia*. This pattern held true for communities exposed to no pesticide ($p < 0.001$), communities exposed to all three concentrations of the three AChE-inhibiting insecticides (all $p \leq 0.016$) and communities exposed to the lowest and highest concentration of permethrin (C1 and C3; both $p \leq 0.004$). Unlike the communities exposed to permethrin, there were no differences in average Gosner stage achieved across any of the three cypermethrin treatments (all $p > 0.409$).

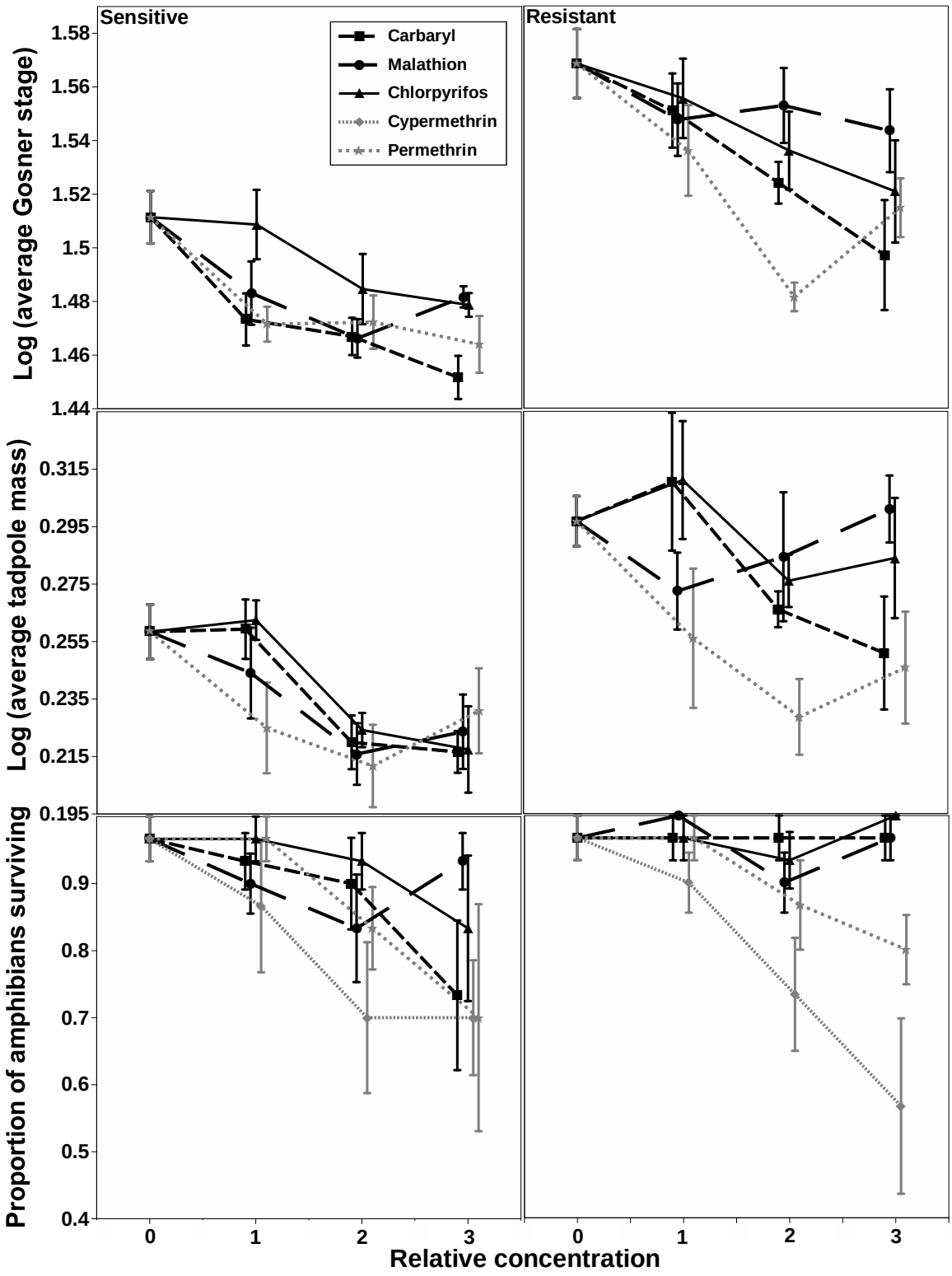

Figure 4.5. Differences in overall survival, average tadpole mass (g), and developmental stage, between communities with resistant or sensitive *Daphnia.*

4.4 DISCUSSION

In this study, we discovered that naturally-occurring populations of *Daphnia* that vary in their resistance to a commonly applied insecticide (chlorpyrifos), also show a pattern of cross resistance to other insecticides with a similar mode of action (carbaryl and malathion). Our LC50 studies in the lab found that *Daphnia* populations collected from ponds near agricultural fields were more resistant to AChE-inhibiting insecticides than populations collected far from agricultural fields. In contrast, the populations did not differ in their resistant to two pyrethroid insecticides, which have a different mode of action. These patterns translated into strikingly similar community-wide effects, when communities were exposed to moderate concentrations of the AChE-inhibiting insecticides. In contrast, communities exposed to the pyrethroid insecticide, showed markedly different effects with no evidence of resistant *Daphnia* populations being able to prevent zooplankton-mediated trophic cascades.

Previous studies have found that cladocerans, such as *Daphnia* are typically the most sensitive class of zooplankton in terms of their exposure to many, but not all, insecticides (Boone and James 2003, Mills and Semlitsch 2004, Relyea and Hoverman 2006, Relyea and Diecks 2008, Relyea 2009). We found that the concentrations used in our experiment ranged from sublethal to lethal and when communities were exposed to the two lowest concentrations of chlorpyrifos, there were more *Daphnia* in communities with resistant populations than in communities with sensitive populations. Given that our previous study (Bendis and Relyea 2014, *in review*) and the current study found consistent variation in resistance to chlorpyrifos, this suggests that the resistance to chlorpyrifos is maintained across multiple years.

The other two AChE-inhibiting insecticides (carbaryl and malathion) had similar effects on *Daphnia* abundance throughout the experiment. In communities exposed to carbaryl and

malathion, there were commonly more *Daphnia* in communities with resistant populations than communities with sensitive populations, although the particular concentrations that exhibited the population difference varied among the pesticides. The highest concentration, which was intended to be lethal for both populations regardless of their sensitivity, caused a marked decline in *Daphnia* abundance. In short, there was clear evidence that the *Daphnia* populations that were previously shown to be resistant to chlorpyrifos were also cross-resistant to malathion and carbaryl. This pattern of cross-resistance was concordant with our hypothesis that cross-resistance between insecticides should be related to their mode of action. Evidence of cross-resistance to insecticides with similar modes of action is relatively common among targeted pest species, but is not commonly described in non-target species (Tabashnik et al. 1987, ffrench-Constant et al. 1993, Daborn et al. 2002, Smirle et al. 2002, Hua et al. 2013).

When we exposed the communities to the two pyrethroid insecticides, which have a different mode of action compared to the three AChE-inhibiting insecticides, we hypothesized that we would not observe cross-resistance in *Daphnia*. For communities exposed to cypermethrin, there was no evidence of more *Daphnia* in communities that contained chlorpyrifos-resistant *Daphnia*. For permethrin, we saw an interesting pattern where *Daphnia* populations that were less resistant to chlorpyrifos were actually more resistant to permethrin. It is unlikely that this was due to insecticide exposure to both types of chemicals in the wild, as both sensitive populations were collected in protected wildlife areas with little to no surrounding agriculture. Some studies have found that cross-resistance can develop for insecticides that have markedly different modes of action (Scott 1989, Zhao et al. 1996, Berengues et al. 2003, Brausch and Smith 2009*a*/*b*, Mitchell et al. 2012), but we found no such evidence here. However, our results are concordant with our pilot LC50 data, which found that the population that was more

sensitive to chlorpyrifos had a higher LC50 value for permethrin compared to the population that was less sensitive to chlorpyrifos (Table A1). This suggests an interesting tradeoff between the evolution of resistance to AChE-inhibiting insecticides and permethrin, which is an unexpected finding that, to our knowledge, has not been found in other previous studies. Because permethrin and cypermethrin share the same mode of action, but have different effects on *Daphnia* populations, this suggests that we cannot group the pyrethroids by modes of action when extrapolating their potential effects on aquatic communities.

There was no evidence of a life history-trade-off between the resistant and sensitive populations of *Daphnia*. Although we did not explicitly track the population growth rates of the *Daphnia* within our communities, our samples of *Daphnia* abundance showed that early in the experiment, communities containing resistant *Daphnia* actually had more *Daphnia* than communities containing sensitive *Daphnia*, which is the opposite outcome expected from a life-history tradeoff as the energetic constrains of maintaining resistance within a population may lead to decreased fecundity or longer generation times. The equivalent numbers of *Daphnia* later in the experiment may reflect all communities achieving a similar carrying capacity for the *Daphnia*. These results are concordant with our previous work that found that these populations, which were sampled a year earlier, also showed no evidence of a tradeoff between insecticide resistance and *Daphnia* population abundance (Bendis and Relyea, *in review*). Many other studies have found that the resistance or tolerance of *Daphnia* species to parasites, disease, anthropogenic chemicals, and other factors are often associated with one or more life-history trade-offs that could potentially lead to reductions in population growth rates (van der Hoeven and Gerritsen 1997, Duffy and Sivars-Becker 2007, Coors and De Meester 2008, Jansen et al. 2011*a*, Auld et al. 2013).

Studies have shown that cladocerans such as *Daphnia* are the primary drivers of phytoplankton abundance in many aquatic ecosystems (Relyea and Diecks 2008). This is particularly important because low and environmentally-relevant concentrations of insecticides can cause complete extirpation of zooplankton assemblages causing a phytoplankton bloom and subsequent trophic cascade (Sierzen et al 1998, Boone et al. 2004, Mills and Semlitsch 2004, Relyea and Diecks 2008). All five insecticides caused increases in phytoplankton and the magnitude of the increase grew with increasing insecticide concentrations across nearly every treatment. Associated with these increases in phytoplankton content were increases in light decay rates, DO, and pH; the latter two variables reflect the increase in photosynthesis that occurs with an increase in phytoplankton.

The differential survivorship among *Daphnia* populations drove changes throughout the community. For example, across the two sampling dates, there was a pattern of lower phytoplankton abundance in communities with resistant *Daphnia* populations that were exposed to low to moderate concentrations of the three AChE-inhibiting insecticides. This indicated that *Daphnia* populations with resistance and cross-resistance to these insecticides could mitigate the trophic cascade from the zooplankton to the phytoplankton. In contrast, because the resistant *Daphnia* populations exhibited no cross-resistance to cypermethrin and permethrin, the resistant *Daphnia* populations were not able to prevent the trophic cascade from the zooplankton to the phytoplankton Indeed, permethrin caused the opposite effect; it caused a higher abundance of the sensitive *Daphnia* and a subsequent lower abundance of phytoplankton. Previous studies have shown the importance of zooplankton in maintaining the stability of aquatic communities through their top-down control on phytoplankton abundance (Mills and Semlitsch 2004, Boone et al. 2004, Hanson et al. 2007, Relyea and Diecks 2008, Bendis and Relyea, *in review*). This

study, however, is the first to show that naturally occurring cross-resistance to these agrochemicals may protect pond communities that are chronically impacted by such stressors.

The trophic cascade caused by insecticides that reduce the zooplankton and increase phytoplankton often causes sufficient light decay as to reduce the abundance of periphyton at the bottom of a body of water (Mills and Semlitsch 2004, Rohr et al. 2006, Hanson et al. 2007, Relyea and Diecks 2008). In our study, there was a clear pattern of increased insecticide concentrations causing a decrease in the abundance of periphyton. However, the effect on the periphyton differed between resistant and sensitive *Daphnia* populations. Periphyton was often more abundant in communities with resistant *Daphnia* compared to communities with sensitive *Daphnia* when exposed to moderate to high concentrations of the three AChE-inhibiting insecticides. This is clear evidence of the far-reaching effects of resistance and cross-resistance of *Daphnia* to the AChE-inhibiting insecticides.

Daphnia sensitivity had no effect on periphyton abundance when exposed to either of the two pyrethroid insecticides for the first sample. During the second sample, however, there was less periphyton in communities with resistant *Daphnia* exposed to the highest concentration of permethrin. This was consistent with there being both fewer *Daphnia* and significantly more phytoplankton within this treatment during this time period. Furthermore, it highlights that the far-reaching effects of the tradeoff between resistance to AChE-inhibiting insecticides and the pyrethroid insecticide permethrin; permethrin not only had direct effects on zooplankton abundance but led to dramatic community-wide effects across trophic levels.

The trophic cascade that was initiated by the direct lethal effects of the insecticides on the zooplankton also affected the leopard frogs. In terms of survivorship, we found a modest increase (3.7%) in survivorship of leopard frogs in communities with resistant *Daphnia* when

survivorship was averaged across all treatments. Furthermore, we were able to rule out pesticide exposure being linked to leopard frog mortality for 3 of the 5 insecticides (chlorpyrifos, carbaryl, and malathion) because overall survivorship across all treatments was not significantly different from survivorship within control communities in communities with resistant *Daphnia*. For the two pyrethroid insecticides, we saw moderate decreases in survivorship, but we attempted to utilize concentrations that had been shown to be sublethal to other anuran species (Paulov 1990, Saha and Kaviraj 2008, Agostini et al. 2009, Biga 2013). Thus, the decline in amphibian survivorship was likely due to declining food availability. Furthermore, while we saw small improvements in amphibian survivorship in communities with resistant *Daphnia* compared to communities with a sensitive population, there were more substantial effects of *Daphnia* cross-resistance on tadpole mass and development.

There was clear evidence that the effects of cross-resistance within *Daphnia* resulted in faster development and higher growth rates of amphibians within communities exposed to several concentrations of all of the AChE-inhibiting insecticides. Given the lack of evidence for cross-resistance of *Daphnia* to the pyrethroids, we expected to see no evidence of faster development in communities exposed to pyrethroids. This was true for cypermethrin, but we found that amphibians in communities exposed to C1 and C3 of permethrin actually developed at a faster rate for reasons that remain unclear. Aside from this one anomaly, our results coincided clearly with our hypothesis that *Daphnia* resistance to chlorpyrifos would lead to cross-resistance to chemicals with a similar mode of action, but not necessarily to insecticides with a different mode of action. *Daphnia* that were cross-resistant to the three AChE-inhibiting insecticides survived to a greater degree when compared to sensitive *Daphnia*. This led to more phytoplankton being removed from the water column and an increase in resources (i.e. light) for

periphyton, and as periphyton abundance increased this led to an increase in average tadpole mass and faster overall developmental rates. We anticipated this finding from previous studies which have shown that concentrations of insecticides that are lethal to zooplankton can cause marked trophic cascades that can eventually affect amphibian growth a development through a series of cascading events (Fleeger et al. 2003, Boone et al. 2004, Mills and Semlitsch 2004, Relyea and Diecks 2008, Hua and Relyea 2012). These studies, however, typically only manipulate one insecticide and then use inference to hypothesize how insecticides with similar chemical structures or modes of action might affect similar aquatic ecosystems. Our study explicitly shows that cross-resistance of *Daphnia* may be common in nature and that insecticides of the same mode of action do indeed cause similar community responses that can affect amphibians at extremely low and environmentally-relevant concentrations. Furthermore, our study is the first to demonstrate that genetic differences between populations that promote insecticide cross-resistance in *Daphnia* can result in the buffering of aquatic communities via a chain of events that can alter the growth and development of grazers such as amphibians.

4.4.1 Conclusions

We have shown that low and environmentally-relevant concentrations of five commonly applied insecticides had direct lethal effects on zooplankton, which lead to a series of indirect effects that cascaded throughout the entire aquatic community. More importantly, we incorporated our pre-existing knowledge of natural variation in *Daphnia* population-level resistance to the insecticide chlorpyrifos to explore the possibility of the evolution of cross-resistance in *Daphnia*. Our results indicate that populations that were resistant to the AChE-inhibiting insecticide

chlorpyrifos were also resistant to carbaryl and malathion, which share the same mode of action. We detected no signs of cross-resistance to cypermethrin, but found an interesting possible tradeoff between resistance to AChE-inhibiting insecticides and sensitivity to permethrin. To further our understanding of the effects of insecticides on aquatic communities, future studies should attempt to utilize more diverse and realistic zooplankton assemblages to determine whether or not the effects of these insecticide induced trophic cascades are driven by the loss of *Daphnia* (as posited by both our previous and current findings). If it is possible that other species of copepods, rotifers or even other genera of cladocerans may have the potential to fill the same ecological niche as *Daphnia*. If these species are more resistant than *Daphnia*, and can potentially consume phytoplankton at an equal or greater rate, then the future for aquatic ecosystems that are at high risk from nearby agricultural chemicals may be brighter than initially anticipated. Only by fully understanding variation within and among both abiotic and biotic factors (particularly those that can have dramatic effects on aquatic food web stability) will we be able to protect these threatened ecosystems from future anthropogenically-induced stressors.

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5.0 KEYSTONE CONSUMERS: A TEST OF FUNCTIONAL REDUNDANCY WITHIN NATURAL ZOOPLANKTON ASSEMBLAGES THAT VARY IN THEIR RESISTANCE TO AGROCHEMICALS

5.1 INTRODUCTION

Attempting to predict the effects of environmental change on community and ecosystem dynamics is a major issue in the field of ecology. A common focus of ecologists over the past few decades has been on biotic perturbations and their cascading effects throughout the food web (Shurin et al. 2002, Finke and Denno 2004, Schmitz et al. 2004, Altieri et al. 2012). However, abiotic stressors, even if they are only periodic, can also have marked impacts on aquatic and terrestrial communities that may rival, if not surpass, the impacts of biotic stressors, particularly if these stressors are anthropogenic (Rohr et al. 2006, Relyea and Hoverman 2006, Kratina et al. 2012). Furthermore, biotic and abiotic stressors can often interact synergistically (Blaustein and Kiesecker. 2002, Crain et al. 2008, Montoya and Raffaelli 2010).

One such abiotic stressor that often interacts with biotic stressors in aquatic systems is the presence of pesticides (Relyea and Mills 2001, Boone and James 2003, Blaustein et al. 2003). Very low concentrations of insecticides can have indirect lethal effects on other organisms (Mills and Semlitsch 2004, Relyea and Diecks 2008, Bendis and Relyea, *in review*). This can happen when pesticides are directly lethal to a sensitive species in the community, such as the high

sensitivity of zooplankton when exposed to most insecticides (Brock et al. 1996, Hanazato 2001, Van Wijngaarden et al. 2005). When zooplankton are reduced or eliminated, it causes a cascading chain of events that starts with a bloom in phytoplankton (Fleeger et al. 2003, Mills and Semlitsch 2004). The bloom in phytoplankton shades out the periphytic algae that lives below the phytoplankton and this causes the periphyton to decline over time. Organisms that consume periphyton (e.g., tadpoles) grow and develop more slowly to the point that those living in vernal pools can fail to metamorphose before their pond dries (Boone et al. 2004, Relyea and Diecks 2008). As a result, pesticide concentrations that are sublethal to grazers such as tadpoles can become lethal through a chain of indirect effects.

Previous studies that have examined pesticide-induced trophic cascades have implicated the loss of zooplankton as the primary cause of the phytoplankton bloom and the resulting chain of effects through the food web (Mills and Semlitsch 2004, Boone et al. 2004, Relyea and Hoverman 2006, Relyea and Diecks 2008). Of the three major groups of freshwater "macro-zooplankton" (cladocerans, copepods and rotifers), cladocerans are generally the largest in body size and they typically consume the most phytoplankton per capita via non-selective filter feeding (Hanazato 1998). Copepods, which are typically smaller than most cladocerans, are mainly omnivorous and will consume phytoplankton, protozoa, detritus, bacteria, rotifers, other copepods and even some smaller-bodied cladocerans (Dagg et al. 1989). Rotifers are the smallest in body size and they feed primarily on phytoplankton and bacteria suspended in the water column, but they can also feed on other rotifers and even juvenile cladocerans (Stemberger and Gilbert 1985). Although these groups of zooplankton differ greatly in their overall body sizes, foraging efficiencies, ability to recycle nutrients and dietary intake, all three groups compete for phytoplankton and, consequently, have an impact on phytoplankton abundance and

diversity (Tilman 1977, DeMott and Kerfoot 1982, Vanni 1986, Hanazato 1998).

The current paradigm is that the loss of cladoceran zooplankton, such as those in the genus *Daphnia*, is the primary cause of these pesticide-induced trophic cascades (Carpenter et al. 1985, Tessier and Woodruff 2002, Korosi et al. 2012). Cladocerans have rapid generation times (~5-7 d for many species) that allow fast population growth rates (Lynch 1989). In contrast, most copepods have longer generation times (Miralto et al. 1999). In rotifers, generation times can range widely (Welch and Meselson 2000). Taking into account the differences in body sizes, resource intake, and generation times, it is not surprising that most studies show that the loss of cladocerans can have a disproportionately large effect on phytoplankton abundance. This suggests that, within these communities, copepods and rotifers have a negligible effect on phytoplankton abundance when they are in competition with cladocerans. However, this hypothesis has yet to be explicitly tested in a community context when subjected to anthropogenic stressors.

An on-going pursuit in ecology is to functional redundancy in ecosystems, such that a missing species can be functionally "replaced" by a similar species that fill a similar ecological role (Naaem 1998). In such situations, the loss of one species has little to no effect on community stability in response to perturbations (Tilman et al. 1998). Evidence of functional redundancy between the three major freshwater zooplankton groups has been noted in previous studies looking at the effects of acidification (Fischer et al. 2001), climate variation (Johnson et al. 2011), eutrophication (Bowszys et al. 2014), and exposure to the fungicide carbendazim (Slijkerman et al. 2004). Large-bodied cladocerans, like *Daphnia* are competitively superior to most other zooplankton and may often outcompete other species, driving the other species to low densities (DeMott and Kerfoot 1982, Vanni 1986, Korosi et al. 2012). The loss of *Daphnia* from

insecticide exposure should therefore release other species from competitive pressure, allowing populations of these other species to increase in abundance. Such a response would then reduce the abundance of phytoplankton to levels that could be similar to when the zooplankton assemblage was dominated by *Daphnia*, which would be evidence of functional redundancy.

We performed a mesocosm study in which we manipulated the composition of zooplankton assemblages, the source of the zooplankton (i.e. ponds that varied in their proximity of agriculture), and a range of environmentally relevant concentrations of the widely-utilized insecticide chlorpyrifos. We tested four hypotheses. First, we hypothesized that the cladocerans that came from ponds far from agriculture would be more susceptible to chlorpyrifos than those collected from a pond near agriculture, as we have seen in previous studies (Bendis and Relyea 2014, *in review*, *in prep.*). Second, we hypothesized that other groups of zooplankton (i.e. rotifers and copepods) that came from ponds far from agriculture would also be more susceptible to chlorpyrifos than those collected from a pond near agriculture. Third, we hypothesized that communities with resistant cladocerans would be buffered from the effects of the chlorpyrifos at low to moderate concentrations, whereas communities with sensitive cladocerans would not be buffered. Fourth, we hypothesized that communities with only a background assemblage should not exhibit functional redundancy in regard to preventing algal blooms and the resulting trophic cascade compared to full assemblages that include cladocerans.

5.2 METHODS

5.2.1 Experimental set up

We conducted the experiment at the University of Pittsburgh's Donald S. Wood Field Laboratory at the Pymatuning Laboratory of Ecology in Linesville, PA. Our design was a full-factorial experiment of identical aquatic communities that crossed six zooplankton treatments with five insecticide treatments. The six zooplankton treatments included partial or complete zooplankton assemblages from a pond that was either close to agriculture (i.e. surrounded by > 30% agricultural land within a 300-m radius) and contained a population of *D. pulex* that was relatively resistant to a commonly applied insecticide (i.e. chlorpyrifos) or a pond that was far from agriculture (i.e. surrounded by <5% agricultural land within a 300-m radius) and contained a population of *D. pulex* that was relatively sensitive to chlorpyrifos (Bendis and Relyea 2014). Using the zooplankton collected from a given pond, we added the following zooplankton assemblages to the mesocosms: only cladocerans, the background assemblage comprised of all zooplankton except cladocerans (i.e. copepods and rotifers), or a full assemblage of zooplankton (cladocerans, copepods and rotifers). For simplicity, we will hereafter refer to this as the "assemblage" treatment.

The five insecticide treatments represented five concentrations of the insecticide chlorpyrifos (0, 0.25, 0.50, 1.0 or 2.5 μg/L), which is commonly used in northwestern Pennsylvania on corn and soybeans (Thelin and Stone 2013). Chlorpyrifos is a widely applied insecticide in U.S. agriculture; approximately 3.6-5.0 x 10^6 kg of active ingredient are applied annually (Grube et al. 2011). In natural lakes and streams, chlorpyrifos is typically found at relatively low concentrations from around 0.01 to 0.65 μg/L (Christensen et al. 2009).

Volatilization of chlorpyrifos, which is the most likely cause of chlorpyrifos breakdown in water, occurs fairly rapidly with estimated half-lives of 3-20 days (Christensen et al. 2009).

We selected the chlorpyrifos concentrations based on our review of published LC50 data for various zooplankton found in northeastern PA, our previous research on LC50 values for chlorpyrifos on these and other populations of *D. pulex* (Bendis and Relyea 2014), and pilot LC50 experiments that we performed prior to the current experiment. These LC50 studies were used to confirm that the wild populations of cladocerans (i.e. *D. pulex*) that were previously reported to be either sensitive or resistant to chlorpyrifos retained their relative susceptibilities to chlorpyrifos when collected in 2014.

The 30 treatment combinations were replicated five times for a total of 150 experimental units. Because the insecticide was dissolved in ethanol (EtOH) prior to being added to the mesocosms, we used two additional replicates to serve as vehicle controls to test for any effects of the highest EtOH concentration. As a result, there was a total of 152 experimental units. There were no differences in any of the variables at any time when comparing communities exposed to EtOH orcontrol conditions, thereby indicating that EtOH had no effect on any of the variables. Therefore, the EtOH treatment was removed from our statistical analysis. . Our experimental units were 75-L garbage cans (58.4 cm x 49.5 cm - Rubbermaid BRUTE^{TM}) that were filled with approximately 65-L of well water on 14 May 2014. Each mesocosm was covered by a 60% shade-cloth lid to prevent organisms from entering or exiting. On 15 May we added 1.5 g of rabbit chow and 20 g of leaf litter (*Quercus* spp.) to each mesocosm to provide an initial source of nutrients. On 16 May we added four unglazed, ceramic tiles (7.5 cm x 15 cm) along the north side of each mesocosm to serve as a standardized measure of periphyton abundance.

Also on this day, we took pond water samples from five local ponds (Love, Mallard, Minnow, Hopscotch and Trailer Park). Two of these ponds (Love and Minnow) were the same ponds from which we collected our zooplankton assemblages for the experiment. We ran each water sample through a series of sieves (1 mm, 25µ um, 64 µm) five times to remove nearly all zooplankton and then treated the pond water with carbonated water to kill any smaller zooplankton, such as rotifers or copepod nauplii that may have passed through the sieves. Once the water from each pond was processed, we combined the five pond water samples and added equal aliquots (~180 mL) to each experimental unit to provide a natural source of bacteria, periphyton and phytoplankton.

5.2.2 Culturing and adding zooplankton

On May 24 we collected and isolated a complete assemblage of zooplankton from one pond located near agriculture (Love Pond, which contains resistant *D. pulex*) and another pond located far from agriculture (Minnow Pond, which contains sensitive *D. pulex*; Bendis and Relyea 2014) using a zooplankton tow with 64-µm mesh. We placed assemblages from each pond into 15, 1.8-L plastic containers. Each container was filled with carbon-filtered, UV-irradiated well water and the zooplankton assemblages were fed lab cultured *Scenedesmus* spp. algae *ad libitum*. As older female cladocerans released their offspring, they were removed from the experimental populations. We did not replicate sensitive or resistant ponds because our past experiments have consistently demonstrated that multiple sensitive populations respond quite similarly to the insecticide chlorpyrifos and other AChE-inhibiting insecticides; the same is true when we have tested multiple resistant populations (Bendis and Relyea 2014, *in review*, *in prep.*).

After approximately 3 weeks in the lab, most of the cladoceran zooplankton populations had produced at least two generations of offspring. At this time we separated cladoceran zooplankton from the background assemblage of zooplankton. We used a 180-um sieve to separate most of the cladoceran zooplankton from the rotifers and copepods. A 64-µm sieve was subsequently used to ensure that only copepod nauplii, copepodites, and rotifers could pass through (i.e. no cladocerans) to serve as our background communities. Samples were inspected at random and we verified that no cladocerans were found in the background assemblage.

All assemblage additions occurred 3 weeks prior to the experimental start date to allow the populations to establish. For the cladoceran-only treatment, we added approximately 400 cladocerans from their respective populations to each randomly assigned mesocosm. For the background-assemblage treatment, we added a standardized amount of water (~350 mL) containing a concentrated amount of rotifers, copepod nauplii, and adult copepods (approximately 800-900 of each). We intentionally added more members of the background assemblage to account for body mass differences between the larger cladocerans and other smaller species of zooplankton. For the full-assemblage treatment, communities received both of the aforementioned zooplankton treatments in an additive design (~400 cladocerans + the mixture of 800-900 background zooplankton). Before adding zooplankton to the mesocosms, we first verified that there were no cladocerans in background-assemblage treatments and no copepods or rotifers in cladoceran-only treatments. However, over time it was apparent that a few individuals of the excluded groups colonized the tanks and then grew to become more abundant. Therefore, when analyzing data for zooplankton abundance we included counts for all three zooplankton groups for every treatment.

5.2.3 Culturing and adding amphibians

Green frog tadpoles (*Lithobates clamitans)* were raised from egg masses that we collected from a single pond in northwestern PA (Geneva Pond #5). We collected 10 partial egg masses on 25 May and reared the hatched tadpoles in 200-L pools containing well water. Once hatched, the tadpoles were fed rabbit chow *ad libitum*. On 10 June, after the algal and bacterial assemblages had developed in the mesocosms for 24 days, we added 10 green frog tadpoles to each mesocosm. We selected the tadpoles for our experiment by mixing individuals from all 10 egg masses and then sorted for individuals of a similar size (initial mass ± SE: 9.5 mg ± 1.4 mg). Survival of the green frog tadpoles after a 24-hour handling test was 100%.

5.2.4 Insecticide additions

On 11 June (defined as day 1; for a timeline, see Figure 5.1), we exposed the mesocosm communities to the chlorpyrifos treatments. We began by creating a stock solution of technical grade chlorpyrifos by dissolving it in ethanol. We dissolved 0.05 g of technical-grade chlorpyrifos in 20 mL of EtOH. We then added 6.3, 12.6, 25.2 or 63 µL of the stock solution to each mesocosm to achieve the respective nominal concentrations (0.25, 0.50, 1.0 and 2.5 µg/L). For control tanks with 0 µg/L chlorpyrifos, we added 63 µL of carbon-filtered, UV-irradiated well water. For the two mesocosms assigned the ethanol treatment, we added 63 µL of EtOH to verify that the largest amount of EtOH added to experimental communities did not affect the community. After the chlorpyrifos was applied to a given mesocosm, the water was stirred to equalize disturbance and to ensure that the insecticide was properly mixed throughout the water

column. Communities exposed to the control and EtOH treatments were similarly stirred to equalize disturbance among all tanks. Chlorpyrifos treatments were applied three times during the course of the experiment (about every 2.5 weeks, Figure 5.1) to simulate natural patterns of exposure and to maintain the concentration of the insecticide within the treatments as chlorpyrifos breaks down fairly rapidly in water (Brock et al. 1996).

To verify the concentrations of chlorpyrifos in our mesocosms, we collected an aliquot of water from each tank within a particular concentration within 1 hour of applying the stock solution. We pooled the samples by concentration in pre-cleaned, 500-mL amber jars containing 2 mL of methylene chloride (CH_2Cl_2) to stabilize the insecticide. We sent these samples to an independent laboratory for chemical analysis using high-performance liquid chromatography (Center for Environmental Services and Engineering, University of Connecticut). Because the laboratory was unable to process our first application (taken on day 1) due to scheduling constraints, we tested the second and third applications. For the second application (day 20), the actual concentrations for the 0.25, 0.50, 1.0 and 2.5 μg/L nominal concentrations of chlorpyrifos were 0.29, 0.52, 0.94 and 2.28 μg/L, respectively. For the third application (day 36), the actual concentrations were 0.35, 0.51, 1.15 and 2.44 μg/L. These results verified that the actual concentrations were within 90% of the nominal values (on average) and that chlorpyrifos was completely breaking down between applications. In both samples tested, our controls had no detectable traces of chlorpyrifos (detection limit = 0.07 μg/L).

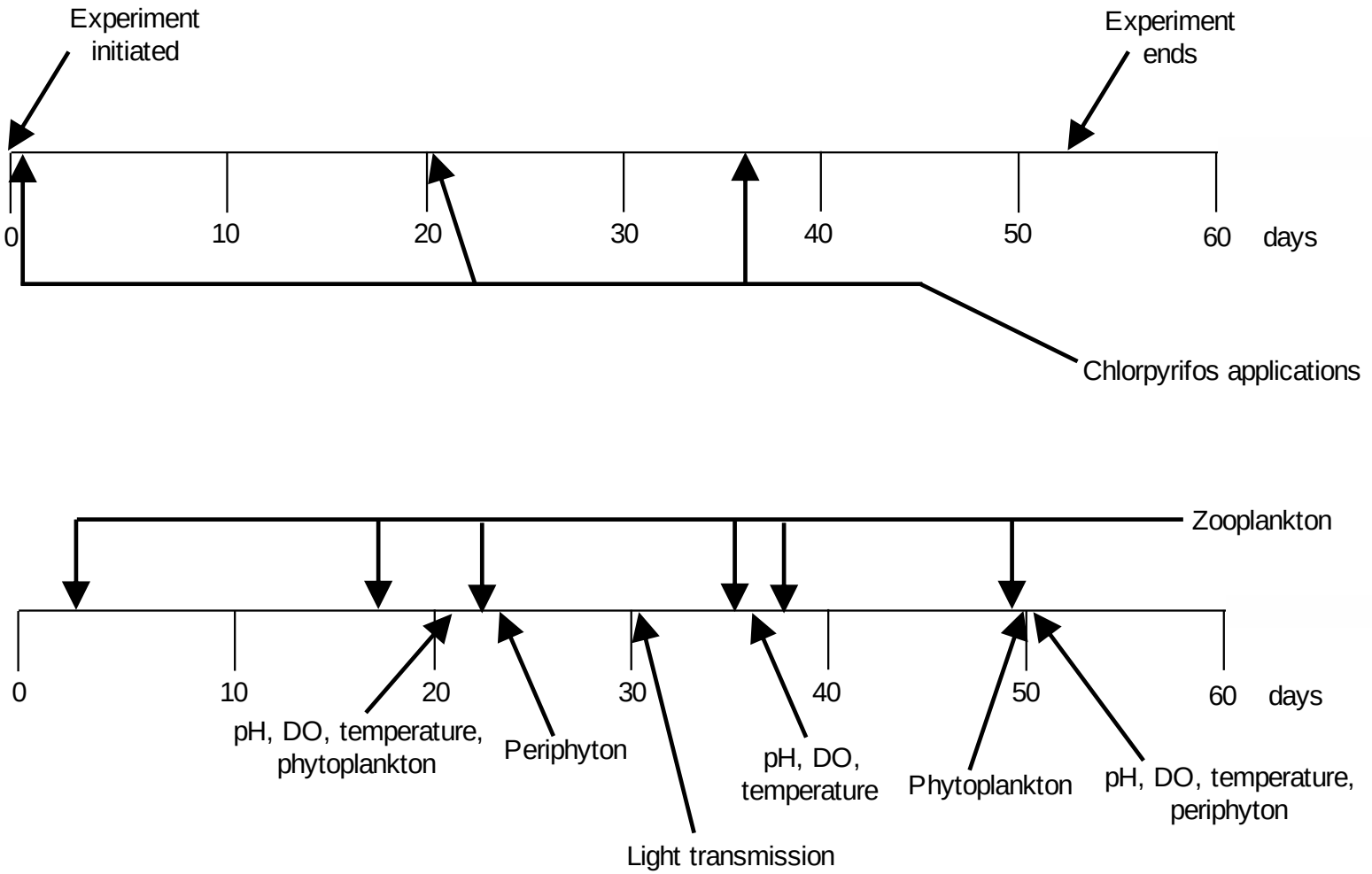

Figure 5.1. Experimental timeline to illustrate when chlorpyrifos treatments were added and when biotic and abiotic variables were measured. In this figure, "DO" stands for dissolved oxygen and "light transmission" indicates when light was measured to quantify light decay rate.

5.2.5 Abiotic response variables

During the course of the experiment, we measured several abiotic response variables to help us understand the effects of chlorpyrifos on the communities. On days 19, 37 and 51, we measured pH, temperature, and dissolved oxygen (DO) content (Figure 5.1). Temperature, pH and DO content readings were taken simultaneously with a calibrated digital aquatic multi-meter (YSI, Yellow Springs, OH, USA). All abiotic readings for all treatments were recorded within a span of 80 minutes so that we did not confound the time of day with the concentrations that were being tested. On days 30 and 31, we measured light transmission through the water column. Both days were cloudless, which is ideal for taking light measurements. Light radiation was measured from the middle of each mesocosm at depths of 10 and 30 cm and we then calculated the decay rate of light with increased water depth (k) using the equation:

$$k = [\ln(L_{10}/L_{30})]/d$$

where L_{10} is the intensity of sunlight from a depth of 10 cm, L_{30} is the intensity of sunlight from a depth of 30 cm, and d is the difference in depth between the two measurements of intensity (Relyea and Diecks 2008). Light attenuation was measured with an underwater light meter (LI-COR. Lincoln, Nebraska, USA).

5.2.6 Biotic response variables

Several biotic response variables were also measured during the experiment. We sampled zooplankton abundance at six times throughout the experiment (Figure 5.1.) Zooplankton were sampled by submerging a 0.2-L plastic sampling tube in the middle of the water column at six

different locations within each mesocosm (north, south and middle quadrants of each mesocosm both towards the surface and near the benthos as different species of zooplankton tend to be found in different regions of the water column). All six samples from a given mesocosm were pooled, the sample was filtered through a 64-µm Nitex cloth screen, and the captured zooplankton were placed into a Whirl-Pak® bag containing 30% EtOH to preserve the samples. This mesh size was selected because it is small enough to capture young and adult cladocerans, copepods, larger copepod nauplii and many species of rotifers. For zooplankton enumeration, the contents of the bags were poured onto a Petri dish with a preset grid. All zooplankton were counted and identified as cladocerans, copepods, or rotifers. Although there is variation in sensitivity to chlorpyrifos among species within each of these groups, there is a clear pattern that indicates that the larger-bodied cladocerans are typically more sensitive than copepods and rotifers (Brock et al. 1996, Lopez-Mancisidor et al. 2008, Daam et al. 2008). Moreover, grouping the zooplankton into these groups takes into account the variation in dietary habits between groups (i.e. cladocerans that specialize on consuming phytoplankton versus copepods which are generalists and consume a wide array of food items). An overall species list was maintained by spot checking samples at random.

Although there were some species that differed between ponds, the similarity in zooplankton diversity between ponds was striking. In both ponds, the cladoceran assemblages were dominated by *Daphnia pulex* (94% and 79% in near agriculture and far from agriculture ponds, respectively). We identified five other species of cladocerans (*Daphnia ambigua*, *Scapholebris mucronata*, *Chydorus sphaericus*, and *Simocephalus vetulus*), with *D. ambigua* found only in the pond far from agriculture and *S. vetulus* only found in the pond near agriculture. We also identified eight species of copepods (*Leptodiaptomus minutus*, *L. siciloides*,

Eurytemora affinis, *Senecella calanoides*, *Skistodiaptomus oregonensis*, *Acanthocyclops robustus*, and *A. vernalis*). *S. calanoides* and *E. affinis* were found only within the pond that was located far from agriculture. Aside from the two predatory cyclopoid copepods that were found in both ponds (*A. robustus* and *A. vernalis*), most copepods were calanoid copepods that feed primarily on phytoplankton, but can be omnivorous (Wong and Chow-Frasier 1985). Finally, we identified five species of rotifers across the two ponds (*Keratella* spp., *Platyias patulus*, *Notholca* spp. *Lecane* spp., and *Brachionus calyciflorus*).

Phytoplankton was quantified three times during the experiment (Figure 5.1). To measure the phytoplankton, we sampled 0.5 L of water from the center of each mesocosm and in the middle of the water column. Each water sample was vacuum-filtered through GF/C Whatman glass microfiber filters (Whatman Industries Inc., Florham Park, New Jersey, USA). After filtering, each filter was individually wrapped in aluminum foil and stored in a freezer at -18 °C. We used a fluorometer (Turner Designs TD-700, Sunnyvale, CA, USA) to measure the concentration of chlorophyll *a*, which we used as our proxy for phytoplankton abundance using the protocol developed by Arar and Collins (1997).

We quantified periphyton abundance three times during the course of the experiment (Figure 5.1) by removing one of the clay tiles from each mesocosm. Once a tile was removed, it was scrubbed with a toothbrush to remove all of the periphyton on the face of the tile and subsequently rinsed with carbon-filtered, UV-irradiated well water. The mixture containing water and periphyton was then vacuum-filtered onto a Whatman GF/C filter that had been previously dried for 24 hours at 70°C and weighed. After the periphyton sample was vacuum filtered, the filters were dried at 70°C for an additional 24 hours and re-weighed. The amount of periphyton biomass was measured as the mass of the filter paper containing the dried periphyton

minus the original dry mass of the unused filter.

On day 52 we terminated the experiment by draining the water from the mesocosms. We then sorted through the leaf litter to recover all surviving green frog tadpoles. For each mesocosm, all tadpoles were massed and were euthanized using a 2% solution MS-222 (tricane mesylate). We then quantified the survival and mean mass of the tadpoles from each mesocosm.

5.2.7 Statistical analysis

All response variables that did not meet the assumption of homogenous variances were transformed. We initially ran a MANOVA on all of the abiotic and biotic response variables collected during the final takedown. Whenever significant effects were found, we ran individual repeated measures ANOVAs (rm-ANOVAs) or univariate ANOVAs depending on whether a variable was measured multiple times. If there were significant effects in any of the rm-ANOVAs, we then further analyzed the data for that particular variable by using ANOVAs at each time point.

For the biotic response variables, we used a MANOVA to test for the effects of chlorpyrifos concentration, assemblage, and proximity to agriculture on the final measurements of periphyton, phytoplankton, and zooplankton (copepod and rotifer) abundances. We did not include cladocerans in the MANOVA because few or no cladocerans were alive at the end of the experiment in the highest chlorpyrifos concentration. Given that phytoplankton and periphyton abundances were measured three times, while zooplankton abundances were measured six times during the experiment, we used separate rm-ANOVAs for each variable. When significant effects were found, we subsequently used univariate ANOVAs at each time point. For

cladocerans, we removed the highest chlorpyrifos treatment from the analysis because few or no cladocerans survived this treatment. Reponses variables for the green frogs (survivorship and average tadpole mass) were analyzed using a separate MANOVA.

5.3 RESULTS

The overall MANOVA of the three abiotic variables (pH, DO, and temperature) and four biotic variables (copepod, rotifer, phytoplankton and periphyton abundances) sampled at the end of the experiment found main effects of assemblage (Wilks' λ, $F_{14,228} = 8.18$, $p < 0.001$), proximity to agriculture (Wilks' λ, $F_{7,114} = 3.48$, $p = 0.002$), chlorpyrifos concentration (Wilks' λ, $F_{28,412} = 11.64$, $p < 0.001$), and several significant interaction terms (Table L.1). We then analyzed each variable separately using rm-ANOVAs (Table L.2).

5.3.1 Abiotic variables

A detailed analysis of the abiotic variables can be found in Appendix K. Temperature fluctuated during the course of the experiment, but was not significantly affected by any of the main effects (Table L.2, Figure M.1). Analyses of DO and pH both showed significant effects of assemblage concentration, and several interactions. In general, pH and DO showed similar patterns across the course of the experiment; as chlorpyrifos concentration increased, so did pH and DO (Figure M.2, M.3). Data for pH and DO showed similar patterns with respect to the assemblages as both showed significant proximity to agriculture-by-concentration interactions (Table L.3A,B) Light decay, which was only sampled once during the experiment, only showed main effects of assemblage and chlorpyrifos concentration (both $p < 0.001$), but no significant interactions

(Table L.3C). In general, light decay rate increased as chlorpyrifos concentration increased and there was a general trend of higher rates of light decay in communities with only a background assemblage of zooplankton (Figure M.4).

5.3.2 Zooplankton

5.3.2.1 Cladocerans – The rm-ANOVA of cladoceran abundance found main effects of assemblage, proximity to agriculture, concentration, time (all $p \leq 0.001$), and several interactions (Table L.4). We then ran individual ANOVAs for each of the six sample dates and found that the three main effects were frequently significant as were the assemblage-by-concentration and the proximity-by-concentration interactions (Table L.5A; Figure 5.2, M.5). In general, we saw a decline in cladocerans with higher chlorpyrifos treatments across all sample dates. As the experiment progressed, cladocerans, which were not originally detected in the background assemblages, began to appear and increase in abundance, particularly in communities exposed to little or no chlorpyrifos. To address our hypothesis concerning variation in cladoceran resistance with proximity to agriculture, below we focus on the abundance of cladocerans in communities containing zooplankton populations collected near to versus far from agriculture.

On the first sample (day 3), there were no clear differences in cladoceran abundance within any of the treatments when comparing between assemblages from either near or far from agriculture. On the second sample (day 17), we found that within the cladoceran-only treatment exposed to 0.5 μg/L, communities with zooplankton collected near agriculture had more cladocerans than communities with zooplankton collected far from agriculture ($p = 0.048$).

On the third sample (day 22), communities with a background assemblage that were

exposed to 0.5 μg/L and communities of cladocerans only or the full assemblage that were exposed to 1.0 μg/L had more cladocerans, if they were collected near agriculture than if collected far from agriculture (all $p \leq 0.004$). On the fourth sample (day 35), there were more cladocerans in the cladoceran-only treatment within communities containing zooplankton collected near agriculture, but only at the 1.0 μg/L concentration.

On the fifth sample (day 38), in communities with a full assemblage, there were more cladocerans in those communities with zooplankton collected near agriculture at the 0.5 μg/L treatment level ($p = 0.004$). Furthermore, in communities with only cladocerans, there were more cladocerans in communities with zooplankton collected near agriculture at the 1.0 μg/L treatment level ($p < 0.001$). There were also more cladocerans in the background assemblage exposed to 1.0 μg/L, compared to communities with background assemblages containing zooplankton collected from the pond that was located near agriculture ($p < 0.001$). On the sixth and final sample (day 49), there were more cladocerans in communities with zooplankton assemblages from near agriculture when these communities were exposed to 0.5 μg/L (background treatment) or 1.0 μg/L (background and cladoceran only treatments, all $p \leq 0.001$) when compared to communities with assemblages collected further from agriculture.

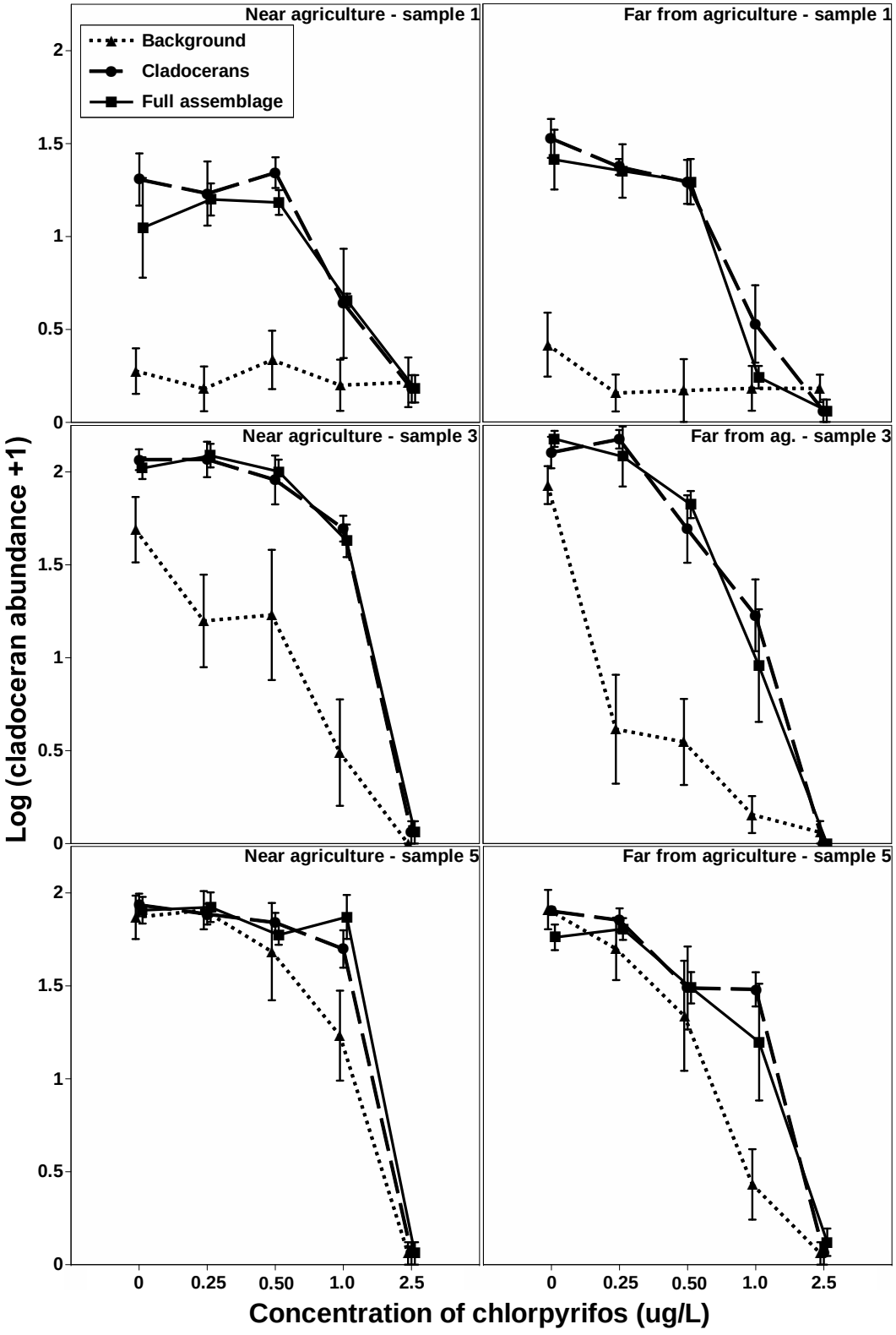

Figure 5.2. Differences in cladoceran abundance within experimental communities across the three sampling dates that immediately followed the three chlorpyrifos additions.

5.3.2.2 Copepods – The rm-ANOVA of copepod abundance indicated that there were main effects of assemblage, concentration, time, as well as several significant interaction terms (all $p < 0.001$, Table L.4). We then ran univariate ANOVAs for each of the six sampling dates and found that the three main effects were frequently significant as were several of the interactions (Table L.5B). Much like the data for cladoceran abundances, copepod abundances tended to decline with increasing chlorpyrifos concentrations. However, copepods were more resistant to the effects of the insecticide (Figure 5.3, M.6). We also observed that while the copepods were not detected in the initial cladoceran-only treatment, there must have been some rare individuals that became abundant over time. To address our hypothesis concerning variation in resistance to chlorpyrifos in copepods collected from ponds near versus far from agriculture, we first focused on whether there were differences in copepod abundance between communities with zooplankton collected near or far from agriculture.

In the first sample, within the cladoceran-only treatment there were more copepods in communities collected from near agriculture, when exposed to the lowest two concentrations and the highest concentrations of chlorpyrifos (all $p \leq 0.043$). In the full-assemblage treatment, there were more copepods in communities collected near agriculture when exposed to the middle three concentrations of chlorpyrifos (all $p < 0.001$). In the second sample of copepod abundance, there were more copepods across all chlorpyrifos treatments in communities containing zooplankton that were collected near agriculture when compared to communities with assemblages collected further from agriculture (all $p < 0.001$; Figure M.6).

In the third sample, there were more copepods in the communities with cladoceran-only and full-assemblage treatments that were collected near agriculture across nearly all treatments (all $p < 0.001$, except for those communities with a full assemblage at highest concentration). In

the fourth sample, there were generally higher copepod abundances under low chlorpyrifos concentrations in communities with zooplankton collected near agriculture. There were more copepods in communities with zooplankton from nearby agriculture within the cladoceran-only treatment at the three lowest concentrations of chlorpyrifos ($p \leq 0.003$).

The fifth sample indicated that in communities exposed to 1.0 μg/L chlorpyrifos, there were more copepods in communities with zooplankton collected near agriculture within the background-assemblage and full-assemblage treatments ($p \leq 0.029$). At the 0.5 μg/L treatment level, there were more copepods in communities with zooplankton collected near agriculture within the full-assemblage treatment ($p = 0.045$). In the sixth and final copepod sample, there were more copepods in communities with zooplankton collected near agriculture within the cladoceran-only treatment at the 0, 0.5 and 1.0 μg/L treatment levels (all $p \leq 0.037$).

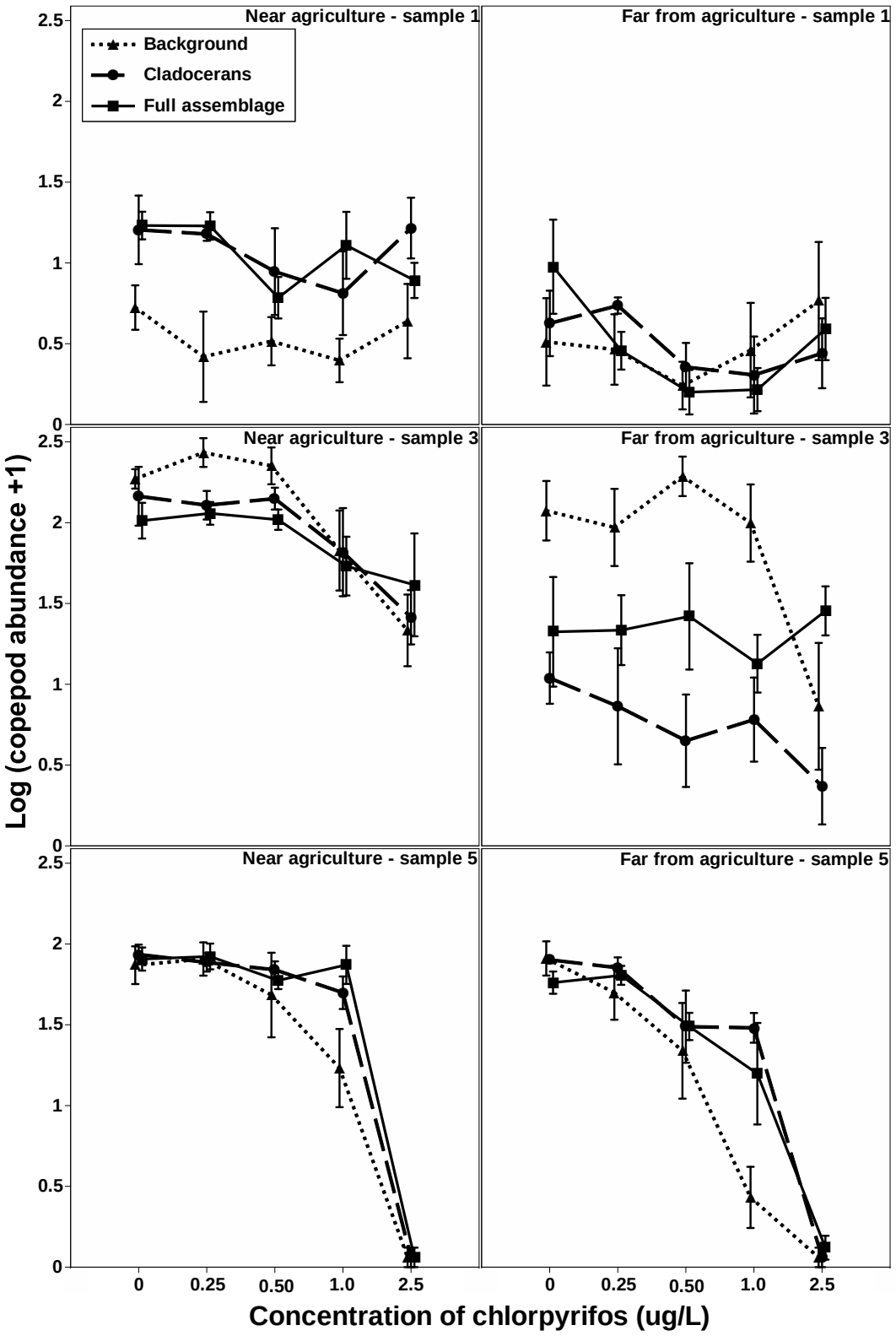

Figure 5.3. Differences in copepod abundance within experimental communities across the three sampling dates that immediately followed the three chlorpyrifos additions.

5.3.2.3 Rotifers – The rm-ANOVA of rotifer abundance found significant effects of assemblage type time (both $p < 0.001$) and several significant interaction terms (Table L.4). We then analyzed each sampling date separately using univariate ANOVAs (Table L.5C). Unlike the data for cladocerans and copepods, rotifers did not respond to increasing chlorpyrifos concentrations in a generalizable way and abundances varied greatly throughout the experiment at most concentrations (Figure 5.4, M.7). To address our hypothesis concerning variation in resistance to chlorpyrifos among rotifer assemblages from ponds near versus far from agriculture, we focused our analysis on differences in rotifer abundance between communities with zooplankton collected near or far from agriculture.

On the first sample date, there were fewer rotifers in the cladoceran-only treatment collected near agriculture, when exposed to 0.25 and 0.5 μg/L (both $p \leq 0.051$). Conversely, there were fewer rotifers in the background-assemblage treatment collected farther from agriculture when exposed to 0.5 μg/L ($p < 0.001$). On the second sampling date (Figure M.7), there was no significant evidence of variation in resistance that could be attributed to proximity to agricultural land use. On the third sampling date, there were more rotifers in communities containing a background assemblage collected farther from agriculture at the 0.25 and 0.5 μg/L treatments levels (both $p < 0.001$). Furthermore, there were more rotifers in communities containing a background assemblage collected near agriculture at the 1.0 μg/L treatment level ($p < 0.001$).

During the fourth sampling date, communities containing cladoceran-only assemblages from near agriculture contained fewer rotifers at the two highest concentrations, when compared to communities with cladoceran only assemblages found near agriculture (both $p \leq 0.006$). There were also fewer rotifers in communities containing a full assemblage from near agriculture

that were exposed to the highest concentration of chlorpyrifos ($p = 0.001$). In the fifth sample, were fewer rotifers in communities containing background assemblages collected near agriculture exposed to 1.0 μg/L chlorpyrifos, when compared to communities with zooplankton collected farther from agriculture ($p = 0.008$). Furthermore, there were more rotifers in communities containing the cladoceran-only assemblages collected near agriculture at the lowest two concentrations when compared to communities with zooplankton collected farther from agriculture ($p \leq 0.002$). Finally, during the sixth sample, there were more rotifers in communities with background-only assemblages from near agriculture at the lowest and highest concentrations of chlorpyrifos, when compared to communities with zooplankton collected farther from agriculture (both $p < 0.001$).

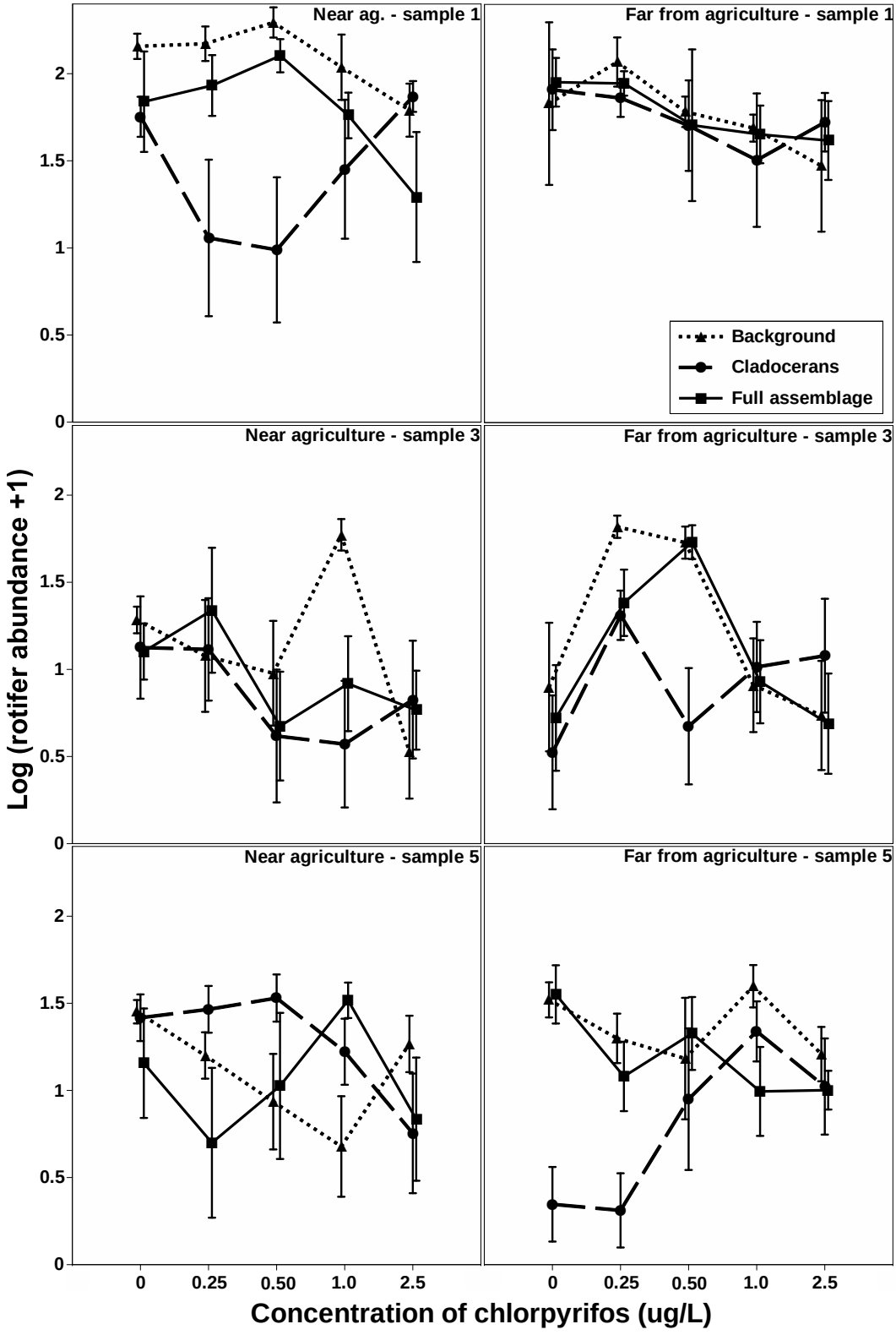

Figure 5.4. Differences in rotifer abundance within experimental communities across the three sampling dates that immediately followed the three chlorpyrifos additions.

5.3.3 Phytoplankton

The rm-ANOVA on phytoplankton abundance revealed main effects of assemblage, proximity to agriculture, concentration, time, and several interactions (Table L.6). We then analyzed each sampling date of phytoplankton abundance independently using univariate ANOVAs and found that all three main effects and most interactions were significant during one or more sample dates (Table L.7A). In general, increasing chlorpyrifos concentrations, which caused a decline in zooplankton, also caused increases in phytoplankton (i.e. an algal bloom, Figure 5.5). We then used the ANOVAs to determine whether any of the zooplankton assemblages that were collected near agriculture were superior in preventing algal blooms compared to those collected far from agriculture across a range of chlorpyrifos concentrations.

We first compared the amount of phytoplankton within communities containing each of the three zooplankton assemblage treatments from either near or far from agriculture. In the first sample, in communities with zooplankton collected near agriculture, there was less phytoplankton in cladoceran-only and full-assemblage treatments exposed to 0.5 μg/L chlorpyrifos, when compared to communities with zooplankton collected farther from agriculture (both $p \leq 0.039$). On the second sampling date, we found that at the 0.5 and the 1.0 μg/L treatment levels, there was also less phytoplankton in communities with cladocerans or a full assemblage of zooplankton from a pond near agriculture when compared to communities with the same assemblages from a pond near agriculture (all $p < 0.009$). On the third and final sampling date, there was more phytoplankton in communities within the cladoceran-only or full-assemblage treatments in communities where the zooplankton were collected farther from agriculture relative to the communities where the zooplankton were collected from near

agriculture (0.5 and 1.0 μg/L treatments, both $p \leq 0.044$). Within communities with zooplankton collected near agriculture, there was less phytoplankton in communities with cladocerans only relative to the full-assemblage treatment at the 0.5 μg/L treatment level ($p < 0.001$).

To address the hypothesis of functional redundancy, we tested whether the background assemblage was able to prevent algal blooms to a similar degree as cladocerans alone and the full assemblage within each agricultural category across a range of chlorpyrifos concentrations. On the first sample date, in communities containing zooplankton from near agriculture, there was more phytoplankton in communities with a background assemblage of zooplankton, when compared to cladoceran-only or full-assemblage treatments at the 0.25, 0.50 and 1.0 μg/L treatments levels (all $p < 0.001$). For communities with zooplankton collected farther from agriculture, there was more phytoplankton in communities with a background assemblage at the 0.25 μg/L treatment level, when compared to the other two assemblages ($p < 0.001$).

On the second sample date, communities with zooplankton collected from near agriculture, there was more phytoplankton in communities containing a background assemblage of zooplankton when compared to communities with cladocerans-only or a full assemblage of zooplankton, across the middle three concentrations (all $p < 0.001$). On the third sample date, there was more phytoplankton in communities with a background assemblage of zooplankton from near agriculture, when compared to the cladoceran- only and full-assemblage treatments across the middle three concentrations of chlorpyrifos (all $p < 0.001$). In those communities with zooplankton collected farther from agriculture, relative to communities with either cladocerans or a full zooplankton assemblage, there was more phytoplankton in those communities with a background assemblage that was exposed to 0.25 μg/L chlorpyrifos ($p = 0.001$).

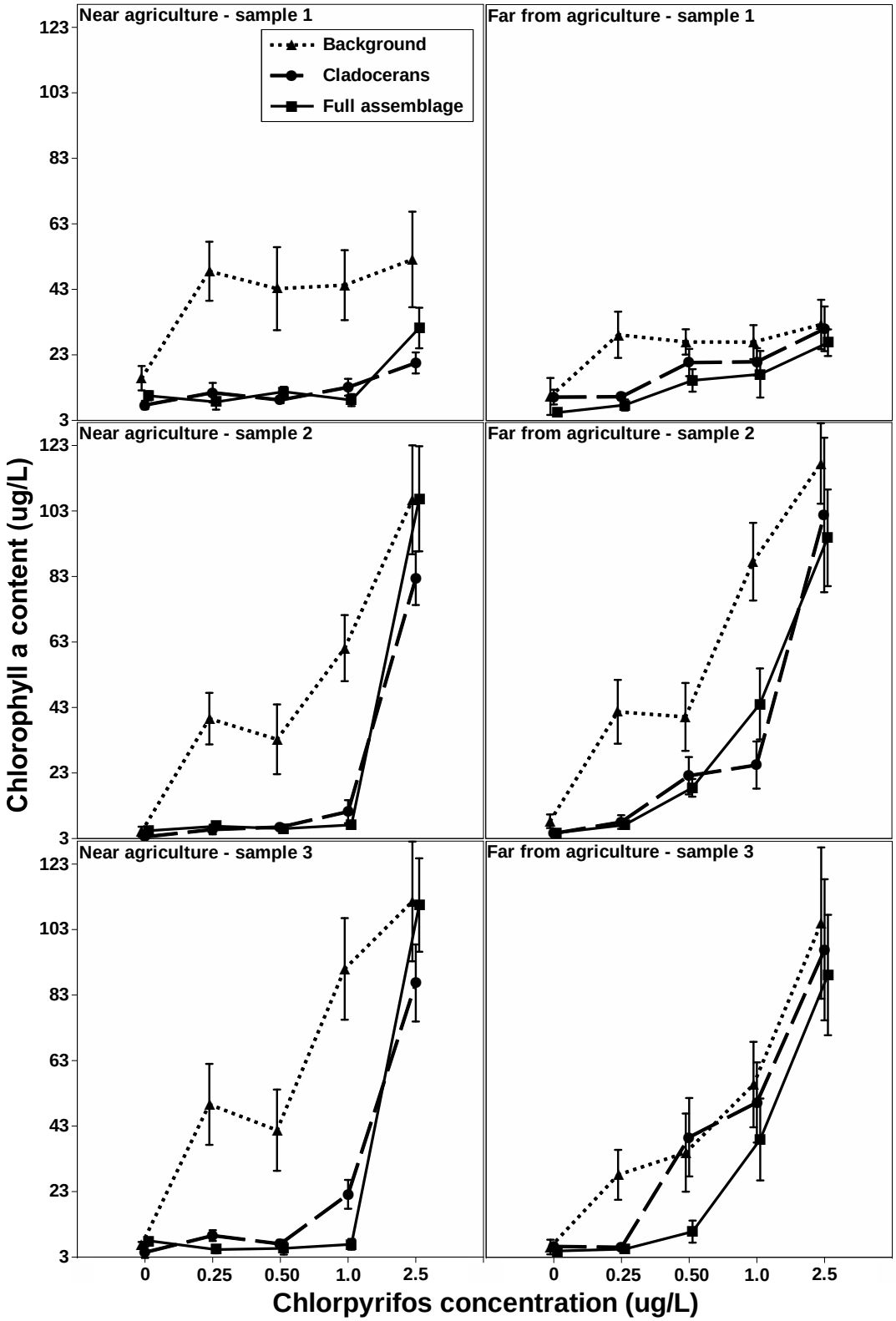

Figure 5.5. Differences in phytoplankton content (measured in chlorophyll *a* content in μg/L) across the three sampling dates of phytoplankton abundance.

5.3.4 Periphyton

The rm-ANOVA of periphyton abundance revealed that there were effects of the assemblage type, concentration and time (all $p < 0.001$), as well as a number of significant interaction terms (all $p \leq 0.036$, Table L.6). We then ran individual ANOVA analyses on periphyton abundance for each sampling date and found multiple main effects and interactions (Table L.7B). Once the zooplankton had died and the phytoplankton began to bloom, we could discern a pattern of increasing chlorpyrifos concentrations causing decreases in periphyton abundance beginning on the second sample date (Figure 5.6). Data was used to determine whether or not declines in cladocerans caused decreases in periphyton, and whether or not cladocerans that were collected near agriculture, were superior in preventing periphyton decline. On the first sample date, there was no evidence of communities with zooplankton assemblages from nearby or far from agriculture differing in their periphyton content.

On the second sample date, there was a clear trend in declining periphyton content within communities with zooplankton collected farther from agriculture. Communities exposed to 0 or 0.25 μg/L chlorpyrifos with either cladocerans or a full assemblage of zooplankton had more periphyton than all other community treatments exposed to the highest three concentrations of chlorpyrifos (all $p \leq 0.043$). There was significantly more periphyton in communities with a cladoceran-only or full assemblage collected near agriculture that was exposed to 0.5 or 1.0 μg/L chlorpyrifos relative to communities with zooplankton collected farther from agriculture (all $p < 0.001$). On the third sample date, in communities with zooplankton from a pond farther from agriculture, we again saw a marked decline in periphyton abundance in communities subjected to more than 0.5 μg/L chlorpyrifos relative to controls. There was also less periphyton in

communities with a background assemblage of zooplankton collected near agriculture when exposed to the four highest concentrations of chlorpyrifos relative to communities with background assemblages collected farther from agriculture (all $p \leq 0.002$).

Furthermore, to address the hypothesis of functional redundancy, we also wanted to determine whether or not a background assemblage was able to prevent periphyton decline to the same degree as cladocerans. On the first sample date, within the communities containing zooplankton collected farther from agriculture, there was more periphyton in communities with a background assemblage exposed to 0.5 μg/L chlorpyrifos and within those communities with a cladoceran-only assemblage exposed to 2.5 μg/L, when compared with communities containing zooplankton collected near agriculture (both $p < 0.001$).

On the second sample date, communities with zooplankton from near agriculture, there was significantly more periphyton in those communities exposed to 0.5 or 1.0 μg/L chlorpyrifos within the cladoceran-only or full-assemblage treatments relative to the background assemblage (all $p < 0.001$). On the third sample date, communities with zooplankton collected near agriculture had more periphyton in the cladoceran-only or full-assemblage treatments, relative to communities with only a background assemblage when exposed to the four highest concentrations of chlorpyrifos (all $p \leq 0.053$). In control communities, there was more periphyton in cladoceran-only communities, when compared to communities with only a background assemblage of zooplankton ($p < 0.001$). However, as insecticide concentration increased, there was no longer any significant differences in periphyton abundance, when comparing the three types of zooplankton assemblages (all $p \geq 0.213$).

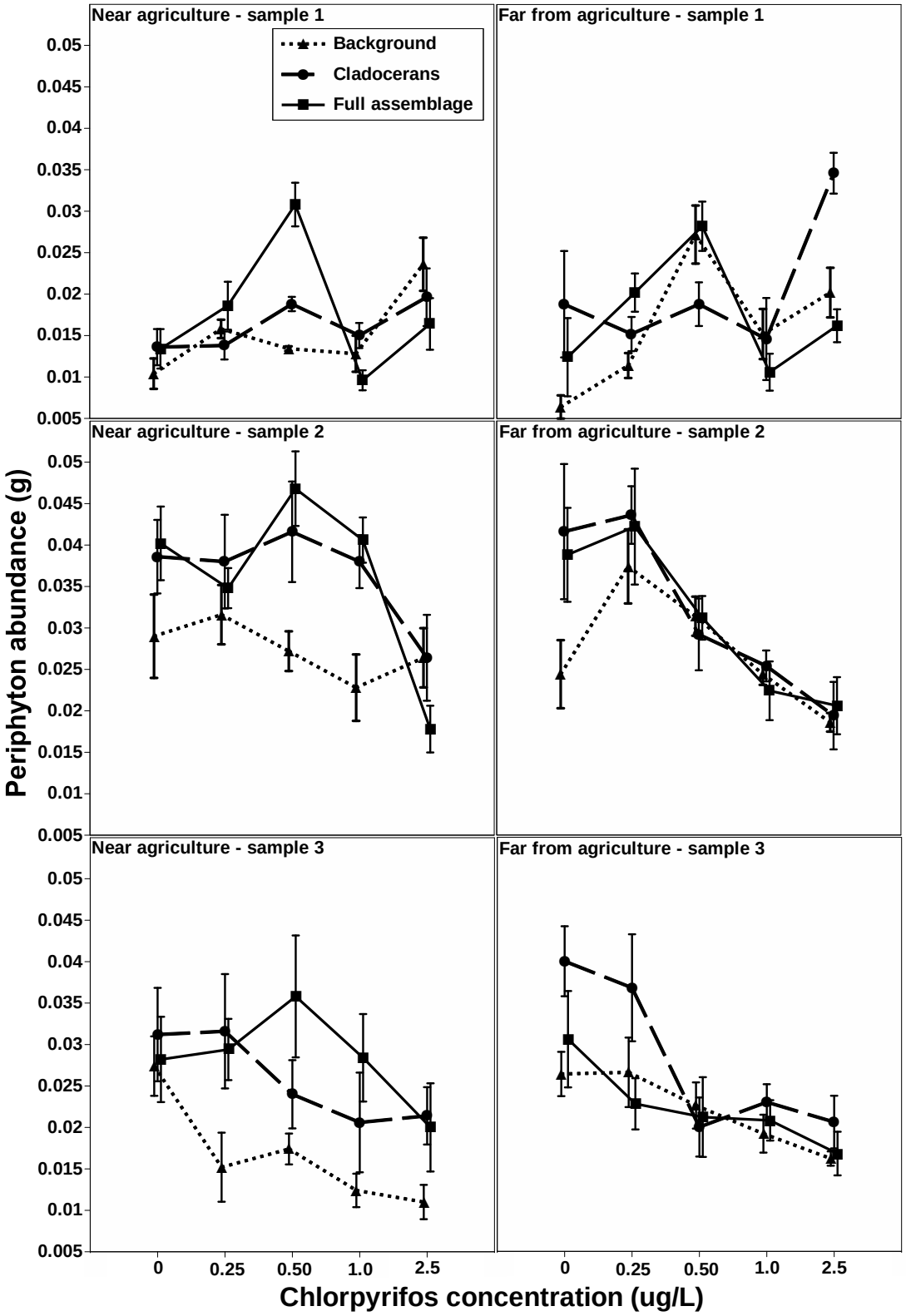

Figure 5.6. Differences in periphyton content (in g) across the three sampling dates of periphyton abundance.

5.3.5 Amphibian responses

The MANOVA on amphibian response variables (average mass of tadpole and survivorship) indicated that there were no significant main effects or any significant interaction terms (Table L.8).

5.4 DISCUSSION

As in previous studies, we were able to demonstrate that naturally occurring populations of cladocerans collected near agriculture were more resistant to a commonly applied insecticide than those collected far from agriculture (Bendis and Relyea 2014, Bendis and Relyea *in review, in prep*). However, we also found that copepods from these same ponds exhibited a similar pattern of resistance to chlorpyrifos, but with rotifers, although there were several instances of population-level differences in resistance, these differences were unrelated to their proximity to agriculture. To our knowledge, this is the first study to examine population-level variation in insecticide resistance among copepods and rotifers based on proximity to agriculture. We also showed that there is little to no evidence of functional redundancy in terms of the ability of copepods and rotifers to buffer vernal pond communities from trophic cascades initiated by insecticide applications. Among the three major groups of zooplankton, previous studies have implicated cladocerans as being one of the most important drivers of phytoplankton abundance in limnetic systems, but this is not often empirically tested in a community context (Carpenter et al. 1985, Hanazato 1998, Tessier and Woodruff 2002, Korosi et al. 2012). This study is the first to combine the effects of insecticide applications on zooplankton assemblages that vary in their resistance to an insecticide, as well as the identities of the zooplankton within the community, to

explicitly test the hypothesis of functional redundancy in phytoplankton consumption among different zooplankton assemblages.

Of the three main groups of zooplankton, cladocerans were the most heavily affected by periodic applications of chlorpyrifos. Sensitivity to a wide range of insecticides is a common theme among many cladoceran species, particularly those within the genus *Daphnia* (Barata et al. 2001, Hanazato 2001, Baird and Van den Brink 2007, Relyea and Hoverman 2008, Daam et al. 2008, Simpson et al. 2014). Moreover, this also confirms previous findings that copepods and rotifers are typically less affected by low, environmentally relevant concentrations of organophosphate insecticides compared to cladocerans (Havens 1994, Hanazato and Kasai 1995, DeLorenzo et al. 2001). Our range of chlorpyrifos treatments spanned the range from having no observable effects on cladoceran abundance (0 – 0.25 μg/L) to completely extirpating all cladocerans by the end of only the first application (2.5 μg/L). More importantly, whereas previous experiments have shown that cladoceran populations can often rebound between applications of an insecticide, the highest concentration of chlorpyrifos was lethal to all cladoceran zooplankton and prevented cladocerans from recolonizing the tanks.

There were clear effects of increasing chlorpyrifos concentrations on both cladocerans collected both near and far from agriculture. During the first two sample dates, there was no clear difference in *Daphnia* abundance, when comparing those cladocerans collected near or far from agriculture, but as time progressed, significant differences in *Daphnia* abundance began to develop in communities exposed to moderate concentrations of chlorpyrifos (0.5 – 1.0 μg/L). In general, there were more *Daphnia* in communities comprised of zooplankton that were collected from the pond found near agriculture. This is consistent with the hypothesis that populations of zooplankton in ponds near agricultural fields can evolve increased resistance to pesticides,

whereas zooplankton from ponds far from agriculture should not evolve such resistance (Coors et al. 2009, Jansen et al. *in press*). Moreover, the differences in cladoceran abundance at these concentrations is consistent with LC50 data for *D. pulex* that we have found in several previous experiments (Bendis and Relyea 2014, Bendis and Relyea *in review*, *in prep*).

The effects of increasing chlorpyrifos concentrations on copepods were similar to cladocerans; increasing concentrations resulted in declining abundance. Where these results differed, however, were that copepods were not completely extirpated from the community, even at the highest concentration, which was lethal to nearly all cladocerans. This finding coincides with previous community studies that have shown that concentrations of several insecticides that are lethal cladocerans are sublethal to copepods (Sierzen and Lozano 1988, DeLorenzo et al. 2001, Van Wijngaarden et al. 2005, Daam et al. 2008).

We also found that there were differences in copepod abundance when they were collected from assemblages near versus far from agriculture. For example, during the first three samples of copepod abundance, there were more copepods in communities with zooplankton collected near agriculture, particularly in communities exposed to the middle three concentrations of chlorpyrifos (0.25 – 1.0 μg/L). Over time, however, this effect diminished and the differences between copepod abundance in communities near and far from agriculture began to disappear.

We hypothesize that the first and second pesticide applications served as major selection events, which removed the especially sensitive copepod genotypes from the community. This allowed more resistant genotypes (even within the assemblages collected far from agriculture) to reproduce and proliferate, thereby increasing the resistance of overall copepod assemblage through time. Conversely, chlorpyrifos applications may have removed particular species of

copepods that were initially predominant in the communities or may have had disproportionately large effects on particularly sensitive size or age classes. Once these more sensitive species or age classes were removed, this may have no longer competitively excluded other species that may have been more resistant, but were also initially less abundant. Though we did not determine the species identity of all copepods within our samples due to time constraints, future work to explicitly examine population-level variation in resistance to insecticides for each species of copepod would be crucial in determining the mechanism of increased resistance during the experiment.

Rotifers, unlike cladocerans and copepods, did not show a clear pattern of increased resistance with a closer proximity to agriculture. Interestingly, rotifers generally survived well across all chlorpyrifos treatments, whereas the highest chlorpyrifos treatments typically caused precipitous declines in survivorship of both copepods and cladocerans. A general pattern that we did see, however, was that there were typically more rotifers in communities exposed to moderate chlorpyrifos concentrations when compared to copepod or cladoceran abundances. We hypothesize that competition with highly abundant cladocerans for access to phytoplankton (and potentially some inadvertent consumption of rotifers by cladocerans) in control treatments may have kept rotifer abundance lower in unexposed communities (Vanni 1986, Hanazato and Kasai 1995). Furthermore, there may have been direct predation on rotifers by omnivorous or predatory copepods, particularly at low to moderate insecticide concentrations. When these communities were exposed to the highest concentrations of chlorpyrifos, cladocerans and copepods were nearly eliminated. However, both copepods and rotifers did not increase in abundance even when freed from competition from the larger-bodied cladocerans. This suggests that perhaps rotifers and copepods may have been near a threshold between sublethal and lethal

concentrations at this particular concentration. It is also possible that copepods and rotifers may have been constrained in development or their reproductive output by sublethal effects of chlorpyrifos at these higher concentrations. Regardless, across the range of chlorpyrifos concentrations that were used, both rotifers and copepods may have been somewhat constrained in the ability to increase in abundance due to possible competitive pressure from cladocerans or predation by copepods in unexposed communities and possible direct or indirect effects of toxicant exposure at higher concentrations. This, however, would need to be more explicitly tested in smaller, more controlled laboratory-based studies.

Although we initially set up communities with zooplankton assemblages that were visually inspected and verified to be either only cladocerans or only copepods and rotifers, over time we observed an increasing number of copepods and rotifers in the cladoceran-only assemblage and an increasing number of cladocerans in the background assemblage. As noted earlier, visual inspections of the samples that were initially added to each mesocosm did not detect any of the undesired taxa, but clearly some small individuals must have been present. Because there was evidence of unexpected zooplankton getting into other treatments in the first sample of zooplankton abundance, and because these animals showed patterns of resistance that were consistent with proximity to agriculture, we are fairly certain that these colonizers entered the mesocosms when we added our initial zooplankton treatments rather from unintended transfers of zooplankton when sampling the mesocosms. Although this is an issue from the standpoint of determining the possible effects of functional redundancy, we argue that the initial abundances of the "invading" zooplankton were extremely low and that competition between some species and direct predation by others may have ameliorated the effects of these invading species.

The direct toxic effects of chlorpyrifos on the zooplankton assemblages led to dramatic increases in phytoplankton. The general trend was that as chlorpyrifos concentrations increased, the amount of phytoplankton in the communities increased. The amount of phytoplankton was also largely determined by the type of zooplankton assemblage and whether the zooplankton were collected near or far from agriculture. Communities containing only a background assemblage of copepods and rotifers had more phytoplankton than communities with only cladocerans or the full assemblage of zooplankton when exposed to moderate concentrations (0.25 – 1.0 μg/L) of chlorpyrifos. The disparity of phytoplankton in communities containing cladocerans-only or the full assemblage of zooplankton was due to the rapid consumption of phytoplankton by cladocerans. Of the three groups of zooplankton, the large-bodied cladocerans consume far more phytoplankton per capita than do copepods and rotifers and therefore should have the most dramatic impact on phytoplankton abundance (Vanni 1986, Hanazato and Kasai 1995, Hanazato 1998). Furthermore, in communities with a background assemblage, there is the additional complexity of there being numerous food sources for copepods. Whereas some copepods are herbivorous, others are predatory and will consume not only other copepods but cladocerans and rotifers as well. Therefore, increases in phytoplankton content within communities with a background assemblage could potentially be exacerbated by predatory copepods that are consuming other herbivorous species of zooplankton.

There were also differences in phytoplankton abundance within treatments that only differed in the source of zooplankton. In communities that were exposed to either 0.5 or 1.0 μg/L, there was more phytoplankton in communities with cladocerans or full assemblages of zooplankton that were collected near versus far from agriculture. This can be directly attributed to the higher abundances of the insecticide-resistant *Daphnia* within these particular

communities. Resistance within these *Daphnia* populations and the associated higher abundances of these competitively dominant zooplankton caused direct declines in phytoplankton. Although the differences in cladoceran abundance between these two community types at these concentrations were significant, they were not dramatically different. This is reflected in the smaller albeit significant differences in the first two samples of phytoplankton content. However, as time progressed the differences in cladoceran abundance and phytoplankton abundance grew to be quite substantial, thereby indicating that there was a pronounced lag effect of the phytoplankton bloom. Additionally, there may have been differences in the nutrient cycling efficiencies of zooplankton assemblages that were collected near agriculture versus those further from agriculture, which would need to be explicitly tested using more controlled laboratory assays. These results are strikingly similar to other studies that have found that the population genetics *Daphnia* populations can have dramatic community-wide effects on aquatic ecosystems that are impacted by anthropogenic stressors such as insecticides (Bendis and Relyea *in review*, *in prep*).

In our experimental communities, the resultant phytoplankton blooms caused declines in periphyton content and these declines were affected by the particular assemblage within the community, as well as whether or not the zooplankton assemblage was from a pond either near or far from agriculture. From previous studies, it is known that relatively low insecticide concentrations in aquatic communities can cause dramatic declines in zooplankton abundance that cause increases in the phytoplankton content, which is the main food for numerous species of zooplankton. As phytoplankton content increases, the rate of light transmission through the water column continually declines, which prevents the attached periphytic algae on the bottom and sides of the pond from access to this now limiting resource (Boone et al. 2004, Mills and

Semlitsch 2004, Relyea and Diecks 2008). Furthermore, as phytoplankton abundance increases, the phytoplankton utilizes the majority of other dissolved nutrients that are available in limited quantity, such as phosphorus, nitrogen, and calcium, thereby exacerbating the decline in periphyton content (Hansson 1988, Korosi et al. 2012).

Concordant with our data of phytoplankton abundance, there was an effect of zooplankton assemblage type on periphyton abundance, but this effect was somewhat less pronounced when compared to the effects on phytoplankton content. In general, there was less periphyton in communities with only a background assemblage of zooplankton, when compared to communities containing cladocerans only or a full assemblage of zooplankton. This is consistent with our hypothesis that a community comprised of mainly copepods and rotifers will experience prolonged phytoplankon blooms, as these groups of zooplankton are not functionally redundant in their abilities to consume and draw down phytoplankton content. In communities with either cladocerans or the full assemblage of zooplankton, the typically more insecticide-sensitive cladocerans are consuming the majority of the phytoplankton content and, in doing so, can competitively exclude copepods and rotifers when exposed to sublethal concentrations of insecticides. Our experiment simulates how press insecticide exposures can control the abundance of, and in some cases completely extirpate, cladoceran zooplankton. This results in higher abundances of phytoplankton available for the more resistant zooplankton groups (copepods and rotifers), when exposed to higher insecticide concentrations. However, evidence here shows that copepods and rotifers are not able to consume the adequate amount of phytoplankton required to re-stabilize these aquatic communities - at least within the timespan of this experiment. Given enough time, however, copepods and rotifers may be able to increase in abundance to a point where they may be able to significantly affect phytoplankton abundance

and this is an area where future studies should be focused.

There were also marked differences in periphyton content, when comparing the same zooplankton assemblages from different native ponds within a given treatment of chlorpyrifos exposure. In communities exposed to 0.5 or 1.0 μg/L chlorpyrifos, there was more periphyton in communities with cladoceran-only and full-zooplankton assemblage treatments that were collected from the pond near agriculture in comparison to those same assemblages that were collected farther from agriculture. Again, this was due to the differential survivorship of zooplankton (primarily *Daphnia*) within these treatments. More *Daphnia* within communities containing zooplankton assemblages collected near agriculture survived when compared to *Daphnia* collected farther from agriculture at these concentrations. This meant that there would be less phytoplankton within these communities and, as a result, a higher abundance of periphyton to be made available to grazers.

The variation in periphyton abundance between community types has the potential to negatively affect the growth, development and survivorship of grazers such as amphibians. In our study, we found no effects of any treatments on green frog tadpole mass or survivorship. A previous study performed under similar conditions demonstrated that low, environmentally-relevant concentrations of chlorpyrifos had significant effects on the survivorship and growth of a different amphibian species (the northern leopard frog, *L. pipiens;* Bendis and Relyea, *in prep*). Survivorship declines in that study were attributed to insufficient periphyton, which prevents amphibians from attaining the nutrients necessary to metamorphose prior to pond desiccation. In the current study, there was reduced periphyton content in those treatments that experienced an algal bloom, but the amount of periphyton available to amphibians was not limiting. When comparing the average masses of leopard frog tadpoles in the previous study and green frog

tadpoles in the current study, we found that the mean mass of our green frog tadpoles were only ~15% of the mass of the leopard frog tadpoles. Previous studies have also shown that green frog tadpoles are typically less active and forage for periphyton less than leopard frog tadpoles (Relyea 2001). Therefore, it is perhaps not surprising that we found no effects on tadpole growth and overall survivorship in our green frogs.

5.4.1 Conclusions

In this study, we found that cladocerans and copepods collected from a pond closer to agriculture were more resistant to chlorpyrifos than those collected from a pond in a more pristine location. Although there was variation in resistance among rotifers collected far and near agriculture, there was no clear evidence that this variation was associated with surrounding land use as we did with cladocerans and copepods. Laboratory-based studies examining the effects of chlorpyrifos on the individual species of copepods and rotifers found in ponds across a gradient of surrounding agricultural land use are needed to determine just how pervasive the phenomenon of insecticide resistance is among zooplankton taxa. We found no evidence that a background community composed of the copepods and rotifers would be able to protect the stability of an aquatic community from environmentally-relevant concentrations of chlorpyrifos. On the contrary, communities with more resistant cladocerans collected from a pond near agriculture, which are typically more sensitive to insecticides, were buffered from the deleterious effects of the chlorpyrifos at moderate to moderately high concentrations of the insecticide. Future studies should aim to isolate individual genotypes of zooplankton and use pure cultures of previously identified zooplankton to populate mesocosms to prevent cross-contamination of zooplankton so

that communities will contain purely cladocerans or background assemblages. This can be paired with smaller laboratory studies that examine differences in foraging efficiencies or elemental content of zooplankton species collected near and far from agriculture. To gain a better understanding of the effects of future anthropogenic perturbations and to protect the stability of these often heavily impacted vernal pond communities, we must fully understand how insecticides can affect the most sensitive and integral members of the community by incorporating as much ecological realisms into studies as possible.

5.5 ACKNOWLEDGMENTS

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6.0 CONCLUSION

In order to protect and conserve vernal pond communities and wetlands from future anthropogenic disturbances, we have to fully understand how the organisms within these communities are adapting to these stressors and how these changes can potentially affect overall ecosystem function. We have found that there is clear variation in population-level resistance to several insecticides in common zooplankton species and that this variation is associated with nearby agricultural land use. The difference in sensitivity between populations is not staggering from a purely toxicological standpoint, but these small differences have been empirically shown to translate into dramatic community-wide effects when aquatic communities are exposed to environmentally-relevant concentrations of commonly applied insecticides. With this in mind, we could hypothesize that ponds that have not historically been located near agriculture are at the highest risk of species loss through the deleterious direct and indirect effects of toxicity. Ponds with a history of high agricultural land use surrounding them would potentially be the most protected from pesticide contamination. From a conservation-based standpoint, if time and resources were limited, one could potentially use historical agricultural land use as a proxy to determine which ponds should be the primary targets for protection.

Consideration should also be given to determining possible differences in foraging efficiencies or preferences among resistant and sensitive *D. pulex* clones. An unanticipated finding in two of the aforementioned experiments was that leopard frog tadpoles in control

communities developed at different rates based on whether or not they were in a community with resistant or sensitive *D. pulex*. There were no differences in *D. pulex* abundance or any associated differences in phytoplankton or periphyton abundance in these communities, which would have led to differences in amphibian growth, but there were still differences in variables such as mass at metamorphosis and average developmental (Gosner) stage. The only differences between the communities were the population genetics of the *D. pulex*, which suggests that resistant and sensitive *D. pulex* clones potentially had differential foraging strategies (i.e. in species of phytoplankton preferentially consumed or the amount of nutrients efficiently recycled, Garcia et al. 2007). For instance, *D. pulex* has been shown to be fairly phosphorous-limited in many ecological contexts (DeMott and Gulati 1999, Elser et al. 2001, Jeyasingh and Weider 2005). If the maintenance of resistance within a population is costly when there are no insecticides present, then perhaps resistant *Daphnia* are selectively feeding on phytoplankton containing high phosphorous content whereas sensitive *Daphnia* can be a more of a generalist grazer. This, in turn, could have subtle albeit significant effects on light transmission and periphyton growth (in terms of quality versus quantity), which would have an effect on tadpole growth and development as well. Clearly, more attention should be paid to determining whether or not resistance to insecticides has a cost in terms of competitive ability or dietary restrictions. There is ample ecological theory in the realm of ecological stoichiometry that could be used to generate predictions as to why these different clones of *D. pulex* may alter their respective communities in these subtle and complex ways (Lind and Jeyasingh 2015).

Although the utilization of mesocosms to study community dynamics is undoubtedly a useful way to simulate natural pond communities while allowing the researcher to manipulate specific conditions and directly test their hypotheses, we need to pair these findings with

observations and *in situ* experiments performed in natural pond or wetland communities. Additionally, one drawback with mesocosms and controlled, large-scale community experiments is that we can collect large amounts of data in a relatively short period of time, but the data is typically more quantitative than qualitative. We can determine how much periphyton or phytoplankton exist within a particular community, but we often do not know anything about the nutritional quality of the algae in questions or what species exist within the typically diverse algal assemblage. Studies explicitly examining how resistant or sensitive *D. pulex* can potentially modify phytoplankton and periphyton species abundance and diversity as well as its nutritional quality are of the utmost importance in order to understand how these different populations can cause divergent community effects even in unexposed communities. Small-scale laboratory experiments that can adequately control zooplankton abundance while providing standardized amounts of phytoplankton (with known or potentially modified nutritional content) to each experimental unit can test for trade-offs in terms of foraging efficiencies or competitive ability may not have been able to be detected within the large-scale mesocosm studies.

Further effort should be made to expand our existing knowledge of zooplankton sensitivity to insecticides and to determine if the phenomenon of insecticide resistance is widespread among zooplankton taxa. Only a handful of previous studies have found naturally existing variation in insecticide resistance in zooplankton and, of those, two recent studies have found that surrounding agricultural land can be used as a proxy for determining whether or not resistance within cladocerans may have previously evolved within these communities (Bendis and Relyea 2014, Jansen et al. *in press*). Furthermore, studies within this thesis are the first to show natural variation in resistance among copepod and rotifer assemblages. To explicitly test for effects of these insecticides on individual species, short-term laboratory toxicity tests are

required to fully discern whether these differences in resistance truly exist. These tests are simple to perform, relatively cost-effective and can greatly enhance our understanding of the effects of chemical stressors on natural zooplankton assemblages. Nonetheless, these findings suggest that a wide array of non-target organisms may be evolving resistance to agrochemicals. For each species that shows the potential for evolved resistance to environmentally-relevant concentrations of these chemicals, the future prospects for these threatened and fragile ecosystems look continually brighter.

Finally, wetlands are often at the lowest topographical points within a specific geographical area which means that gravitational run off from surrounding agriculture will continually drain directly into them (Bedford 1999, Stoler 2013). Vernal ponds typically exist in relative isolation and are not directly connected with any source of flowing water meaning that any chemical contaminants that enter the water remain there until they are broken down (Heimbach et al. 1992). Thus, these ecosystems are arguably some of the most threatened in terms of their exposure to pesticides, as pesticide use is not likely to end in the foreseeable future. The data presented in this thesis suggests that future perturbations on aquatic communities may be buffered by the presence of insecticide resistant zooplankton. However, the concentrations that these organisms can withstand are extremely low (albeit environmentally-relevant), leaving these communities in a somewhat precarious state in terms of their future exposures to these chemicals. By continuing to deepen our understanding of ecological theory and pairing theory with innovative empirical experiments that build in more of the complexity that often exists in nature, the studies presented here can undoubtedly contribute to advancing our understanding of the effects of anthropogenic disturbance on aquatic communities.

APPENDIX A

CHAPTER TWO: SUPPLMENTAL TABLES

Table A.1. The location of *S. vetulus* and *D. pulex* populations in northwest Pennsylvania.

Pond Name	Latitude	Longitude
Blackjack	41.65564	80.51270
Graveyard	41.68436	80.04728
Hopscotch	41.86568	80.47036
Log	41.96912	79.59869
Love	41.41064	80.30460
Minnow	41.41121	80.25302
Mallard	41.69198	80.50071
Road	41.88464	79.60533
Staub	41.58932	80.43100
Trailer Park	41.56900	80.45248
Turkey Track	41.63039	79.91281

APPENDIX B

CHAPTER TWO: SUPPLMENTAL FIGURES

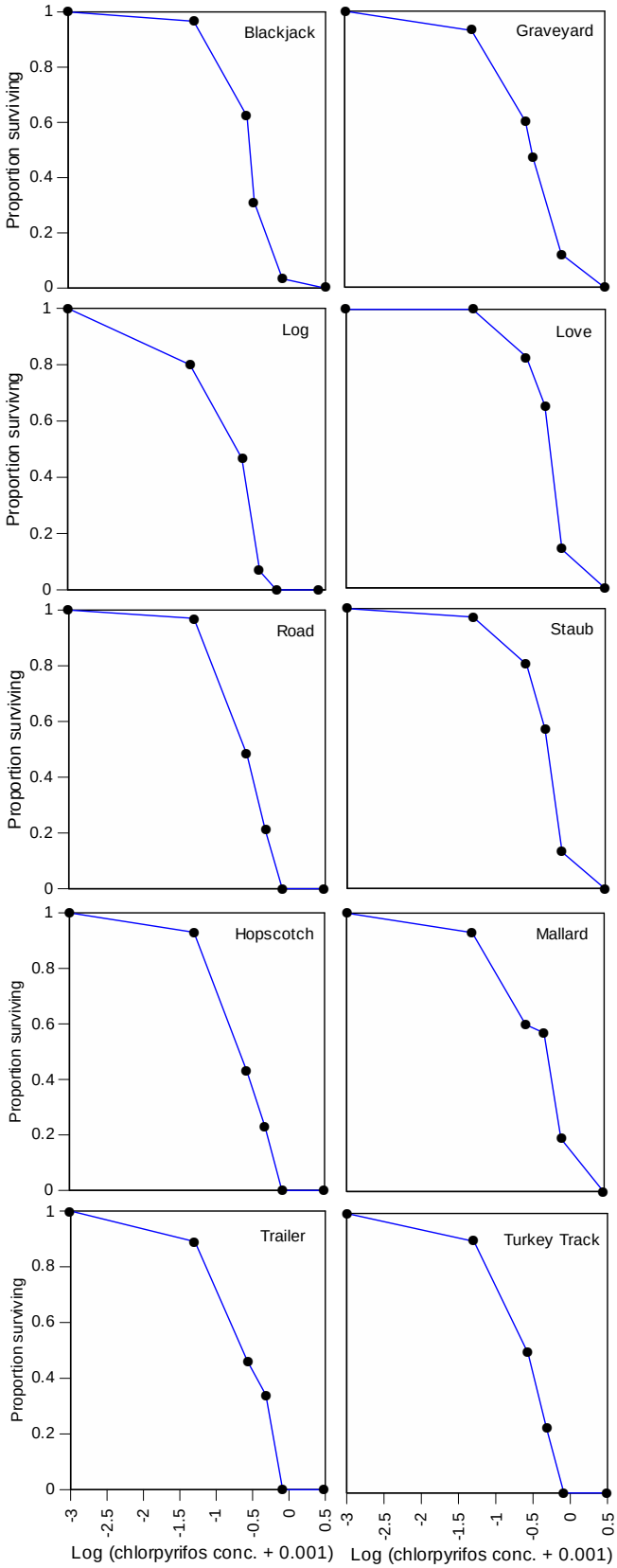

Figure B.1. Survivorship curves for the 10 populations of *S. vetulus* across a span of 48 hours.

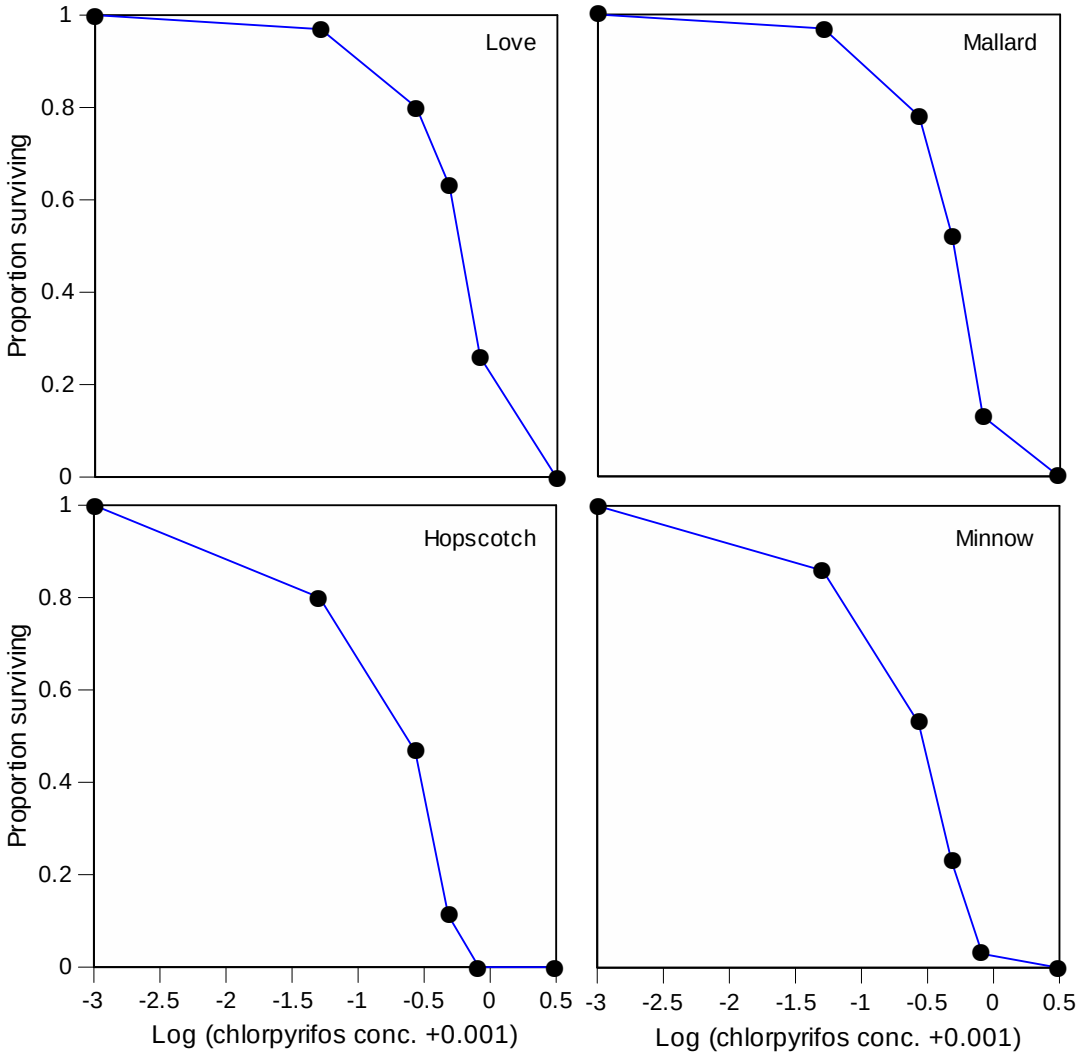

Figure B.2. Survivorship curves for the four populations of *D. pulex* across a span of 48 hours. The upper two populations (Love and Mallard) were collected from ponds near agriculture whereas the bottom two populations (Hopscotch and Minnow) were collected from ponds further from agriculture.

APPENDIX C

CHAPTER THREE: SUPPLMENTAL METHODS

C.1 PESTICIDE APPLICATIONS

We began by creating a stock solution of chlorpyrifos containing 0.2 g of technical grade chlorpyrifos and 100 mL of ethanol. From this stock solution, we added 71, 142 or 284 µL of the stock solution to each mesocosm to achieve the respective nominal concentrations (0.25, 0.50, and 1.0). For control tanks with 0 µg/L chlorpyrifos, we added 284 µL of carbon-filtered, UV-irradiated well water. After the pesticide treatment was applied to a given mesocosm, we stirred and agitated the water in each mesocosm to equalize disturbance and to ensure that the pesticide was spread throughout the water column.

To verify the actual concentrations of chlorpyrifos in our experimental communities, we collected 0.125 L of water from each of the tanks 2 hours after applying the pesticides and pooled the samples by concentration. We sent these samples to an independent laboratory for chemical analysis using high-performance liquid chromatography (Agricultural and Environmental Services Laboratory, University of Georgia, Georgia, USA). The actual concentrations for the 0.25, 0.50 and 1.00 µg/L nominal concentrations were 0.17, 0.76 and 1.04

μg/L, respectively. Our control samples had < 0.1 μg/L, which was the laboratory's detection limit. We reapplied the insecticide concentrations three times on a schedule of every 2.5 weeks. We re-tested our nominal concentrations on two of the three additional applications. When the nominal concentrations were analyzed after the third application, the actual concentrations for the 0.25, 0.50 and 1.0 μg/L treatments were 0.23, 0.46 and 0.80 μg/L, respectively. When the nominal concentrations were analyzed after the fourth application, the actual concentrations for the same three nominal concentrations were 0.36, 0.69 and 2.01 μg/L. We did not retest the control samples as the first sample indicated that we had no detectable amounts of chlorpyrifos within our control tanks.

C.2 ABIOTIC RESPONSE VARIABLES

During the course of the experiment, we measured several abiotic response variables to help us understand the effects of chlorpyrifos on the communities. At four times during the experiments, we measured pH, temperature, and dissolved oxygen (DO; Figure F.1). We chose to measure the abiotic variables on these days because they immediately preceded our four pesticide applications. Temperature, pH and DO content readings were taken with a calibrated digital water meter (YSI, Yellow Springs, OH, USA) whereas light attenuation was measured with an underwater light meter (LI-COR. Lincoln, Nebraska, USA). On days 33, 41, 62, and 82 we took light measurements, primarily because these days followed pesticide applications and were all clear, cloudless days, which are ideal for taking light measurements. We measured light radiation from the middle of each mesocosms at depths of 10 and 30 cm and calculated the decay rate of light with increased water depth (k) using the equation:

$$k = [\ln(L_{10}/L_{30})]/d$$

where L_{10} is the intensity of sunlight from a depth of 10 cm, L_{30} is the intensity of sunlight from a depth of 30 cm, and d is the difference in depth between the two measurements of intensity (Relyea and Diecks 2008).

C.3 BIOTIC RESPONSE VARIABLES

We sampled the *Daphnia* by submerging a 0.2-L plastic sampling tube in the middle of the water column at five different locations within each mesocosm (north, south, east and west quadrants as well as the center). All five samples within each mesocosm were pooled and the sample was filtered through a 60-µm Nitex cloth screen and into a Whirlpak bag containing 30% ethanol to preserve the samples for subsequent enumeration. For zooplankton enumeration, we poured the ethanol from the Whirlpaks containing our zooplankton samples onto a Petri dish with a preset grid. We counted all *D. pulex* individuals in each grid and summed the total to get a count for each sample. We also identified and enumerated any zooplankton that were not *D. pulex*.

Phytoplankton was sampled just prior to each pesticide application. To measure phytoplankton, we sampled 0.5-L of water from the center of each tank. The water samples were poured through a vacuum-filtration system and through GF/C Whatman glass microfiber filters (Whatman Industries Inc., Florham Park, New Jersey, USA). After each sample had been vacuum-filtered, each sample was wrapped in aluminum foil and stored in a freezer at -18 °C. These samples were analyzed later using the protocol developed by Arar and Collins (1997). To assess phytoplankton abundance, we used the concentration of chlorophyll *a* as our proxy, which was quantified using a fluorometer (Model ED-700, Turner Designs, Sunnyvale, California).

Periphyton was sampled within one or two days from our phytoplankton samples by removing one of the clay tiles (Figure F.1) because the abundance of phytoplankton can affcet how much periphyton is within each tank. Once a tile was removed, it was vigorously scrubbed with a toothbrush to remove all of the periphyton on the front face of the tile and subsequently rinsed with carbon-filtered, UV-irradiated well water. The slurry containing water and periphyton was then vacuum-filtered onto a Whatman GF/C filter that had been previously dried for 24 hours at 80°C and weighed. After the periphyton sample was vacuum filtered, the filters were again dried at 80°C for an additional 24 hours and weighed. The amount of periphyton biomass was measured as the mass of the filter paper containing the dried periphyton subtracted by the original mass of the dry, unused filter.

C.4 STATISTICAL ANALYSIS

Because we observed similar responses between communities the two sensitive populations of *D. pulex* and similar responses between communities with the two resistant populations of *D. pulex*, initially conducted nested analyses of variance (ANOVAs) that included the four populations and the four pesticide treatments. Using the generalized linear model (GLM) for each variable to determine if the two populations within each sensitivity category ever differed, we nested the two sensitive populations within the category "sensitive" and the two resistant populations within the category "resistant." The sensitive and resistant populations were defined *a priori* by Bendis and Relyea (2014). Because these nested analyses never found significant differences between the two sensitive populations or between the two resistant populations for any of our response variables (all p-values > 0.05), we pooled the two resistant populations and we pooled the two

sensitive populations for all subsequent analyses.

We used multivariate analysis of variance (MANOVA) to test for effects of the chlorpyrifos concentration and sensitivity of the *D. pulex* populations (resistant or sensitive) on the three abiotic response variables (pH, DO, and temperature) at each of the four time points. When we found significant multivariate effects, we performed univariate repeated-measures ANOVAs (rm-ANOVA) to determine how each abiotic variable was affected by the treatments. Light attenuation was measured at three time points, which were different from the days when other abiotic variables were measured due to inclement weather conditions. Light attenuation was analyzed using rm-ANOVA. We log-transformed any response variables that did not meet the aforementioned assumption.

We used a MANOVA to test for effects of chlorpyrifos concentration and *Daphnia* population sensitivity on the final measurements of six biotic response variables: periphyton, phytoplankton and *D. pulex* abundance, and the three leopard frog life-history variables (i.e. time to metamorphosis, mass at metamorphosis and survival to metamorphosis). To gain an understanding of which response variables were responsible for any significant multivariate effects, we conducted ANOVAs on variables that were measured only at the end of the experiment (leopard frog survivorship, mass at metamorphosis and size at metamorphosis) and rm-ANOVAs on other individual variables that were measured at several time points throughout the experiment (zooplankton, phytoplankton, and periphyton abundance.) All statistics were calculated using SPSS statistical software (IBM, version 22).

APPENDIX D

CHAPTER THREE: SUPPLMENTAL RESULTS

D.1 ABIOTIC VARIABLES

The MANOVA from the final sample on water temperature, pH and DO indicated that there was a significant effect of insecticide concentration (Wilks' λ, $F_{9,73} = 5.575$, $p < 0.001$) but no effect of sensitivity of the *D. pulex* population (Wilks' λ, $F_{9,73} = 0.658$, $p = 0.774$) or any significant interaction (Wilks' λ, $F_{27,88} = 1.286$, $p = 0.190$). Subsequent univariate tests revealed effects on pH ($F_{3,32} = 19.042$, $p < 0.001$) and DO ($F_{3,32} = 15.352$, $p < 0.001$), but not on water temperature ($F_{3,32} = 1.453$, $p = 0.246$). We then conducted individual rm-ANOVAs on pH, DO and temperature.

D.1.1 pH and DO content

The rm-ANOVA of pH revealed that there were significant effects of time, insecticide

concentration, and a time-by-concentration interaction. (Table E.1). The general pattern was that there was an increase in pH as chlorpyrifos concentration increased (Figure F.2). The rm-ANOVA of DO also indicated that there were significant effects of time, insecticide concentration and a time-by-concentration interaction (Table E.1; Figure F.3).

D.1.2. Temperature

The rm-ANOVA of temperature revealed significant effects of time, insecticide concentration, and a time-by-concentration interaction. Unlike pH and DO, there were also significant time-by-*Daphnia* sensitivity and time-by-*Daphnia* sensitivity-by-insecticide interactions (Table E.1). The general trend was that there was an increase in temperature as chlorpyrifos concentration increased (Figure F.4). The 1.0 μg/L treatment showed the greatest amount of variation and indicated that communities with resistant *D. pulex* had significantly higher water temperatures when compared to communities with sensitive D. *pulex* ($p < 0.001$). As the experiment progressed, specifically during the third and fourth samples (days 61 and 82), there was little to no effect of any of the variables on water temperature (Table E.2C)

D.1.3 Light attenuation

The rm-ANOVA of the rate of light decay indicated that there were significant effects of time, insecticide concentration, and a time-by-concentration interaction (Table E.3, Figure F.5).

APPENDIX E

CHAPTER THREE: SUPPLMENTAL TABLES

Table E.1. Results of repeated-measures ANOVAs to determine the effects of experimental manipulations on the three abiotic variables (pH, DO, and temperature) that were measured simultaneously at four time points throughout the experiment. Because the analyses of pH and temperature used Greenhouse-Geisser corrections due to lack of sphericity, these two response variables have different adjusted degrees of freedom. *F* values for each factor are followed by *p* values in parentheses (significant *p* values in bold.)

Factor	*pH*	*df*	DO	*df*	Temperature	*df*
Conc. (C)	40.3 (<**0.001**)	3,40	38.5 (<**0.001**)	3,40	4.1 (**0.012**)	3,40
Sensitivity (S)	1.3 (0.264)	1,40	0.1 (0.719)	1,40	3.1 (0.083)	1,40
C x S	1.1 (0.350)	3,40	0.6 (0.636)	3,40	1.4 (0.267)	3,40
Time (T)	109.5 (<**0.001**)	2,86	40.7 (<**0.001**)	3,120	558 (<**0.001**)	3,102
T x C	4.0 (**0.001**)	6,86	4.8 (<**0.001**)	9,120	3.2 (**0.003**)	8,102
T x S	0.1 (0.886)	2,86	0.6 (0.630)	3,120	3.4 (**0.027**)	3,102
T x C x S	1.2 (0.370**)**	6**,**86	0.8 **(**0.606**)**	9,120	2.5 (**0.016)**	8,102

Table E.2. Results of univariate analyses of variance for each sample date for A) pH, B) DO, and C) temperature. Values in the table are *p* values (significant *p* values in bold.)

A) pH	**Day 20**	**Day 40**	**Day 61**	**Day 81**
Concentration (C)	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**

B) DO	**Day 20**	**Day 41**	**Day 62**	**Day 82**
Concentration (C)	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**

C) Temperature	**Day 20**	**Day 41**	**Day 61**	**Day 82**
Concentration (C)	**0.024**	0.143	**< 0.001**	0.302
Sensitivity (S)	**0.010**	0.574	0.053	0.864
C x S	0.124	**0.041**	0.973	0.929

Table E.3. Results of the A) repeated-measures ANOVAs and B) univariate ANOVAs at each sample time to determine the effects of experimental manipulations on light attenuation (measured three times throughout the experiment). Because the analysis of light attenuation utilized Greenhouse-Geisser corrections due to lack of sphericity, this response variable has adjusted degrees of freedom. *F* values for each factor are followed by *p* values in parentheses.

A) rm-ANOVA	**Light decay (K)**	***df***
Concentration (C)	9.1 (<**0.001**)	3,40
Sensitivity (S)	0.6 (0.454)	1,40
C x S	0.4 (0.398)	3,40
Time (T)	755 (<**0.001**)	2,65
T x C	74.2 (<**0.001**)	5,65
T x S	0.1 (0.895)	2,65
T x C x S	1.2 (0.301)	5,65

B) ANOVAs	**Day 41**	**Day 62**	**Day 82**
Concentration (C)	**< 0.001**	**< 0.001**	**< 0.001**

Table E.4. Results of repeated-measures ANOVAs to determine the effects of experimental manipulations on the abundance of *Daphnia*, periphyton, and phytoplankton. Because the analyses for *Daphnia* and periphyton abundance used Greenhouse-Geisser corrections due to lack of sphericity, these two response variables have different adjusted degrees of freedom. *F* values for each factor are followed by *p* values in parentheses (significant *p* values in bold.)

Factor	*Daphnia* abundance	*df*	Phytoplankton abundance	*df*	Periphyton abundance	*df*
Conc. (C)	91.7 (<**0.001**)	3,40	47.0 (<**0.001**)	3,40	24.5 (<**0.001)**	3,40
Sensitivity (S)	40.3 (<**0.001**)	1,40	8.5 (**0.006**)	1,40	8.1 (**0.007**)	1,40
C x S	9.9 (<**0.001)**	3,40	2.4 (0.078)	3,40	0.3 (0.859)	3,40
Time (T)	7.0 (<**0.001**)	4,160	8.2 (<**0.001**)	3,120	115.5 (<**0.001**)	2,86
T x C	1.9 (**0.044**)	12,160	6.3 (<**0.001**)	9,120	1.8 (0.104)	6,86
T x S	2.3 (0.058)	4,160	1.2 (0.306)	3,120	2.4 (0.098)	2,86
T x C x S	1.9 **(0.039)**	12,160	1.7 (0.096)	9,120	1.0 (0.429)	6,86

Table E.5. Results of univariate analyses of variance for each sample date for A) *Daphnia* abundance and B) phytoplankton abundance. Values in table are *p* values (significant *p* values in bold.) Univariate analyses of periphyton were not preformed since neither of the main effects (concentration or sensitivity) interacted with time.

A) *Daphnia*	**Day 14**	**Day 21**	**Day 28**	**Day 42**	**Day 50**	**Day 62**	**Day 83**
Concentration (C)	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
Sensitivity (S)	**0.003**	**0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**0.001**
C x S	0.328	**0.035**	**< 0.001**	**< 0.001**	**0.001**	**< 0.001**	**0.003**

B) Phytoplankton	**Day 20**	**Day 40**	**Day 61**	**Day 81**
Concentration (C)	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
Sensitivity (S)	**0.019**	**0.041**	0.104	**0.004**

Table E.6. Results of a A) multivariate analysis of variance (using Pillai's Trace) and B) subsequent univariate analyses of the effects of insecticide concentration and *Daphnia* sensitivity on overall survivorship, mass at metamorphosis, and time to metamorphosis of leopard frog tadpoles (significant *p* values in bold.)

A) MANOVA	***df***	***F* value**	***p* value**
Concentration (C)	9,117	0.7	0.683
Sensitivity (S)	3,37	2.3	**< 0.001**
C x S	9,117	3.0	**0.039**

B) ANOVAs	**Overall survivorship**	**Mass at metamorphosis**	**Time to metamorphosis**
Sensitivity (S)	**0.007**	**<0.001**	0.101
C x S	0.385	0.143	**< 0.001**

APPENDIX F

CHAPTER THREE: SUPPLMENTAL FIGURES

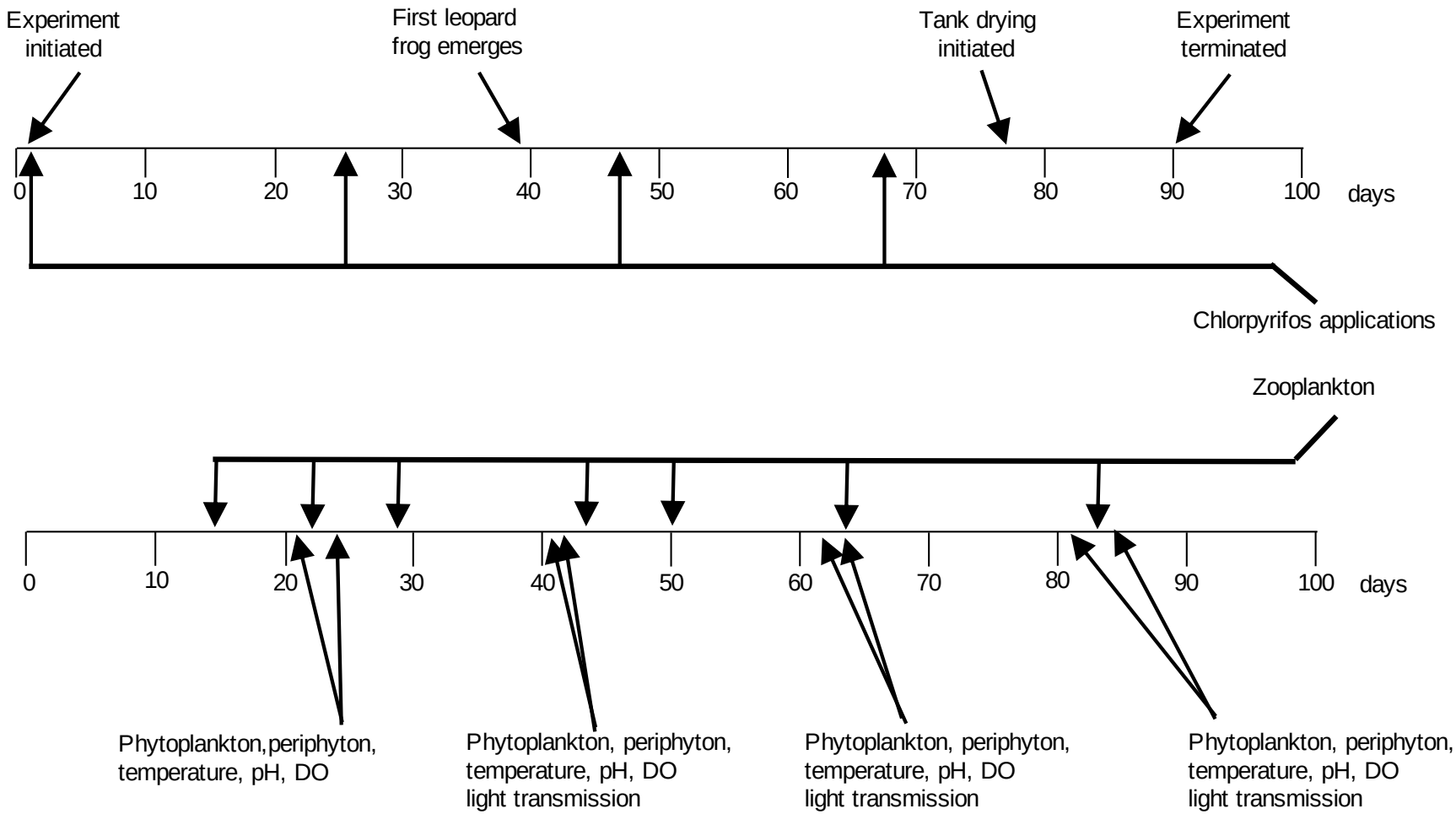

Figure F.1. Experimental timelines to indicate when chlorpyrifos was added and when variables were measured. "DO" stands for dissolved oxygen and "light transmission" indicates when light transparency was measured for light decay rate calculations.

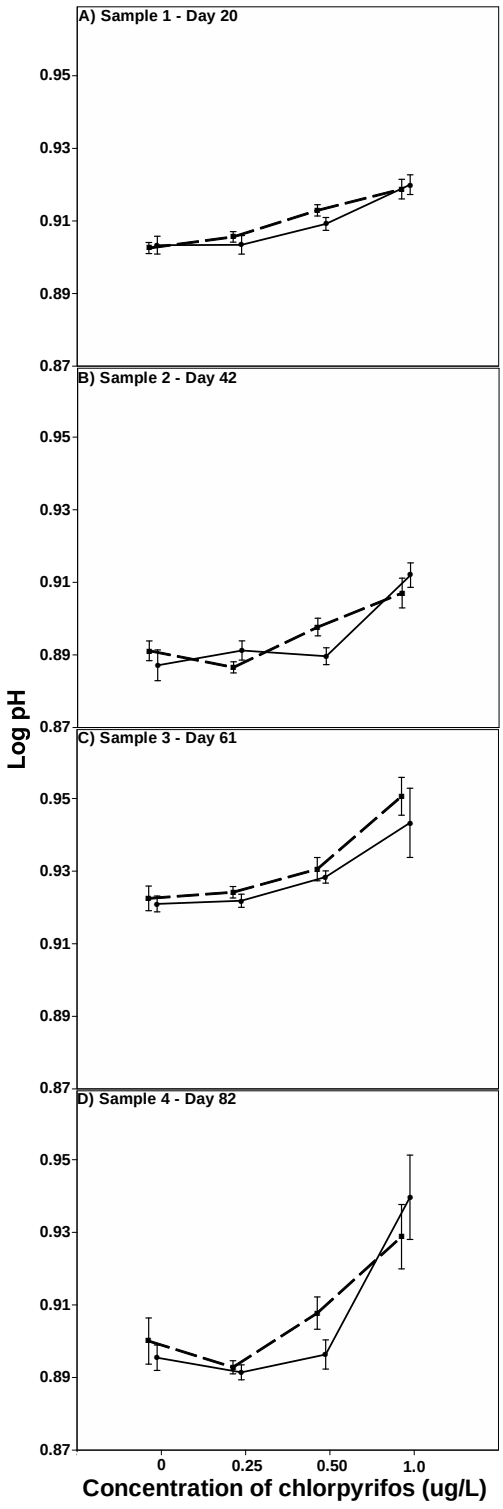

Figure F.2. Differences in pH within experimental communities across the four sampling dates of abiotic variables. The solid line indicates communities with resistant *D. pulex* whereas the dashed line indicates communities with sensitive *D. pulex*.

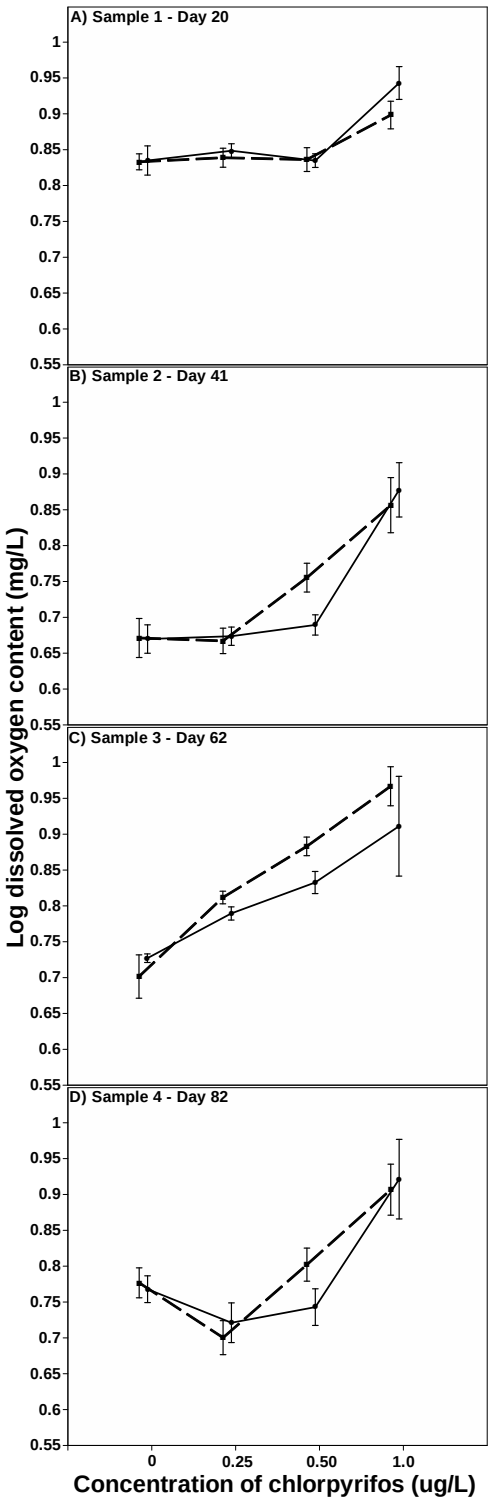

Figure F.3. Differences in DO within experimental communities across the four sampling dates of abiotic variables. The solid line indicates communities with resistant *D. pulex* whereas the dashed line indicates communities with sensitive *D. pulex*.

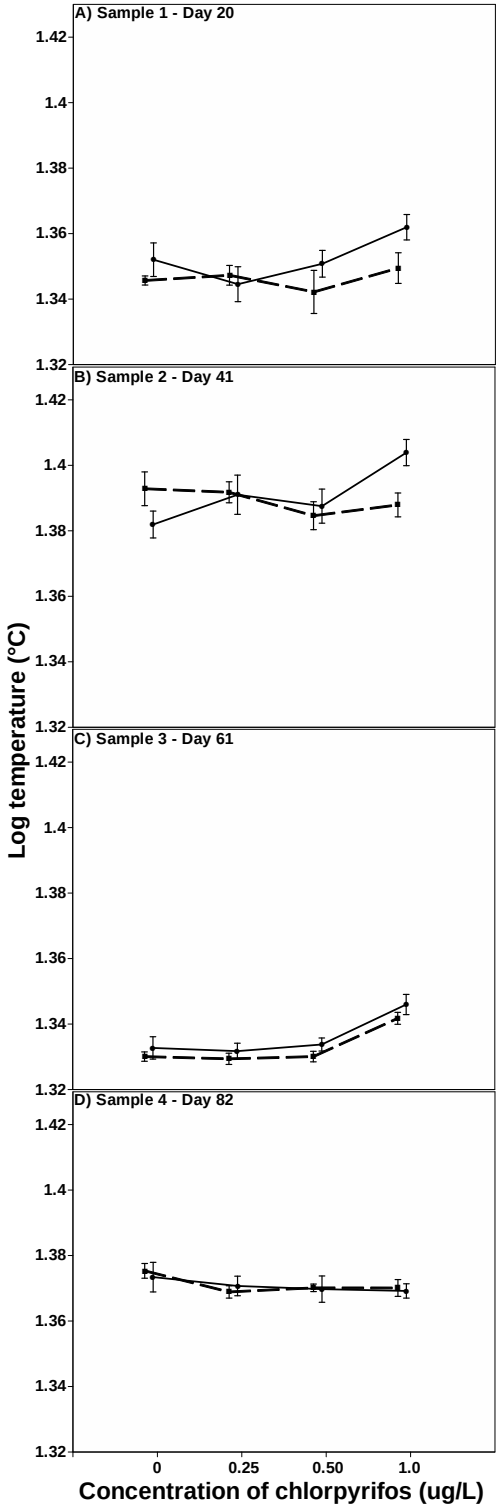

Figure F.4. Differences in temperature within experimental communities across the four sampling dates of abiotic variables. The solid line indicates communities with resistant *D. pulex* whereas the dashed line indicates communities with sensitive *D. pulex.*

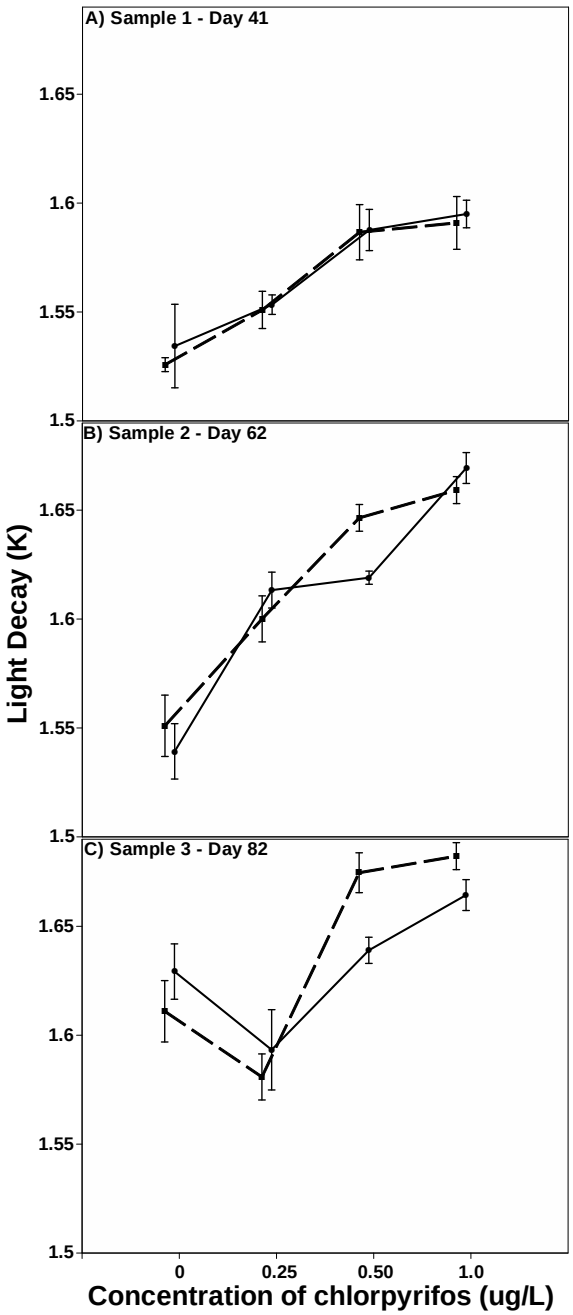

Figure F.5. Differences in light rate decay (K) within experimental communities across the three sampling dates for light transmission. The solid line indicates communities with resistant *D. pulex* whereas the dashed line indicates communities with sensitive *D. pulex*.

APPENDIX G

CHAPTER FOUR: SUPPLMENTAL METHODS

G.1 PESTICIDE PREPARATION AND TESTING

G.1.1 Preparation of stock solutions

For chlorpyrifos, we dissolved 0.05 g of technical-grade chlorpyrifos in 20 mL of ethanol (EtOH). From this stock solution, we added 6.5, 13 or 26 µL of the stock solution to each mesocosm to achieve the respective nominal concentrations (0.25, 0.50, and 1.0 µg/L). For malathion, permethrin and cypermethrin, we dissolved 0.05 g of the respective technical-grade chemicals in 20 mL of EtOH to create separate stock solutions. From these stock solutions, we added 13, 26 or 52 µL of stock solution to each mesocosm to achieve the respective nominal concentrations (0.50, 1.0 and 2.0 µg/L). For carbaryl, we dissolved 0.1 g of technical-grade carbaryl in 10 mL of EtOH. From this stock solution, we added 82, 164, or 328 µL of stock solution to each mesocosm to achieve nominal concentrations of 12.5, 25 and 50 µg/L.

G.1.2 Determination of the actual concentrations of each insecticide

For application #1: For chlorpyrifos, the actual concentrations for the 0.25, 0.50 and 1.0 μg/L nominal concentrations were 0.3, 0.5 and 1.0 μg/L, respectively. For malathion, the actual concentrations for the 0.5, 1.0 and 2.0 μg/L nominal concentrations were 0.7, 1.0 and 2.3 μg/L, respectively. For carbaryl, the actual concentrations for the 12.5, 25 and 50 μg/L nominal concentrations were 14.6, 25, and 51.9 μg/L. For cypermethrin, our 0.50 μg/L treatment fell below the laboratory's detection limits for cypermethrin (0.84 μg/L), so it was not tested. The actual concentrations of the 1.0 and 2.0 μg/L nominal concentrations were 0.2 and 0.7 μg/L. For permethrin, our lowest two concentrations (i.e. 0.5 and 1.0 μg/L) fell below the laboratory's detection limits for permethin (1.02 μg/L) and were not tested. The actual concentration of the 2.0 μg/L nominal concentration was 0.1 μg/L.

For application #2: For chlorpyrifos, the actual concentrations for the 0.25, 0.50 and 1.0 μg/L nominal concentrations were 0.4, 0.8 and 1.1 μg/L, respectively. For malathion, the actual concentrations for the 0.5, 1.0 and 2.0 μg/L nominal concentrations were 0.4, 0.6 and 1.1 μg/L, respectively. For carbaryl, the actual concentrations for the 12.5, 25 and 50 μg/L nominal concentrations were 11.9, 35.8, and 61.7 μg/L. For cypermethrin, our 0.50 μg/L treatment again fell below the detection limits of the equipment of the laboratory and was not tested. The actual concentrations of the 1.0 and 2.0 μg/L nominal concentrations were 0.4 and 0.7 μg/L, respectively. For permethrin, our lowest two concentrations, again, fell below the minimum detection levels of the equipment and were not tested. The actual concentration of the 2.0 μg/L nominal concentration was 0.9 μg/L.

APPENDIX H

CHAPTER FOUR: SUPPLMENTAL RESULTS

H.1 ABIOTIC DATA

The MANOVA from the final sample on the four abiotic variables (water temperature, pH, DO, and light transmission) and the three biotic variables (zooplankton, phytoplankton and periphyton abundance) indicated that there were significant effects of insecticide treatment (Wilks' λ, $F_{112,1044} = 9.946$, $p < 0.001$), sensitivity of the *D. pulex* population (Wilks' λ, $F_{7,160} = 19.191$, $p < 0.001$) and an interaction (Wilks' λ, $F_{112,1044} = 2.284$, $p < 0.001$). We then conducted individual rm-ANOVAs on all of the aforementioned variables.

H.1.1 Temperature

The rm-ANOVA of temperature revealed significant effects of time, insecticide treatment, and a time-by-insecticide treatment interaction. Unlike pH and DO, there were no significant time-by-*Daphnia* sensitivity and time-by-*Daphnia* sensitivity-by-insecticide interactions (Table I.2). The

general trend was that there were increases in temperature as insecticide concentration increased.

H.1.2 pH

The rm-ANOVA of pH revealed that indicated that there were significant effects of all of the main effects (sensitivity, insecticide treatment, and time) as well as all possible interactions. (Table I.2). The general pattern was that there was an increase in pH as chlorpyrifos concentration increased (Figure J.2). The driving force of these significant effects were the differences in DO content during the third and final sample (day 48; Table I.3B). Communities with resistant *D. pulex* that were exposed to the two highest concentrations of the three AChE-inhibiting insecticides (chlorpyrifos, carbaryl and malathion), all had significantly lower (all $p < 0.002$) DO when compared to communities with sensitive *D. pulex*.

H.1.3 DO Content

The rm-ANOVA of DO also indicated that there were significant effects of time, sensitivity, and insecticide treatment and significant interactions with any factor that interacted with time. The only non-significant interaction was the interaction between insecticide treatment and *D. pulex* sensitivity (Table I.2). On day 4, there was a significant difference in pH between communities with resistant and sensitive *D. pulex* that were exposed to the first and second highest concentrations of chlorpyrifos, as well as the second highest concentration of carbaryl (all $p < 0.024$). Communities containing resistant *D. pulex* had lower pH than communities with sensitive *D. pulex* (Table I.3C). On day 48, communities with resistant *D. pulex* all had

significantly lower pH, when compared to communities with sensitive *D. pulex*, but only when exposed to the three AChE-inhibiting insecticides (all $p < 0.045$; Figure J.3).

H.1.4 Light attenuation

The rm-ANOVA of the rate of light decay indicated all main effect variables as well as all interaction terms were significant (Table I.2). Generally, the pattern was that the rate of light decay increased over time. More specifically, the effects on light attenuation were mainly driven by the differences on the final sampling date of light transmission (day 50; Table I.3D). Communities with resistant *D. pulex* that were exposed to the second highest concentration of carbaryl or the two highest concentrations of either chlorpyrifos or malathion had significantly lower rates of light decay, when compared to communities with sensitive *D. pulex* at the same concentrations (all $p < 0.019$; Figure J.4).

APPENDIX I

CHAPTER FOUR: SUPPLMENTAL TABLES

Table I.1. Calculated LC50 values and associated 84% confidence intervals for the resistant (Love pond) and sensitive (Minnow pond) populations of *D. pulex* based on a 24 hour laboratory study. These values were used to infer the median concentrations of each of the five insecticides to use in our large outdoor mesocosm experiment. Data for chlorpyrifos taken from Bendis and Relyea 2014. All values are in μg/L.

Insecticide	**Resistant *D. pulex* LC50**	**84% CI lower bound**	**84% CI upper bound**	**Sensitive *D. pulex* LC50**	**84% CI lower bound**	**84% CI Upper bound**
Chlorpyrifos	**0.59**	0.53	0.65	**0.33**	0.28	0.35
Malathion	**3.35**	1.27	5.35	**0.70**	0.025	1.12
Carbaryl	**50.51**	41.84	56.97	**32.42**	28.98	34.45
Permethrin	**1.47**	0.74	1.87	**2.05**	1.09	2.86
Cypermethrin	**2.16**	1.24	2.57	**1.56**	0.78	1.95

Table I.2. Results of repeated-measures ANOVAs to determine the effects of experimental manipulations on the four abiotic variables (pH, DO, temperature and light transmission) that were measured simultaneously at three time points throughout the experiment. Because the analyses of DO, temperature and light transmission used Greenhouse-Geisser corrections due to lack of sphericity, these two response variables have different adjusted degrees of freedom. *F* values for each factor are followed by *p* values in parentheses (significant *p* values in bold.)

Factor	**Temp.**	***df***	**pH**	***df***	**DO**	***df***	**Light decay**	***df***
Insecticide (I)	7.8 (<**0.001**)	16,166	12.8 (<**0.001**)	16,166	16.9 (<**0.001**)	16,166	22.7 (<**0.001**)	16,166
Sensitivity (S)	0.5 (0.477)	1,166	43.9 (<**0.001**)	1,166	38.9 (<**0.001**)	1,166	8.7 (**0.004**)	1,166
I x S	0.2 (0.999)	16,166	2.5 (**0.002**)	16,166	1.4 (0.169)	16,166	3.1 (<**0.001**)	16,166
Time (T)	2587 (<**0.001**)	1.5,220	458.9 (<**0.001**)	2,332	354.3 (<**0.001**)	2,312	285.9 (<**0.001**)	2,300
T x I	20.4 (<**0.001**)	21,220	7.6 (<**0.001**)	32,332	11.2 (<**0.001**)	30,312	29.9 (<**0.001**)	29,300
T x S	0.1 (0.799)	1.5,220	8.9 (<**0.001**)	2,332	26.1 (<**0.001**)	2,312	9.1 (<**0.001**)	2,300
T x I x S	0.7 (0.855)	21,220	2.6 (<**0.001)**	32**,**332	1.6 **(0.024)**	30,312	1.7 (**0.015)**	29,300

Table I.3. Results of univariate analyses of variance for each sample date for the abiotic variables sampled during the course of the experiment: A) temperature, B) pH, and C) DO content and D) light decay. Values in the table are *p* values (significant *p* values in bold.)

A) Temperature	Day 4	Day 26	Day 48
Insecticide (I)	**< 0.001**	0.597	**< 0.001**

B) pH	Day 4	Day 26	Day 48
Insecticide (I)	**< 0.001**	**< 0.001**	**< 0.001**
Sensitivity (S)	**< 0.001**	0.115	**< 0.001**
I x S	0.515	0.154	**< 0.001**

C) DO content	Day 4	Day 26	Day 48
Insecticide (I)	**< 0.001**	**< 0.001**	**< 0.001**
Sensitivity (S)	**< 0.001**	0.520	**< 0.001**
I x S	0.131	0.656	**0.001**

D) Light decay	Day 8	Day 30	Day 50
Insecticide (I)	**< 0.001**	**< 0.001**	**< 0.001**
Sensitivity (S)	**0.011**	0.269	**< 0.001**
I x S	**0.047**	0.228	**< 0.001**

Table I.4. Results of repeated-measures ANOVAs to determine the effects of experimental manipulations on three biotic variables that were measured over time (zooplankton, phytoplankton, and periphyton abundance) that were measured at two time points during the experiment. *F* values for each factor are followed by *p* values in parentheses (significant *p* values in bold.)

Factor	Zooplankton	*df*	Phytoplankton	*df*	Periphyton	*df*
Insecticide (I)	79.2 (**<0.001**)	15,160	26.4 (**<0.001**)	15,160	12.5 (**<0.001**)	15,160
Sensitivity (S)	89.6 (**<0.001**)	1,160	22.2 (**<0.001**)	1,160	30.9 (**<0.001**)	1,160
I x S	6.2 (**<0.001**)	16,166	3.7 (**<0.001**)	16,166	2.2 (**0.010**)	16,166
Time (T)	124 (**<0.001**)	3,480	12.3 (**0.001**)	1,160	5.1 (**0.025**)	1,160
T x I	7.1 (**<0.001**)	45,480	6.6 (**<0.001**)	15,160	2.4 (**0.004**)	15,160
T x S	4.9 (**0.002**)	3,480	0.8 (0.402)	1,160	0.5 (0.484)	1,160
T x I x S	1.5 (**0.027**)	45**,**480	1.7 (0.061)	15,160	1.6 **(**0.067)	15,160

Table I.5. Results of univariate analyses of variance for each sample date for A) *D. pulex* abundance B) phytoplankton abundance and C) periphyton abundance. Values in tables are *p* values (significant *p* values in bold.)

A) *Daphnia*	**Day 3**	**Day 24**	**Day 33**	**Day 48**
Insecticide (I)	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
Sensitivity (S)	**< 0.001**	**0.007**	**< 0.001**	**< 0.001**
I x S	**< 0.001**	0.199	**< 0.001**	**0.002**

B) Phytoplankton	**Day 22**	**Day 51**
Insecticide (I)	**< 0.001**	**< 0.001**
Sensitivity (S)	**0.009**	**< 0.001**
I x S	**0.042**	**< 0.001**

C) Periphyton	**Day 26**	**Day 53**
Insecticide (I)	**< 0.001**	**< 0.001**
Sensitivity (S)	**< 0.001**	**< 0.001**
I x S	0.145	**0.010**

Table I.6. Results of a multivariate analysis of variance (using Pillai's Trace) of the effects of insecticide treatment and *Daphnia* sensitivity on survivorship, tadpole mass, and developmental (Gosner) stage (significant *p* values in bold.)

Factor	***df***	***F* value**	***p* value**
Insecticide (I)	48,483	2.6	**< 0.001**
Sensitivity (S)	3,159	39.4	**< 0.001**
I x S	48,483	1.3	0.102

Table I.7. Results of univariate ANOVAs of the effects of insecticide treatment and *Daphnia* sensitivity on: A) tadpole survivorship, B) tadpole mass, and C) tadpole developmental (Gosner) stage. Values in tables are *p* values (significant *p* values in bold.)

A) Factor	Survivorship
Insecticide (I)	**<0.001**
Sensitivity (S)	0.072
I x S	0.861

B) Factor	Mass at metamorphosis
Insecticide (I)	**<0.001**
Sensitivity (S)	**<0.001**
I x S	**0.037**

C) Factor	Developmental stage
Insecticide (I)	**<0.001**
Sensitivity (S)	**<0.001**
I x S	**0.002**

APPENDIX J

CHAPTER FOUR: SUPPLMENTAL FIGURES

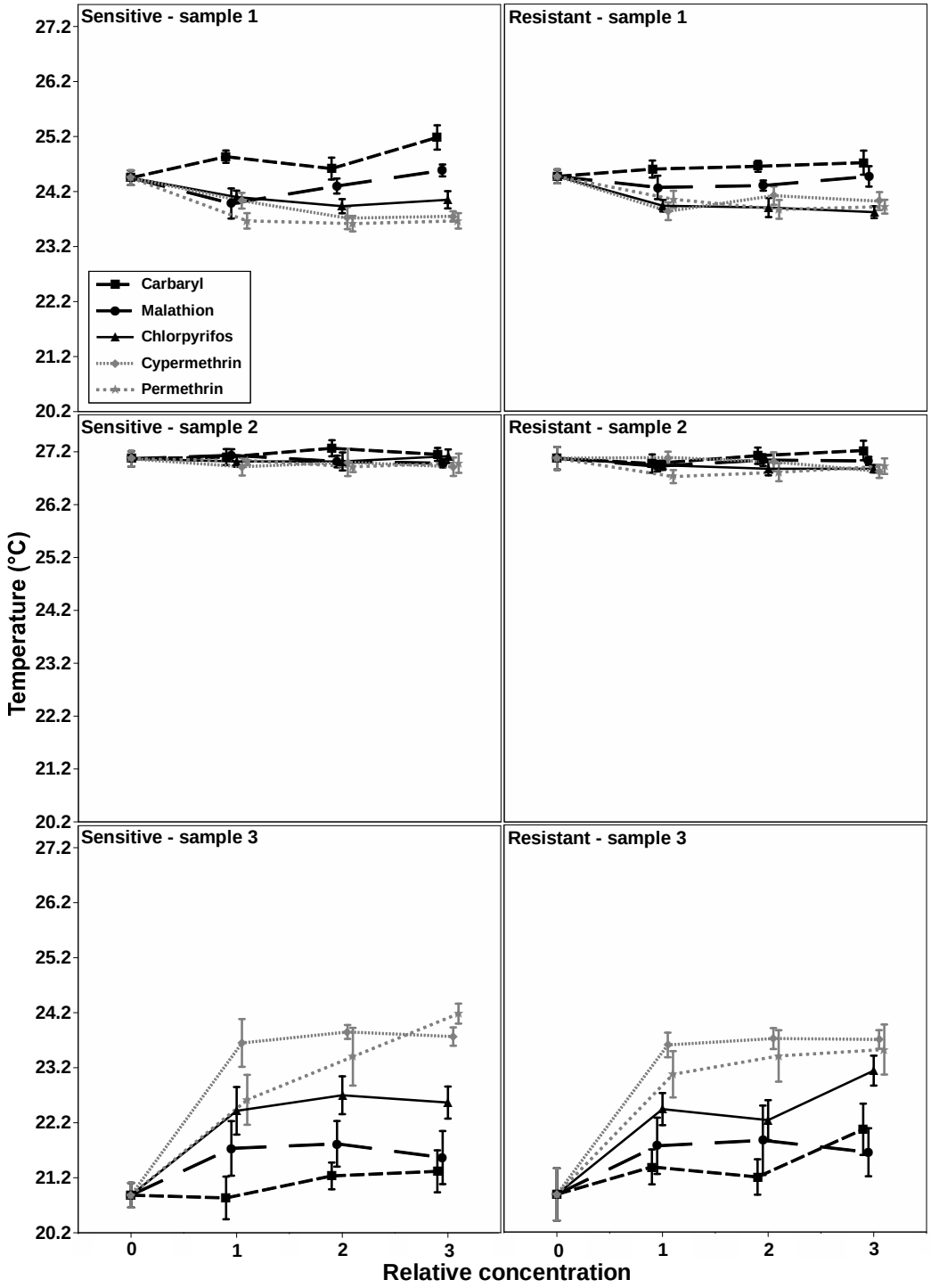

Figure J.1. Differences in temperature (in °C) within experimental communities across the three sampling dates for abiotic variables during the course of the experiment. For all figures, communities containing *D. pulex* populations that are sensitive to chlorpyrifos are on the left, populations that are resistant to chlorpyrifos are on the right.

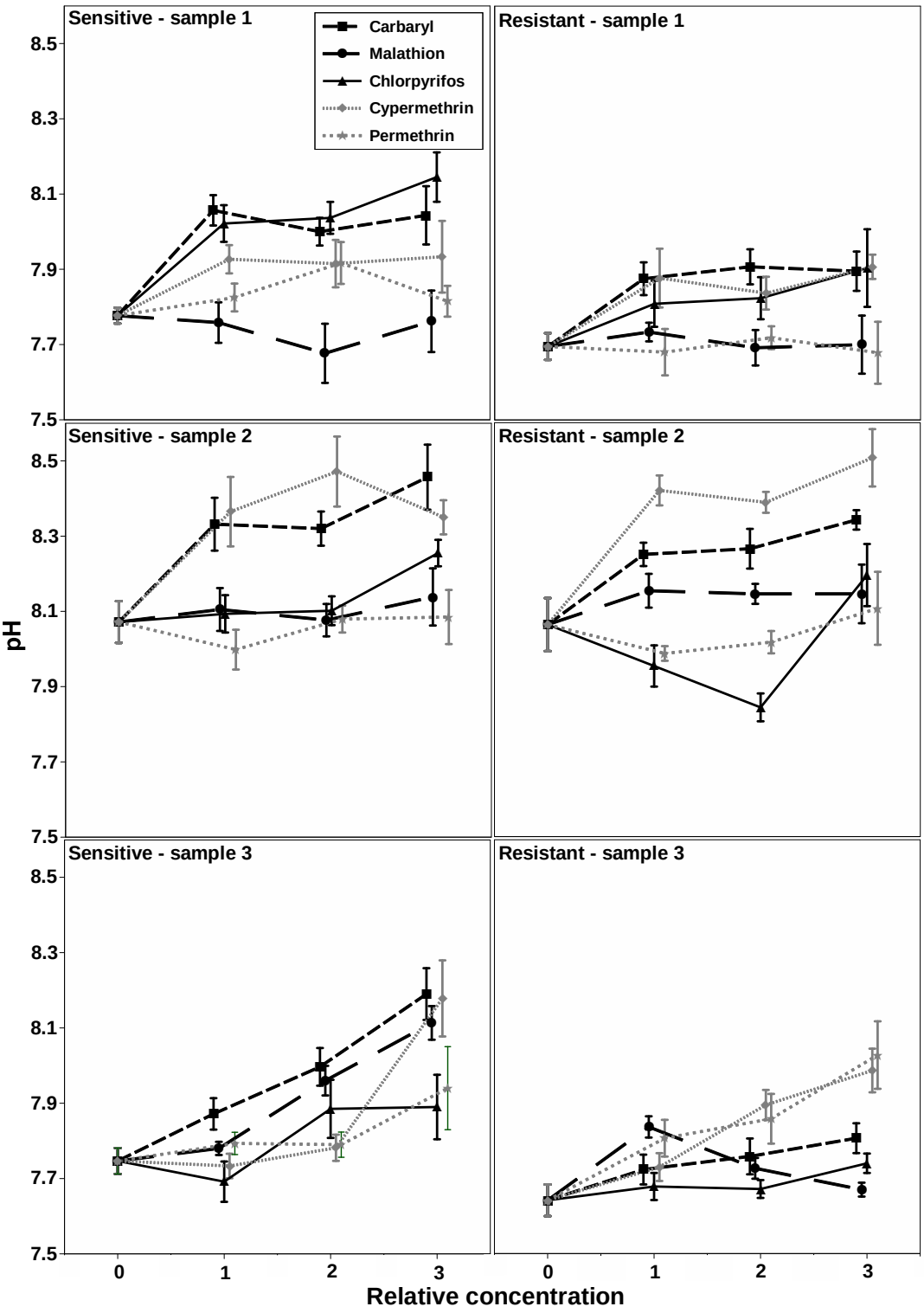

Figure J.2. Differences in pH within experimental communities across the three sampling dates for abiotic variables during the course of the experiment.

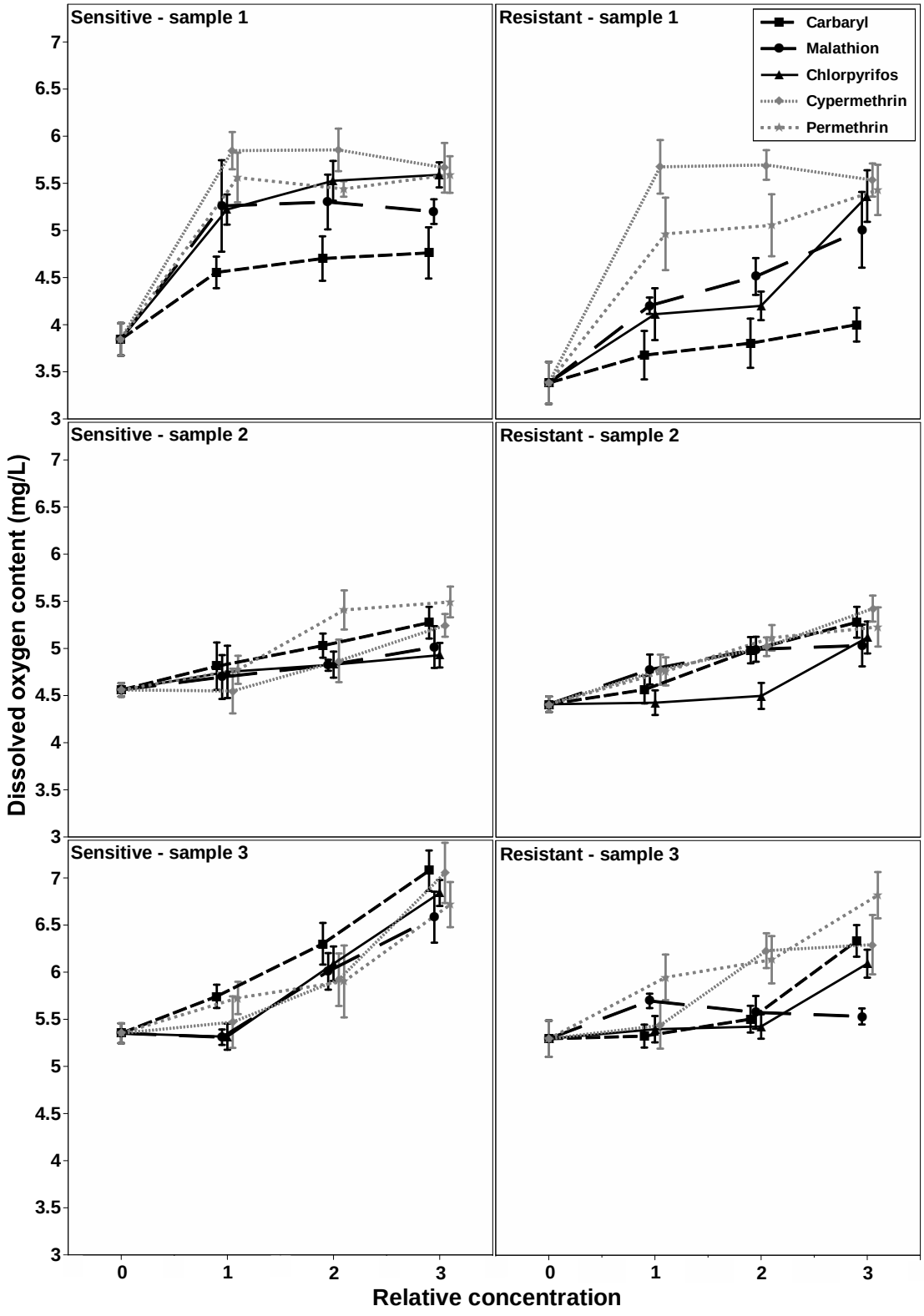

Figure J.3. Differences in DO content (in mg/L) within experimental communities across the three sampling dates for abiotic variables during the course of the experiment.

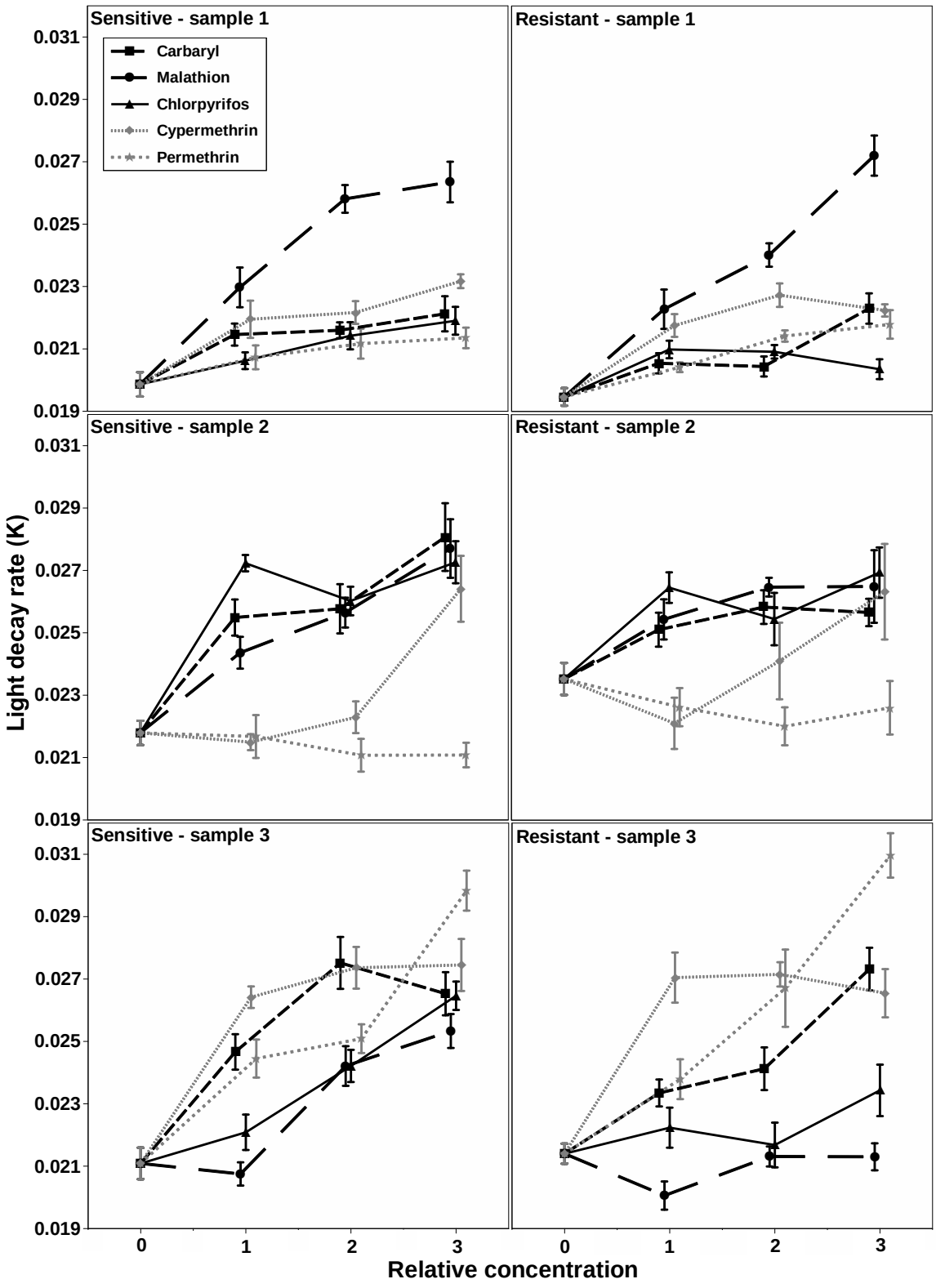

Figure J.4. Differences in light decay rate (K) within experimental communities across the three sampling dates for abiotic variables during the course of the experiment.

APPENDIX K

CHAPTER FIVE: SUPPLMENTAL RESULTS

K.1.1 Temperature

The rm-ANOVA of temperature revealed only significant effects of time and a time-by-chlorpyrifos concentration interaction (both $p < 0.001$; Table L.2). Unlike pH and DO, there were no significant main effects of the zooplankton assemblage type, proximity to agriculture or chlorpyrifos concentration (all $p > 0.084$). There were no discernable patterns in temperature fluctuations throughout the experiment (Figure M.1).

K.1.2 pH

The rm-ANOVA of pH indicated that there were significant effects of three of the four main effects (zooplankton assemblage type, concentration of chlorpyrifos, and time – all $p < 0.001$), but no significant main effect of proximity to agriculture (Table L.2). There were also several significant interaction terms. The general pattern throughout the course of the experiment was that there was an increase in pH as chlorpyrifos concentration increased (Figure M.2). During the first sampling date of the abiotic factors, there was only a significant effect of zooplankton

assemblage type ($p = 0.007$) and no significant interaction terms (Table L.3A). In the second and third samples, however, there were significant effects of both zooplankton assemblage type, as well as chlorpyrifos concentration (all $p < 0.002$), but no significant effect of proximity to agriculture in either case (both $p > 0.069$). In the second sample, there were significant zooplankton assemblage type-by-chlorpyrifos concentration ($p < 0.001$) and proximity to agriculture-by-chlorpyrifos concentration ($p = 0.016$) interactions. During this sampling date, the pH in control communities with a full assemblage of zooplankton collected from near agriculture (cladocerans and the background assemblage) was higher than in communities with a full assemblage collected further from agriculture ($p < 0.001$). In the third sample of pH, there were significant proximity to agriculture-by-zooplankton assemblage ($p = 0.002$) and proximity to agriculture-by-chlorpyrifos concentration ($p = 0.025$) interactions. Communities with a resistant background assemblage had a higher pH when exposed to 0.5 or 1 μg/L chlorpyrifos (both $p < 0.021$), whereas communities with a sensitive full assemblage of zooplankton had higher pH at the same two concentrations (both $p < 0.005$).

K.1.3 DO content

The rm-ANOVA of DO also revealed that there were significant effects of zooplankton assemblage type, chlorpyrifos concentration time (all $p < 0.001$) and a number of significant interaction terms (Table L.2). Like the data for pH, the general trend throughout the course of the experiment was that there was an increase in DO content as chlorpyrifos concentration increased (Figure M.3). In the first sample, there was only a significant effect of zooplankton assemblage ($p < 0.001$; Table L.3B). During the second and third samples, however, there were

significant main effects of zooplankton assemblage and chlorpyrifos concentration (both $p < 0.001$), as well as a significant zooplankton assemblage-by-concentration interactions for both sampling dates and a significant proximity to agriculture-by-concentration interaction for the third and final sampling date (all $p < 0.001$) . In the second sample of DO, there was a sharp increase in DO content in communities with only the background assemblage of zooplankton, when compared to other zooplankton assemblage types, but this difference was not affected by their proximity to agriculture ($p > 0.548$). Furthermore, communities with a full zooplankton assemblage far from agriculture had higher DO content when compared to communities with a full zooplankton assemblage from near agriculture ($p = 0.034$). In the third sample, communities with a full assemblage from near agriculture had higher DO content ($p < 0.001$) when compared to communities with full assemblages far from agriculture, whereas communities with cladoceran-only treatments far from agriculture had higher DO content (both $p < 0.019$).

K.1.4 Light decay

The univariate ANOVA of light attenuation indicated that there were only significant effects of zooplankton assemblage type and chlorpyrifos concentration (both $p < 0.001$; Table L.3C). There were no significant interactions nor was there a significant effect of proximity to agriculture (all $p > 0.148$). Generally, the pattern was that the rate of light decay increased as chlorpyrifos concentration increased (Figure M.4).

APPENDIX L

CHAPTER FIVE: SUPPLMENTAL TABLES

Table L.1. Results of the MANOVA for the treatment effects on the final measurements of three abiotic variables (pH, DO content, and temperature) and five biotic variables (cladoceran, copepod, rotifer, phytoplankton and periphyton abundances) that were measured at the end of the experiment. Multivariate statistics were analyzed using a Wilks' Lambda distribution (all significant *p* values are in bold.)

Factor	***F* value**	***df***	***p* value**
Zooplankton assemblage (A)	8.18	14,228	**<0.001**
Proximity to agriculture (P)	3.48	7,114	**0.002**
Concentration (C)	11.64	28,412	**<0.001**
A x P	3.91	14,228	**<0.001**
P x C	1.76	28,412	**0.011**
A x C	1.93	56,619	**<0.001**
A x P x C	1.44	56,619	**0.023**

Table L.2. Results of repeated-measures ANOVAs to determine the effects of experimental manipulations on the three abiotic variables (pH, DO, and temperature) that were measured simultaneously at three time points throughout the experiment. Because data for temperature violated the assumptions of sphericity, Greenhouse-Geisser corrections and adjusted degrees of freedom were used. *F* values for each factor are followed by *p* values in parentheses.

Factor	**pH**	***df***	**DO**	***df***	**Temp.**	***df***
Zooplankton assemblage (A)	10.6 (**<0.001**)	2,120	36.6 (**<0.001**)	2,120	1.1 (0.325)	2,120
Proximity to agriculture (P)	0.1 (0.755)	1,120	0.1 (0.831)	1,120	0.6 (0.447)	1,120
Conc. (C)	107.7 (**<0.001**)	4,120	24.2 (**<0.001**)	4,120	2.1 (0.084)	4,120
Time (T)	163.8 (**<0.001**)	2,240	194.6 (**<0.001**)	2,240	469.4 (**<0.001**)	2,181
A x P	2.3 (0.103)	2,120	0.8 (0.466)	2,120	0.9 (0.400)	2,120
P x C	3.9 (**0.005**)	4,120	3.1 (**0.019**)	4,120	0.4 (817)	4,120
A x C	1.0 (0.459)	8,120	3.4 (**0.002**)	8,120	1.0 (0.458)	8,120
T x A	2.7 (**0.029**)	4,240	6.3 (**<0.001**)	4,240	0.6 (0.607)	3,181
T x P	4.2 (**0.016**)	2,240	0.2 (0.824)	2,240	1.7 (0.197)	2,181
T x C	112.7 (**<0.001**)	8,240	6.4 (**<0.001**)	8,240	5.9 (**<0.001**)	6,181
A x P x C	1.0 (0.424)	8,120	0.8 (0.602)	8,120	0.6 (0.759)	8,120
T x A x P	4.5 (**0.002**)	4,240	0.4 (0.796)	4,240	0.8 (0.518)	3,181
T x A x C	3.7 (**<0.001**)	16,240	3.0 (**<0.001**)	16,240	1.0 (0.475)	12,181
T x P x C	1.4 (0.179)	8,240	2.4 (**0.017**)	8,240	1.3 (0.254)	6,181
A x P x C x T	0.9 (0.578)	16,240	0.8 (0.710)	16,240	0.6 (0.824)	12,181

Table L.3. Results of univariate analyses of variance for each sample date for the abiotic variables sampled during the course of the experiment: A) pH, B) DO content and C) light decay. Values presented in the tables are *p* values (all significant *p* values in bold.)

A) pH	**Sample 1**	**Sample 2**	**Sample 3**
Zooplankton assemblage (A)	**0.007**	**0.001**	**0.002**
Proximity to agriculture (P)	0.682	0.069	0.130
Concentration (C)	0.893	**<0.001**	**<0.001**
A x P	0.376	0.233	**0.002**
A x C	0.632	**<0.001**	0.130
P x C	0.258	**0.016**	**0.025**
A x P x C	0.954	0.548	0.200

B) DO	**Sample 1**	**Sample 2**	**Sample 3**
Zooplankton assemblage (A)	**<0.001**	**<0.001**	**<0.001**
Proximity to agriculture (P)	0.498	0.900	0.831
Concentration (C)	0.117	**<0.001**	**<0.001**
A x P	0.982	0.324	0.700
A x C	0.993	**0.001**	**<0.001**
P x C	0.392	0.230	**0.001**
A x P x C	0.925	0.824	0.284

Table L.3. (continued)

C) Light decay	Sample 1
Zooplankton assemblage (A)	**<0.001**
Proximity to agriculture (P)	0.148
Concentration (C)	**<0.001**
A x P	0.212
A x C	0.601
P x C	0.228
A x P x C	0.985

Table L.4. Results of rm-ANOVAs to determine the effects of experimental manipulations on the three biotic variables (cladoceran, copepod and rotifer abundances) that were measured simultaneously at six time points during the experiment. Because data for cladoceran and copepod abundances violated the assumptions of sphericity, Greenhouse-Geisser corrections and adjusted degrees of freedom were used. *F* values for each factor are followed by *p* values in parentheses (all significant *p* values are in bold.)

Factor	**Cladoceran abundance**	***df***	**Copepod abundance**	***df***	**Rotifer abundance**	***df***
Zooplankton assemblage (A)	72.4 (**<0.001**)	2,96	12.0 (**<0.001**)	2,120	8.5 (**<0.001**)	2,120
Proximity to agriculture (P)	11.0 (**0.001**)	1,96	175.4 (**<0.001**)	1,120	0.1 (0.748)	1,120
Conc. (C)	46.7 (**<0.001**)	3,96	22.3 (**<0.001**)	4,120	1.3 (0.246)	4,120
Time (T)	128.9 (**<0.001**)	5,440	154.5 (**<0.001**)	5,546	49.3 (**<0.001**)	5,600
A x P	0.6 (0.557)	2,96	15.6 (**<0.001**)	2,120	1.1 (0.340)	2,120
P x C	6.4 (**0.001**)	3,96	1.3 (0.272)	4,120	0.8 (0.542)	4,120
A x C	2.4 (**0.032**)	6,96	1.0 (0.430)	8,120	2.1 (**0.049**)	8,120
T x A	16.8 (**<0.001**)	9,440	7.4 (**<0.001**)	9,546	3.9 (**<0.001**)	10,600
T x P	4.9 (**<0.001**)	5,440	28.4 (**<0.001**)	5,546	2.7 (**0.020**)	5,600
T x C	3.1 (**<0.001**)	14,440	5.4 (**<0.001**)	18,546	2.0 (**0.007**)	20,600
A x P x C	0.8 (0.564)	6,96	0.9 (0.560)	8,120	1.4 (0.220)	8,120
T x A x P	0.8 (0.640)	9,440	1.6 (0.102)	9,546	3.8 (**<0.001**)	10,600
T x A x C	3.4 (**<0.001**)	28,440	1.1 (0.322)	36,546	1.5 (**0.019**)	40,600
T x P x C	0.5 (0.920)	14,440	1.0 (0.451)	18,546	2.0 (**0.008**)	20,600
A x P x C x T	0.7 (0.848)	28,440	1.0 (0.520)	36,546	1.5 (**0.021**)	40,600

Table L.5. Results of univariate analyses of variance for each sample date for the three zooplankton abundance variables sampled during the course of the experiment: A) cladocerans, B) copepods and C) rotifers. Values presented in tables are *p* values (significant values in bold.)

A) Cladocerans	**Sample 1**	**Sample 2**	**Sample 3**	**Sample 4**	**Sample 5**	**Sample 6**
Zoop. assemblage (A)	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.058
Proximity to ag. (P)	0.647	0.767	**0.002**	**0.045**	**0.003**	**<0.001**
Concentration (C)	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
A x P	0.866	0.734	0.482	0.625	0.475	0.198
A x C	**0.001**	0.080	**0.002**	**0.001**	**0.004**	0.346
P x C	0.087	**0.025**	**0.011**	0.256	**0.016**	**0.028**
A x P x C	0.724	0.753	0.339	0.747	0.743	0.444

B) Copepods	**Sample 1**	**Sample 2**	**Sample 3**	**Sample 4**	**Sample 5**	**Sample 6**
Zoop. assemblage (A)	**0.004**	0.156	**<0.001**	**<0.001**	**0.033**	**0.004**
Proximity to ag. (P)	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.001**	**0.003**
Concentration (C)	**0.008**	**0.005**	**<0.001**	**<0.001**	**0.001**	**<0.001**
A x P	**0.005**	0.172	**<0.001**	**0.010**	**0.018**	0.105
A x C	0.695	0.266	**0.039**	0.616	0.702	0.763
P x C	0.952	0.460	0.730	**0.026**	0.274	0.507
A x P x C	0.685	0.497	0.783	0.426	0.797	0.114

Table L.5. (continued)

C) Rotifers	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
Zoop. assemblage (A)	**0.010**	0.456	0.066	**<0.001**	0.187	**0.051**
Proximity to ag. (P)	0.964	0.091	0.334	0.247	0.749	**0.012**
Concentration (C)	0.310	0.308	**0.010**	0.177	0.282	0.134
A x P	**0.021**	0.615	0.986	**0.001**	**0.002**	0.085
A x C	0.221	0.273	0.308	**<0.001**	0.275	0.626
P x C	0.751	0.141	0.010	0.159	0.494	0.274
A x P x C	0.587	0.217	0.169	0.232	**0.009**	0.247

Table L.6. Results of repeated-measures ANOVAs to determine the effects of experimental manipulations on the two biotic variables (phytoplankton and periphyton abundance) that were measured simultaneously at three time points throughout the experiment. *F* values for each factor are followed by *p* values in parentheses (all significant *p* values in bold.)

Factor	**Phytoplankton abundance**	***df***	**Periphyton abundance**	***df***
Zooplankton assemblage (A)	96.3 (**<0.001**)	2,120	13.5 (**<0.001**)	2,120
Proximity to agriculture (P)	9.2 (**0.003**)	1,120	0.1 (0.844)	1,120
Concentration (C)	180.1 (**<0.001**)	4,120	10.7 (**<0.001**)	4,120
Time (T)	8.6 (**<0.001**)	2,240	115.2 (**<0.001**)	2,240
A x P	10.9 (**<0.001**)	2,120	3.8 (**0.025**)	2,120
P x C	7.2 (**<0.001**)	2,120	0.9 (0.484)	4,120
A x C	10.5 (**<0.001**)	8,120	2.6 (**0.013**)	8,120
T x A	4.5 (**0.002**)	4,240	0.7 (0.563)	4,240
T x P	12.3 (**<0.001**)	2,240	2.7 (0.069)	2,240
T x C	27.0 (**<0.001**)	8,240	15.6 (**<0.001**)	8,240
A x P x C	2.9 (**0.005**)	8,120	2.2 (**0.031**)	8,120
T x A x P	2.1 (0.087)	4,240	2.6 (**0.036**)	4,240
T x A x C	1.5 (0.102)	16,240	1.9 (**0.024**)	16,240
T x P x C	1.4 (0.187)	8,240	1.7 (0.096)	8,240
A x P x C x T	1.3 (0.179)	16,240	1.0 (0.415)	16,240

Table L.7. Results of univariate analyses of variance for each sample date for the biotic variables sampled during the course of the experiment: A) phytoplankton and B) periphyton. Values presented in the tables are *p* values (all significant *p* values in bold.)

A) Phytoplankton abundance	**Sample 1**	**Sample 2**	**Sample 3**
Zooplankton assemblage (A)	**<0.001**	**<0.001**	**<0.001**
Proximity to agriculture (P)	0.426	**<0.001**	**0.034**
Concentration (C)	**<0.001**	**<0.001**	**<0.001**
A x P	**<0.001**	0.169	**<0.001**
A x C	**0.002**	**<0.001**	**<0.001**
P x C	0.173	**<0.001**	**0.001**
A x P x C	0.956	**0.031**	**<0.001**

B) Periphyton abundance	**Sample 1**	**Sample 2**	**Sample 3**
Zooplankton assemblage (A)	0.100	**0.003**	**0.001**
Proximity to agriculture (P)	0.914	**0.022**	0.223
Concentration (C)	**<0.001**	**<0.001**	**<0.001**
A x P	0.484	0.304	**0.003**
A x C	**0.001**	0.124	0.484
P x C	0.330	**0.026**	0.473
A x P x C	0.063	0.057	0.605

Table L.8. Results of the MANOVA to determine the effects of experimental manipulations on the green frog tadpole average mass and survivorship. Multivariate statistics were analyzed using a Wilks' Lambda distribution.

Factor	***F* value**	***df***	***p* value**
Zooplankton assemblage (A)	0.74	4,238	0.564
Proximity to agriculture (P)	0.72	2,119	0.490
Concentration (C)	0.67	8,238	0.722
A x P	1.12	4,238	0.346
A x C	0.64	16,238	0.846
P x C	1.04	8,238	0.406
A x P x C	0.66	16,238	0.829

APPENDIX M

CHAPTER FIVE: SUPPLMENTAL FIGURES

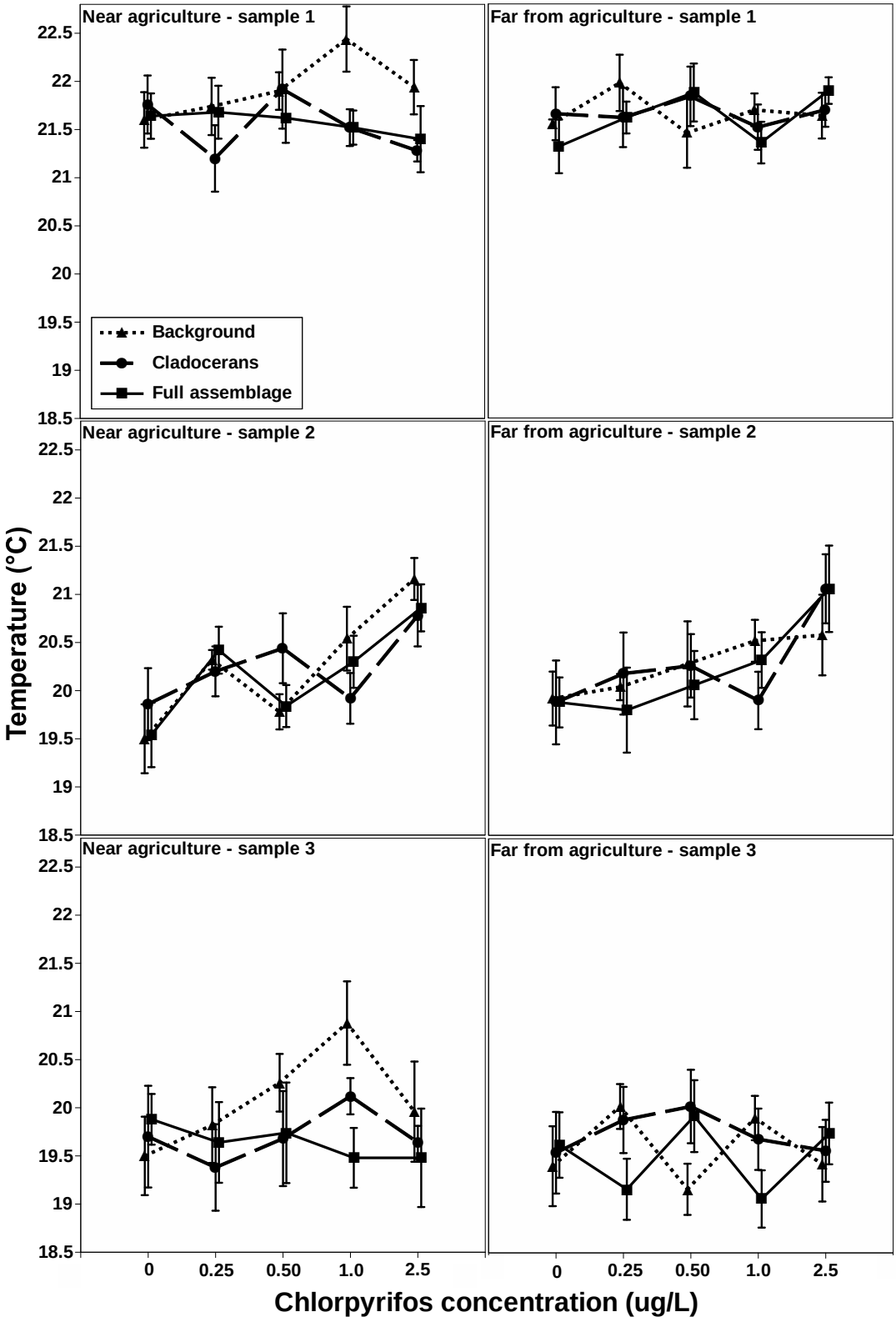

Figure M.1. Differences in temperature (in °C) within experimental communities across the three sampling dates of abiotic variables.

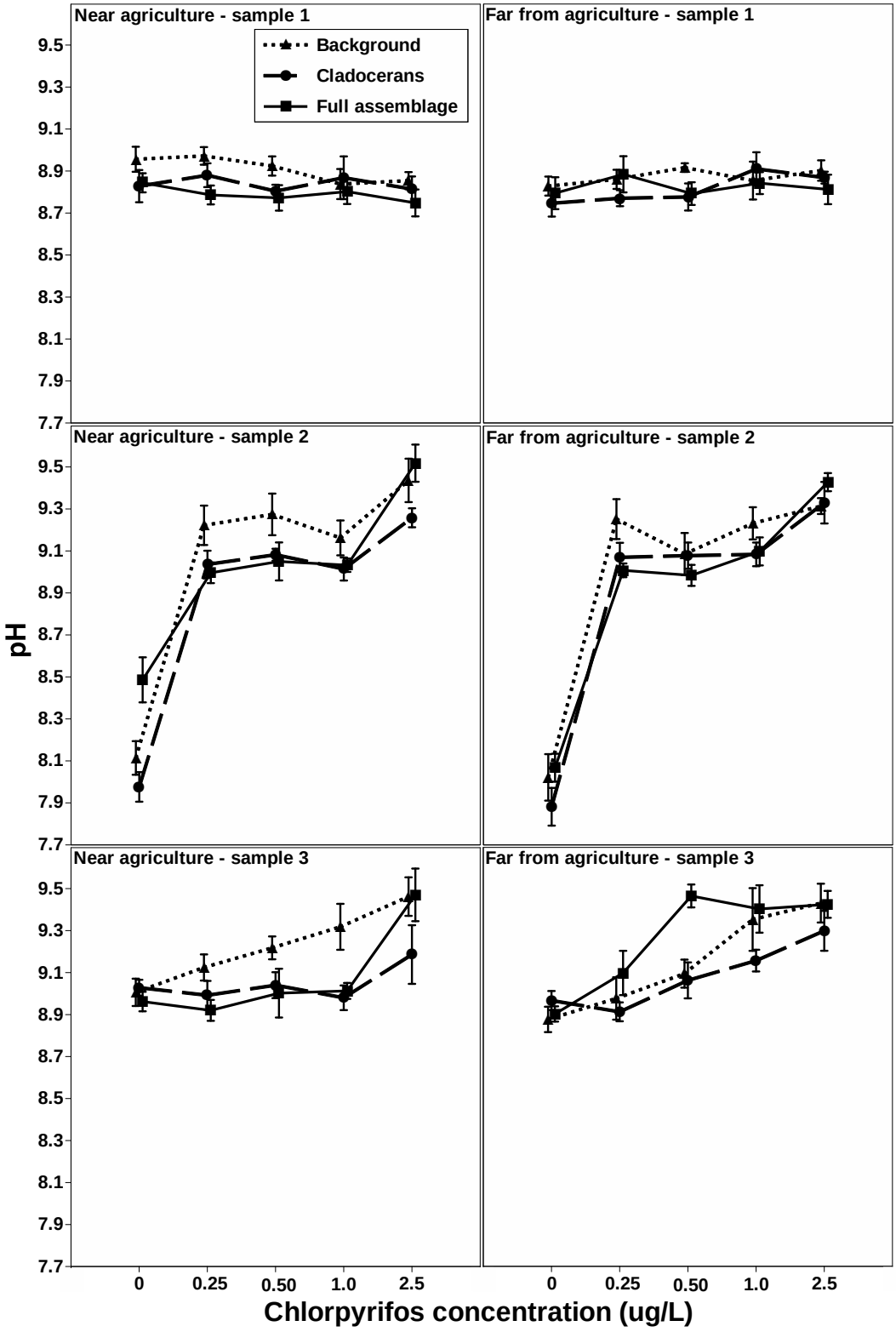

Figure M.2. Differences in pH within experimental communities across the three sampling dates of abiotic variables.

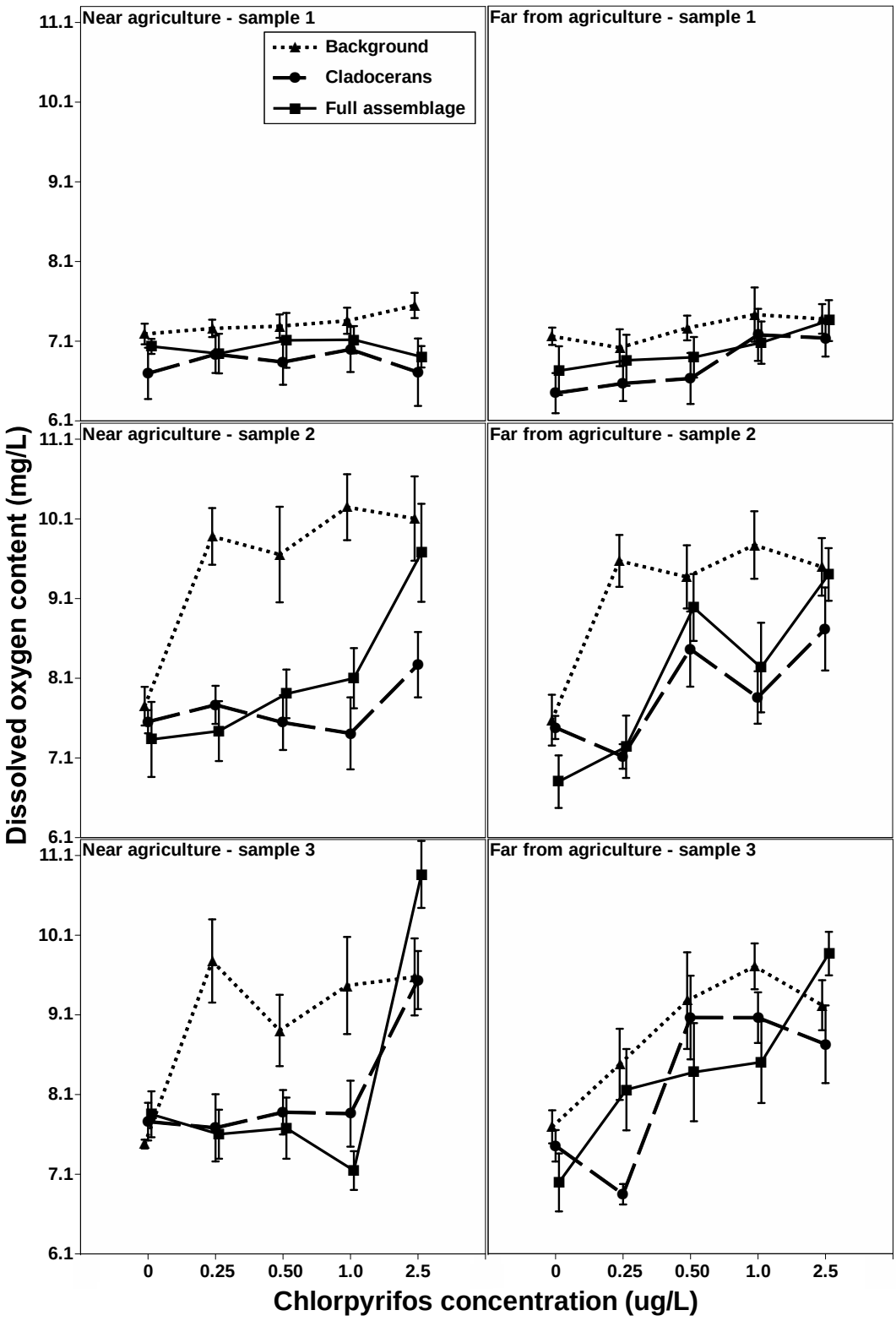

Figure M.3. Differences in dissolved oxygen content (in mg/L) within experimental communities across the three sampling dates of abiotic variables.

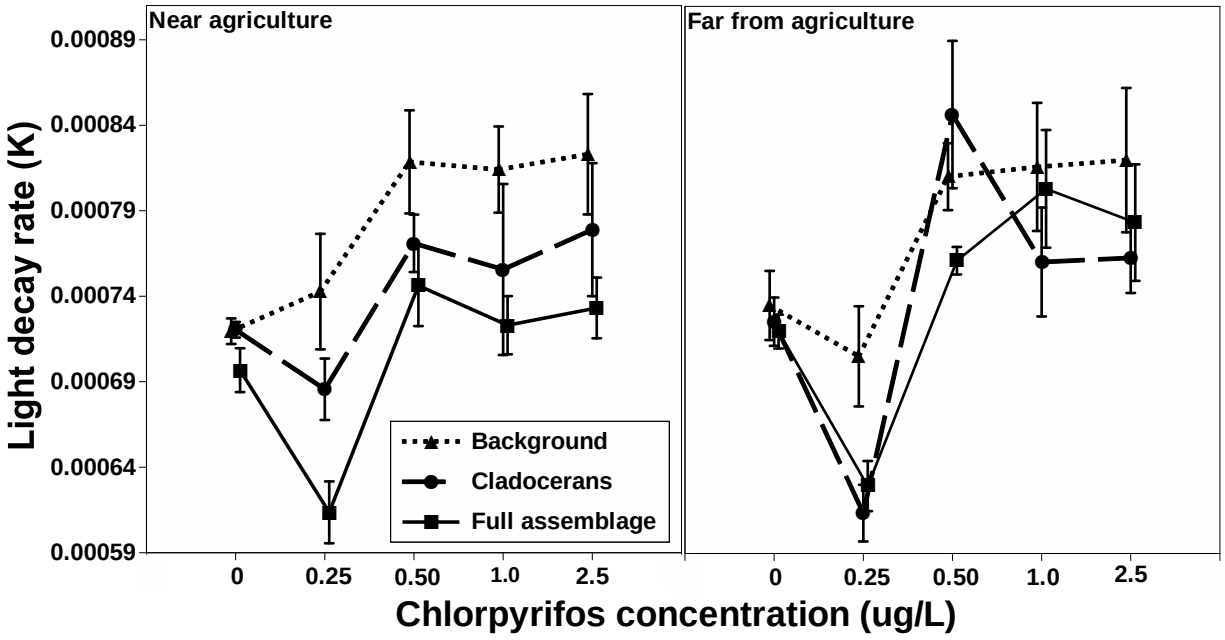

Figure M.4. Differences in light transmission rates within experimental communities across the three sampling dates of abiotic variables.

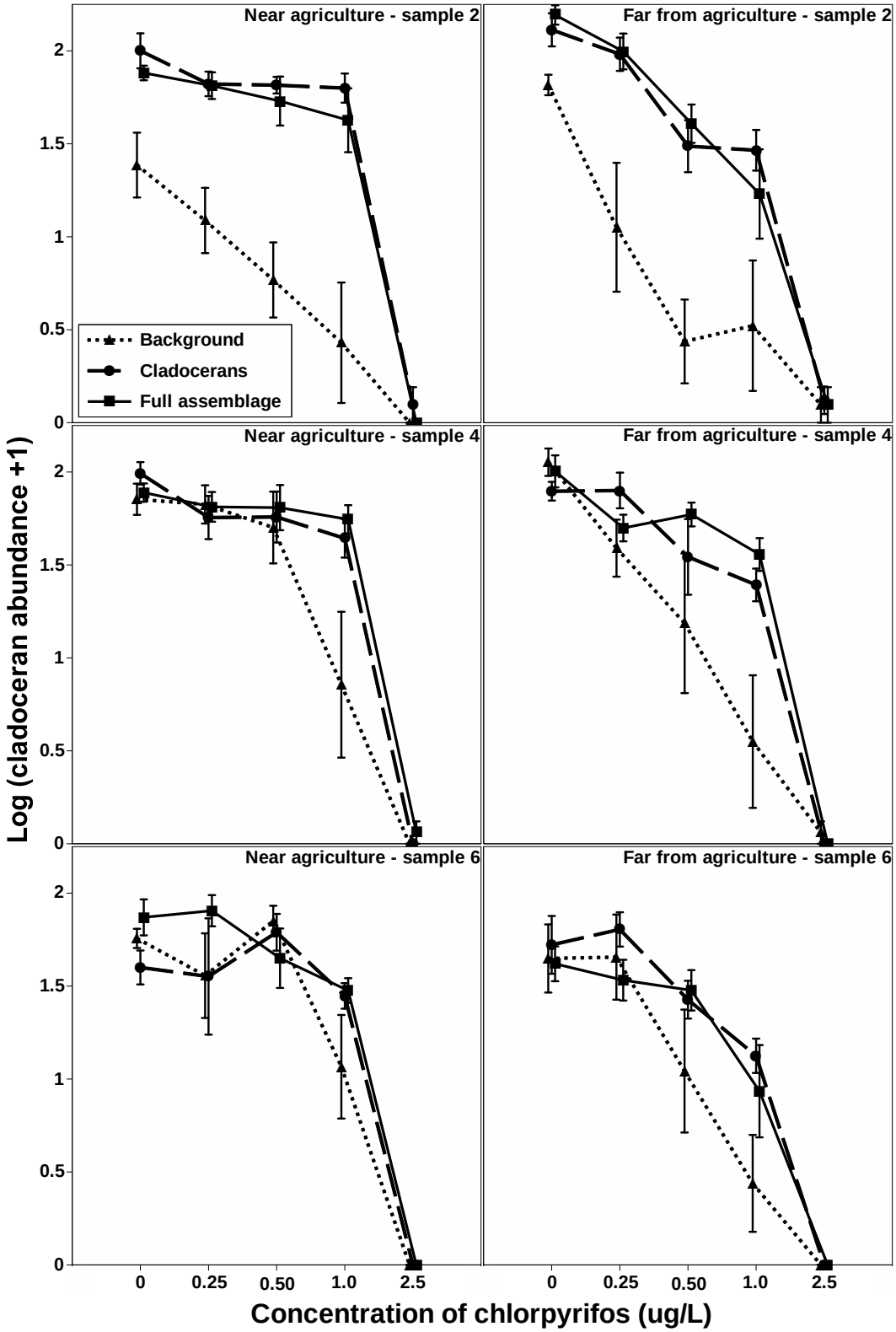

Figure M.5. Differences in cladoceran abundance during the two samples immediately preceeding the second and third chlorpyrifos applications as well as the experimental takedown.

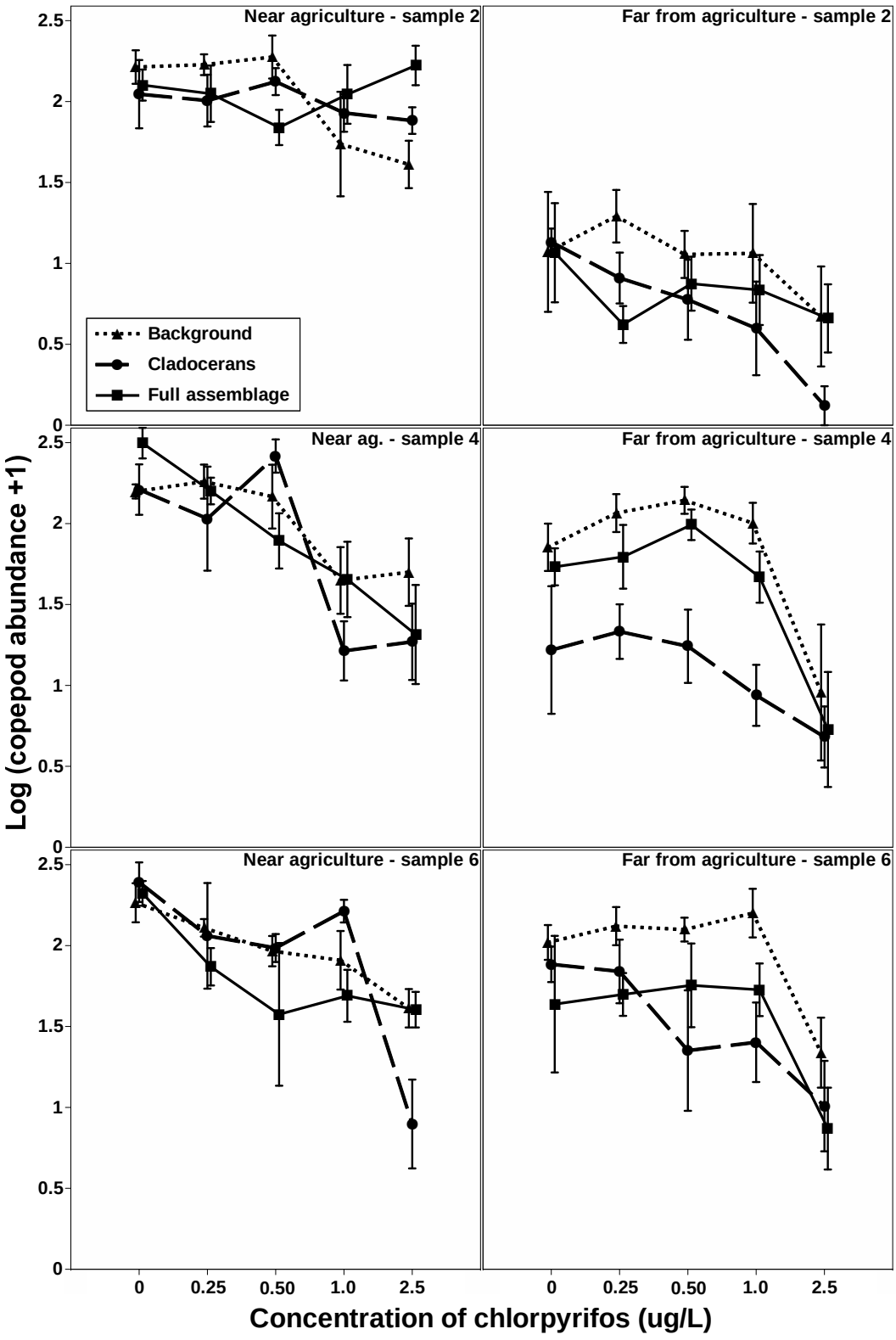

Figure M.6. Differences in copepod abundance during the two samples immediately preceeding the second and third chlorpyrifos applications as well as the experimental takedown.

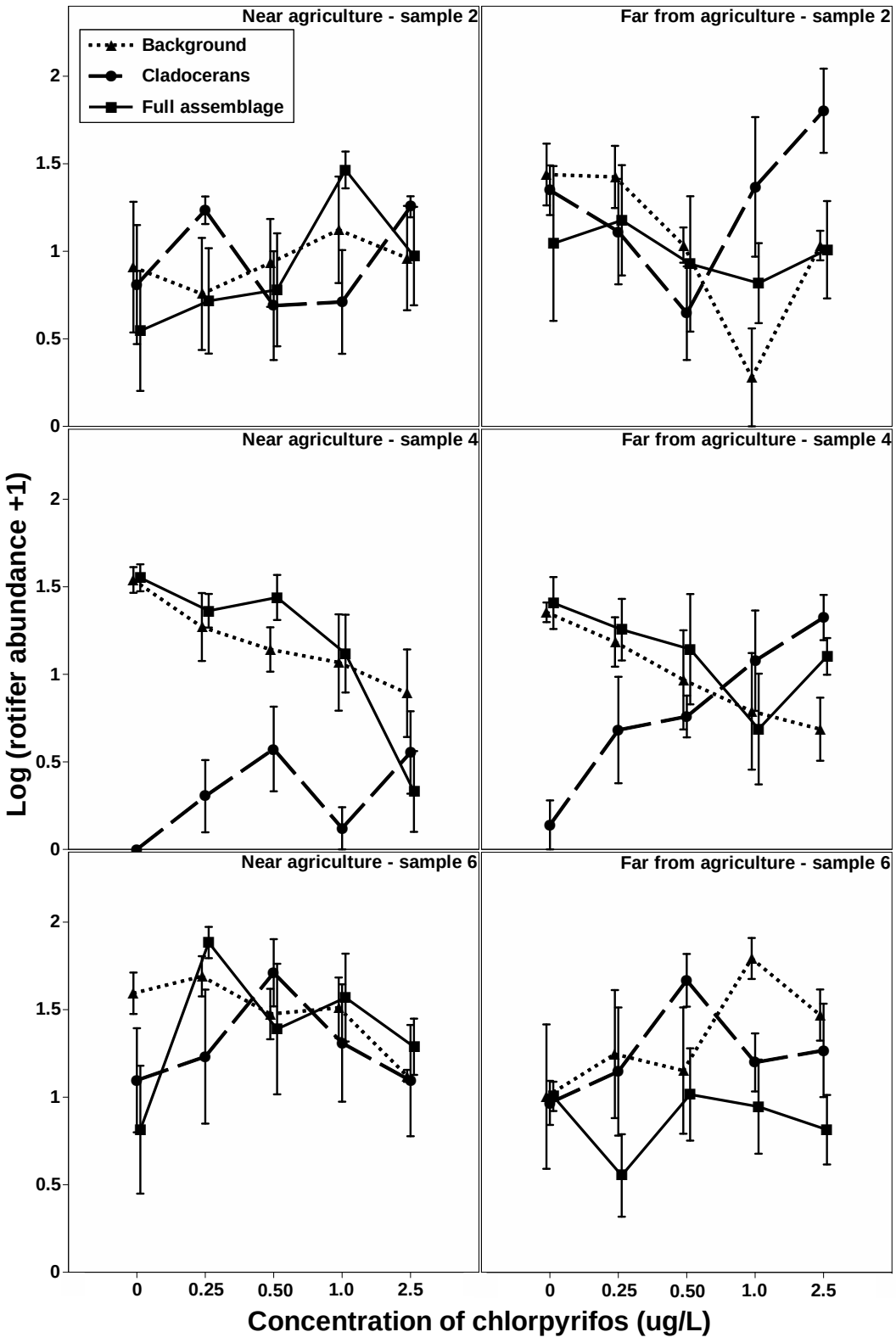

Figure M.7. Differences in rotifer abundance during the two samples immediately preceeding the second and third chlorpyrifos applications as well as the experimental takedown.

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