Running head: *Cross resistance in zooplankton populations promotes food web stability*

**If you see one, have you seen them all?: Community-wide effects of insecticide
cross-resistance in zooplankton populations near agriculture**

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ABSTRACT

1 The worldwide use of pesticides has led to increases in agricultural yields by reducing
2 crop losses. However, increased pesticide use has resulted in pesticide-resistant pest species and
3 recent studies have discovered pesticide-resistance in non-target species living close to farms.
4 Such increased tolerance not only affects the species, but can alter the entire food web. Given
5 that some species can evolve not only resistance to a single pesticide, but also cross-resistance to
6 other pesticides that share the same mode of action, one would predict that cross-resistance to
7 pesticides would also have effects on the entire community and affect community stability. To
8 address this hypothesis, we conducted an outdoor mesocosm experiment comprised of 200
9 identical aquatic communities with phytoplankton, periphyton, and leopard frog (*Lithobates*
10 *pipiens*) tadpoles. To these communities, we added one of four *Daphnia pulex* populations that
11 we previously discovered were either resistant or sensitive to the insecticide of chlorpyrifos as a
12 result of living close to or far from agriculture, respectively. We then exposed the communities
13 to either no insecticide or three different concentrations of AChE-inhibiting insecticides
14 (chlorpyrifos, malathion or carbaryl) or sodium channel-inhibiting insecticides (permethrin or
15 cypermethrin). We discovered that communities containing sensitive *D. pulex* experienced
16 phytoplankton blooms and subsequent cascades through all trophic groups including amphibians
17 at moderate to high concentrations of all five insecticides. However, communities containing
18 resistant *D. pulex* were buffered from these effects at low to moderate concentrations of all
19 AChE-inhibiting insecticides, but were not buffered against the pyrethroid insecticides. These
20 data suggest that a simple change in the population-level resistance of zooplankton to a single
21 insecticide can have widespread consequences for community stability and that the effects can be
22 extrapolated to a wide variety of pesticides that share the same mode of action.

Capsule: This is one of the first studies showing the evidence of cross-resistance among wild populations of zooplankton and direct evidence that this resistance can prevent trophic cascades.

Key words: cross-tolerance, cascade, acetylcholine-inhibitor, amphibians, indirect effect, pesticides

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 U.S. 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. The pest species that are typically targeted by these chemicals can range broadly depending on the chemical formula of chosen pesticide. For instance, in agriculture, the most common pest species for which pesticides are used as a control measure would include an enormous array of insects such as beetles, weevils, grasshoppers, crickets, aphids, and thrips (Oerke 2006). However, widespread pesticide use can also cause direct, indirect, and sublethal effects on non-target organisms, as well as lead to the evolution of resistance. Evolved resistance in targeted pest species has received a lot of attention by researchers across the globe (Georghiou 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 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 2016).

Today there are more than 1,055 active ingredients that are registered as insecticides in the United States (Goldman 2007). Due to this large number of chemicals, insecticides 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, (Brenques 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

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

70 Pond communities are ideal for studying the community-wide effects of cross-resistance
71 to insecticides because they are found across a wide range of distances from agricultural areas
72 and are therefore subjected to a variety of pesticide types and application frequencies (De
73 Meester et al. 2005). Habitats with higher proportions of surrounding agriculture and closer
74 distances to agricultural areas have proven to be useful proxies of historic pesticide exposures
75 that lead to populations evolving higher pesticide resistance (Coors et al. 2009, Cothran et al.
76 2013, Bendis and Relyea 2014). For example, recent studies have shown that wood frog
77 populations (*Lithobates sylvaticus*) living closer to agricultural areas have higher resistance to
78 the insecticide carbaryl (Cothran et al. 2013). A subsequent study found that these populations
79 that were not only resistant to this one acetylcholine esterase (AChE)-inhibiting insecticide, but
80 they also exhibited cross resistance to two additional AChE-inhibiting insecticides, thereby
81 indicating that cross-resistance may be common among non-target species (Hua et al. 2013).
82 AChE-inhibiting insecticides, like these, inhibit the acetylcholinesterase enzyme from breaking
83 down the neurotransmitter acetylcholine at neuromuscular junctions. This causes
84 overstimulation of the muscles which then leads to paralysis and, eventually, death (Fukuto
85 1990). Recent studies have also shown that zooplankton populations in the genus *Daphnia* can
86 vary in their resistance to commonly applied insecticides such as carbaryl (*D. magna*, Coors et
87 al. 2009) and chlorpyrifos (*D. pulex*, Bendis and Relyea 2014) and that these patterns of
88 resistance are also related to agricultural land use surrounding the ponds. Such evolved
89 resistance is important because zooplankton are one of the most sensitive taxonomic groups to
90 insecticides, and they play a key role in the function of pond communities as consumers, as prey,

and as cyclers of nutrients (Hanazato 1988, 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). However, we recently discovered that communities containing resistant populations of *D. pulex* (hereafter referred to as ‘*Daphnia*’ for simplicity) can buffer the entire aquatic communities from the impacts of insecticides (Bendis and Relyea 2016). 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 *Daphnia* 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 *Daphnia* 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 *Daphnia* 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, two pyrethroid insecticides which have a different mode of action (i.e. Na^+ channel-inhibitors which disrupt the normal transmission of nerve impulses by blocking Na^+ channels in nerve cells – Vijverberg et al. 1982), should not

113 exhibit any differences in resistance to these insecticides, regardless of the *Daphnia* population
114 included and should therefore not be buffered from its effects.

115 METHODS

116 We conducted a mesocosm experiment at the University of Pittsburgh's Donald S. Wood
117 Field Laboratory at the Pymatuning Laboratory of Ecology. Using mesocosms allowed us to
118 replicate aquatic communities, while simultaneously subjecting these communities to a range of
119 pesticide applications (Relyea and Diecks 2008). The experimental design was a full factorial
120 using four *Daphnia* populations: two resistant populations from ponds with surrounding
121 agriculture (>30% agricultural land within a 300-m radius) and two sensitive populations from
122 ponds with little or no surrounding agriculture (<5% agricultural land within a 300-m radius).
123 These four populations were each exposed to 16 insecticide treatments ([0.25, 0.50 and 1.0 ug/L
124 chlorpyrifos], [12.5, 25 and 50 ug/L carbaryl], [0.5, 1.0, and 2.0 ug/L malathion], [0.5, 1.0, and
125 2.0 ug/L permethrin], [0.5, 1.0 and 2.0 ug/L cypermethrin], and a negative control).

126 The insecticide concentrations were determined from our review of published data on
127 recorded LC50s for *Daphnia* for each insecticide, a comparison of LC50 values from other LC50
128 pilots performed within our lab, and from a series of LC50 experiments that we performed prior
129 to the setup of the mesocosm experiment (Bendis and Relyea 2014, Table A1). For the LC50
130 pilot, we exposed 10 juvenile female *Daphnia* from either a resistant (Love pond) or sensitive
131 (Minnow pond) to a range of concentrations of malathion, carbaryl, cypermethrin or permethrin
132 for a period of 24 hrs. Additionally, we utilized a negative control containing only UV-filtered
133 water and an ethanol vehicle control to ensure that the highest concentration of ethanol used to
134 dissolve the pesticides was not directly responsible for *Daphnia* mortality. We knew that these
135 two populations significantly varied in their natural resistance to chlorpyrifos from previous

studies (Bendis and Relyea 2014, 2016). 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 A1).

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 had no observed effect on 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 *Daphnia* 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 *Daphnia* 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.

Mesocosm set up

These experimental units were 75-L garbage cans (58.4 cm x 49.5 cm - Rubbermaid BRUTE™) 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 biomass. On 24 May, we took pond water samples from each of the four ponds where our *Daphnia* 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 μ m, 64 μ m) 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.

Daphnia population collection and rearing

On 6 May, we collected and isolated *Daphnia* 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.

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). 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 $\pm$ SE: 76.5 mg $\pm$ 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. Survival of the leopard frog tadpoles 24 hours after being handled was 100%.

Pesticide additions

We allowed the tadpoles to acclimate to experimental conditions for 6 d 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 1). For control mesocosms with 0 ug/L chlorpyrifos, we added 626 uL of carbon-filtered, UV-irradiated well water. For the eight mesocosms assigned the ethanol treatment, we added 626 uL 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

204 to a given mesocosm, we stirred the water in the mesocosm to equalize disturbance and to ensure
205 that the pesticide was mixed throughout the water column. All actual pesticide concentrations
206 were independently verified by the Center for Environmental Services and Engineering,
207 (University of Connecticut, Connecticut, USA - Appendix 1). Three weeks after applying the
208 insecticides (day 23), we re-applied the insecticide concentrations and re-tested our nominal
209 concentrations for the second application (Appendix 1).

210 *Abiotic response variables*

211 During the course of the experiment, we measured several abiotic response variables to
212 help us understand the effects of the various insecticides on the communities. On days 4, 26 and
213 48, we measured pH, temperature, and dissolved oxygen (DO). Days 4 and 26 immediately
214 followed our two pesticide applications and the day 48 was immediately before initiating the
215 water drawdown on day 50. Temperature, pH and DO content readings were taken with a
216 calibrated digital water meter (YSI, Yellow Springs, OH, USA). On days 8, 30 and 50 we took
217 light measurements primarily because these days followed pesticide applications, were close to
218 the days in which we sampled other abiotic factors and were clear, cloudless days, which are
219 ideal for taking light measurements. We measured light radiation from the middle of each
220 mesocosms at depths of 10 and 30 cm and calculated the decay rate of light with increased water
221 depth (k) using the equation

$$222 \quad k = [\ln(L_{10}/L_{30})]/d$$

223 where L_{10} and L_{30} are the intensities of sunlight from a depth of 10 and 30 cm, respectively, and
224 d is the difference in depth between the two measurements of intensity (Relyea and Diecks
225 2008). Light attenuation was measured with an underwater light meter (LI-COR. Lincoln,
226 Nebraska, USA).

227 *Biotic response variables*

228 We quantified several biotic response variables during the experiment. We sampled
229 *Daphnia* abundance at four time points during the experiment (Fig. 1). We sampled the *Daphnia*
230 by submerging a 0.2-L plastic sampling tube in the middle of the water column at six different
231 locations within each mesocosms (north, south and middle quadrants of each mesocosm both
232 towards the surface and near the benthos). All six samples within each mesocosm were pooled
233 and the sample was filtered through a 64-um Nitex cloth screen and poured into a Whirlpak bag
234 containing 30% ethanol to preserve the samples. For *Daphnia* enumeration, we poured the
235 ethanol from the Whirlpaks containing our zooplankton samples onto a Petri dish with a preset
236 grid to enumerate. We also identified and enumerated any zooplankton that were not *Daphnia*.

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

248 Periphyton biomass was also quantified twice during the course of the experiment (days
249 26 and 53) by removing one of the four clay tiles from each mesocosm. Periphyton was always

250 sampled within two days of our phytoplankton samples. Once a tile was removed, it was
251 vigorously scrubbed with a toothbrush to remove all of the periphyton on the face of the tile and
252 subsequently rinsed with carbon-filtered, UV-irradiated well water. The slurry containing water
253 and periphyton was then vacuum-filtered onto a Whatman GF/C filter that had been previously
254 dried for 24 hrs at 80°C and weighed. After the periphyton sample was vacuum-filtered, the
255 filters were again dried at 80°C for an additional 24 hours and weighed. The amount of
256 periphyton biomass was measured as the mass of the filter paper containing the dried periphyton
257 subtracted by the original mass of the dry, unused filter.

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

265 On day 50 (31 July) we began a week-long drying protocol to simulate the natural drying
266 that occurs in wetlands in our region during the late summer. It is important to conduct water
267 drawdowns gradually because amphibians can sense the drying of a pond by detecting a reduced
268 volume and respond by speeding up their development (Denver et al. 1998). To simulate the
269 gradual drying of a pond, we removed 5 L of water each day for 1 wk; on day 57 we terminated
270 our experiment when approximately 30 L of water remained in each mesocosm. On that day we
271 drained the remainder of water from the mesocosms and sorted through the litter and detritus to
272 recover all amphibians that had not metamorphosed yet. If we collected amphibians that had at

273 least one forelimb, we allowed that individual to complete metamorphosis (Gosner stage 46,
274 Gosner 1960) in the lab. All other amphibians that did not have any forelimbs were euthanized
275 using MS-222 and preserved in a 10% formalin solution. Most animals (94.4%) did not
276 metamorphose by the end of the experiment. For those individuals that metamorphosed, we
277 recorded time to metamorphosis, mass at metamorphosis, and survival to metamorphosis. For
278 those individuals that did not metamorphose, we recorded the mass of the tadpoles and
279 developmental (Gosner) stage.

280 *Statistical analysis*

281 We conducted initial ANOVA analyses to determine whether we could group the two
282 resistant *Daphnia* populations and the two sensitive *Daphnia* populations. Of the 21 response
283 variables, including those measured over time, 19 showed no significant differences between the
284 two resistant or between the two sensitive populations after a Bonferroni adjustment. As a result,
285 and because the populations did not differ in the previous two studies that we had performed, we
286 pooled the two resistant and two sensitive populations in all subsequent analyses as we have
287 done in a previous study (Bendis and Relyea 2016).

288 We transformed any response variables that did not meet the assumption of homogenous
289 variances. Data for temperature, pH and DO content were analyzed simultaneously on three
290 separate occasions allowing us to conduct a repeated-measures MANOVA on our abiotic data.
291 When we found significant multivariate effects, we then performed univariate repeated-measures
292 ANOVAs (rm-ANOVA) on those variables that were measured multiple times throughout the
293 experiment to determine how each variable was affected by the treatments. Whenever significant
294 effects were found, particularly treatment-by-time interactions, we conducted independent
295 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.

RESULTS

To display the results of the current study, we have chosen to report the effects on the main variables in separate sections (abiotic variables, zooplankton, phytoplankton, periphyton and leopard frogs). We first report the results of the overall rm-MANOVA for the variable in question. Then, within each section for the biotic variables, we discuss the following: 1) differences in community responses based on varying sensitivities in *Daphnia* resistance when aquatic communities are exposed to AChE-inhibiting insecticides, and then 2) the lack of mitigation of the trophic cascade when both sensitive and resistant populations of *Daphnia* are

exposed to the pyrethroid insecticides.

Abiotic variables

A detailed analysis of all abiotic response variables can be found in the Appendix. 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 A2). 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 A3-A, Fig. A1). 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* led to an increase in pH, whereas adding AChE-inhibiting pesticides to communities with resistant *Daphnia* led to a decrease in pH (Table A3-B, Fig. A2). We observed a similar pattern of ANOVA results across the samples dates for DO (Table A3-C, Fig. A3). Light attenuation generally increased with higher concentrations of the five insecticides and this increase grew in magnitude over time (Table A3-D, Fig. A4).

Zooplankton

The rm-ANOVA of *Daphnia* abundance indicated that there were significant effects of insecticide treatment, *Daphnia* sensitivity, time, and several interactions (Table A4). All sample dates exhibited effects of the insecticides and *Daphnia* sensitivity and three of the four sample dates exhibited insecticide-by-sensitivity interactions (Table A5-A). We then compared whether sensitive and resistant populations of *Daphnia* differed in abundance when exposed to each of the insecticides.

342 When communities are exposed to AChE-inhibitors: On the first sampling date (day 3;

343 Fig. 2), we found that there was a difference in control communities; there were significantly
344 more *Daphnia* (approximately 35%) in communities with resistant *Daphnia* ($p = 0.004$). We
345 also found that communities containing resistant *Daphnia* had more zooplankton than
346 communities containing sensitive zooplankton if they were exposed to the two lowest
347 concentrations of chlorpyrifos (C1 and C2; C3 was marginally non-significant, $p = 0.057$), the
348 two lowest concentrations of carbaryl (C1 and C2), and the two highest concentrations of
349 malathion (C2 and C3; all $p \leq 0.004$). On the second sampling date (day 24), unlike the first
350 sample, in the absence of pesticides there was no significant difference in *Daphnia* abundance
351 between communities with resistant or sensitive *Daphnia* ($p = 0.623$). Communities containing
352 resistant *Daphnia* populations had higher abundances of zooplankton when exposed to the
353 middle concentration of carbaryl (C2; $p = 0.045$) and the highest concentrations of malathion and
354 chlorpyrifos compared to communities containing sensitive *Daphnia* (C3; both $p \leq 0.023$). On
355 the final two sampling dates (days 33 and 48), in the absence of AChE-inhibitors, there were no
356 differences in *Daphnia* abundance between communities with resistant or sensitive *Daphnia* ($p =$
357 0.504 and 0.854 for samples 3 and 4, respectively). There was, however, more *Daphnia* in
358 communities containing resistant *Daphnia* that were exposed to the three carbaryl and
359 chlorpyrifos concentrations (C1, C2, C3) across both dates (all $p \leq 0.026$), compared to
360 communities containing sensitive *Daphnia*. The same pattern was observed with the second
361 highest malathion concentration on day 33 (C2; $p = 0.10$) and the highest malathion
362 concentration on day 48 (C3; $p < 0.001$).

363 When communities are exposed to pyrethroids: On the first sampling date, *Daphnia*

364 sensitivity had no effect on the abundance of *Daphnia* when exposed to either pyrethroid

insecticide (all $p > 0.323$). This trend continued as on the second sampling date there were no effects of *Daphnia* sensitivity when communities were exposed to either pyrethroid insecticide (all $p > 0.478$). On the third and fourth sampling dates, there were fewer *Daphnia* in communities containing resistant *Daphnia* that were exposed to permethrin than communities containing sensitive *Daphnia* at the middle concentration during the third sample (C2; $p = 0.038$). In contrast, there were more *Daphnia* in communities containing resistant *Daphnia* at the highest concentration of permethrin on the final sampling date (C3; $p = 0.027$). For cypermethrin, there were no differences between the two community types at any concentration (all $p > 0.098$).

Phytoplankton

The rm-ANOVA of the phytoplankton data revealed significant effects of insecticide treatment, *Daphnia* sensitivity, time, and multiple interactions with time (Table A4). On both samples, there were significant effects of insecticide treatment, *Daphnia* sensitivity, and their interaction (Table A5-B).

When communities are exposed to AChE-inhibitors: On the first sampling date (day 22; Fig. 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 non-significant ($p = 0.063$) and *Daphnia* sensitivity had no effect on the abundance of phytoplankton when exposed to carbaryl ($p = 0.210$). On the second sampling date (day 51), 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 $p \leq 0.002$).

When communities are exposed to pyrethroids: On the first sampling date, there was no difference in phytoplankton abundance in communities exposed to any concentrations of either permethrin or cypermethrin regardless of *Daphnia* sensitivity (all $p > 0.061$). During the second sampling date, and consistent with the surprising observation that permethrin exposures led to fewer *Daphnia* to be present in communities with resistant zooplankton, 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; $p = 0.003$).

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 A4). 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 A5-C).

When communities are exposed to AChE-inhibitors: On the first sampling date (day 26; Fig. 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; $p = 0.010$). On the second sample (day 53), there was more periphyton in communities with resistant *Daphnia* than communities with sensitive *Daphnia* when exposed to the lowest two

411 concentrations of chlorpyrifos (C1, C2), the middle concentration of malathion (C2) and the
412 highest concentration of (C3) carbaryl (all $p \leq 0.045$).

413 When communities are exposed to pyrethroids: During the first sampling date, *Daphnia*
414 sensitivity had no effect on the abundance of periphyton when exposed to either of the pyrethroid
415 insecticides (all $p > 0.076$). On the second sampling date, and consistent with the observation
416 that the highest permethrin treatment led to fewer zooplankton and more phytoplankton in
417 communities containing resistant *Daphnia*, communities with resistant *Daphnia* had less
418 periphyton when exposed to the highest concentration of permethrin (C3) when compared to
419 communities with sensitive *Daphnia* ($p = 0.001$).

420 *Leopard frogs*

421 We analyzed leopard frog survivorship, average tadpole mass and average developmental
422 (Gosner) stage using a MANOVA. We found multivariate effects of insecticide treatment and
423 *Daphnia* sensitivity (both $p < 0.001$), but no significant interaction (Table A6). We then ran
424 separate univariate analyses of the aforementioned leopard frog variables.

425 *Leopard frogs – survivorship*

426 We began by analyzing survivorship (Fig. 5). The ANOVA of survivorship indicated
427 that there was an effect of insecticide treatment ($p < 0.001$), a non-significant effect of *Daphnia*
428 sensitivity ($p = 0.072$), and a non-significant interaction ($p = 0.861$, Table A7-A). Averaged
429 across all pesticide treatments, overall survivorship of leopard frogs was slightly higher (3.7%)
430 in communities with resistant *Daphnia*.

431 When communities are exposed to AChE-inhibitors: Exposure to chlorpyrifos did not
432 affect mortality beyond that of the control at any of the three concentrations in either community
433 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* ($p = 0.011$).

When communities are exposed to pyrethroids: 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 $p < 0.001$).

Leopard frogs – tadpole mass

For tadpole mass, there was an effect of *Daphnia* sensitivity, insecticide treatment, and their interaction (all $p \leq 0.037$, Table A7-B; Fig. 5). In control treatments, there was a marginal difference in average tadpole mass when comparing communities with sensitive versus resistant *Daphnia* ($p = 0.076$).

When communities are exposed to AChE-inhibitors: Exposure to the AChE-inhibitors 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 $p \leq 0.035$).

When communities are exposed to pyrethroids: 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 $p < 0.103$).

Leopard frogs – developmental (Gosner) stage

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 A7-C; Fig. 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 also held true for communities that were not exposed to pesticides ($p < 0.001$).

When communities are exposed to AChE-inhibitors: In communities with resistant *Daphnia*, leopard frogs generally developed more quickly in those communities exposed to any of three concentrations of all three AChE-inhibiting insecticides when compared to communities that contained sensitive *Daphnia* (all $p \leq 0.016$).

When communities are exposed to pyrethroids: In communities with resistant zooplankton, tadpoles developed more quickly than those in communities with sensitive *Daphnia* when 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 regardless of the sensitivity of the *Daphnia* population (all $p > 0.409$).

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 resistance 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 little to 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 studies (Bendis and Relyea 2014, 2016) 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 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, French-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 2009a-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 necessarily 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 constraints 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 2016). 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 & Sivars-Becker 2007, Coors and De Meester 2008, Jansen et al. 2011, 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 penetration, 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 led to 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 2016). 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 biomass 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

593 survivorship was likely due to declining food availability (i.e. periphyton biomass).
594 Furthermore, while we saw small improvements in amphibian survivorship in communities with
595 resistant *Daphnia* compared to communities with a sensitive population, there were more
596 substantial effects of *Daphnia* cross-resistance on tadpole mass and development. These results
597 are important as they suggest that cross-resistance in organisms within an entirely different
598 trophic level of an aquatic community may yield substantial benefits for grazers such as
599 amphibians through the mitigation of anthropogenically-induced trophic cascades.

600 There was clear evidence that the effects of cross-resistance within *Daphnia* resulted in
601 faster development and higher growth rates of amphibians within communities exposed to
602 several concentrations of all of the AChE-inhibiting insecticides. Given the lack of evidence for
603 cross-resistance of *Daphnia* to the pyrethroids, we expected to see no evidence of faster
604 development in communities exposed to pyrethroids. This was true for cypermethrin, but we
605 found that amphibians in communities exposed to C1 and C3 of permethrin actually developed at
606 a faster rate for reasons that remain unclear. Aside from this one anomaly, our results coincided
607 clearly with our hypothesis that *Daphnia* resistance to chlorpyrifos would lead to cross-resistance
608 to chemicals with a similar mode of action, but not necessarily to insecticides with a different
609 mode of action. *Daphnia* that were cross-resistant to the three AChE-inhibiting insecticides
610 survived to a greater degree when compared to sensitive *Daphnia*. This led to more
611 phytoplankton being removed from the water column and an increase in resources (i.e. light) for
612 periphyton, and as periphyton biomass increased this led to an increase in average tadpole mass
613 and faster overall developmental rates. We anticipated this finding from previous studies which
614 have shown that concentrations of insecticides that are lethal to zooplankton can cause marked
615 trophic cascades that can eventually affect amphibian growth a development through a series of

cascading events (Fleege 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 potential 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.

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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Figure Captions

Figure 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 was measured to quantify light decay rates. The top figure shows important time points in our overall experimental protocol whereas the bottom figure shows the various sampling time points of both the abiotic and biotic variables during the course of the experiment.

Figure 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.

Figure 3. Differences in chlorophyll *a* abundance (in ug/L) within experimental communities across the two sampling dates of phytoplankton abundance.

Figure 4. Differences in periphyton abundance (in g) within experimental communities across the two sampling dates of periphyton abundance.

Figure 5. Differences in overall survival, average tadpole mass (g), and developmental stage, between communities with resistant or sensitive *Daphnia*. Due to the fact that there was significant amphibian mortality in the higher cypermethrin treatments, cypermethrin treatments were removed from the both the analyses and graphs of average mass and developmental stage.

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.