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Are there fitness costs of adaptive pyrethroid resistance in the amphipod, *Hyalella azteca*?☆

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ABSTRACT

Pyrethroid-resistant *Hyalella azteca* with voltage-gated sodium channel mutations have been identified at multiple locations throughout California. In December 2013, *H. azteca* were collected from Mosher Slough in Stockton, CA, USA, a site with reported pyrethroid (primarily bifenthrin and cyfluthrin) sediment concentrations approximately twice the 10-d LC50 for laboratory-cultured *H. azteca.* These *H. azteca* were shipped to Southern Illinois University Carbondale and have been maintained in pyrethroid-free culture since collection. Even after 22 months in culture, resistant animals had approximately 53 times higher tolerance to permethrin than non-resistant laboratory-cultured *H. azteca.* Resistant animals held in culture also lacked the wild-type allele at the L925 locus, and had non-synonymous substitutions that resulted in either a leucine-isoleucine or leucine-valine substitution. Additionally, animals collected from the same site nearly three years later were again resistant to the pyrethroid permethrin. When resistant animals were compared to non-resistant animals, they showed lower reproductive capacity, lower upper thermal tolerance, and the data suggested greater sensitivity to, 4, 4′-dichlorodiphenyltrichloroethane (DDT), copper (II) sulfate, and sodium chloride. Further testing of the greater heat and sodium chloride sensitivity of the resistant animals showed these effects to be unrelated to clade association. Fitness costs associated with resistance to pyrethroids are well documented in pest species (including mosquitoes, peach-potato aphids, and codling moths) and we believe that *H. azteca* collected from Mosher Slough also have fitness costs associated with the developed resistance.

1. Introduction

Pyrethroids are a major class of synthetic insecticides that target the voltage-gated sodium channel (*vgsc*) of the nervous system and are highly toxic to invertebrates and fish, while having relatively low mammalian toxicity. These insecticides are used in and around homes, on pets, for the treatment of head lice and scabies, in disease vector control, and in agriculture (Rinkevich et al., 2013). Intensive use of pyrethroids for pest control has caused many instances of resistance in pest species globally; often associated with single nucleotide polymporphisms (SNPs) in the *vgsc* although other mutations are possible and have been documented (e.g. metabolic resistance mutations) (Feyereisen et al., 2015). As of 2013, more than 50 resistance-associated mutations had been noted in a variety of pest species (Rinkevich et al., 2013). These SNPs result in amino acid substitutions that cause conformational changes of the pyrethroid binding sites that inhibit pyrethroid binding and ultimately decrease pyrethroid sensitivity (Rinkevich et al., 2013). Toxicity in resistant animals appears to be associated with oxidative stress (Weston et al., 2013), similar to pyrethroid toxicity in mammals that have sodium channels insensitive to pyrethroids (Giray et al., 2001).

Most mutations found on the *vgsc* have been identified in terrestrial target species including the whitefly (*Bemisia tabaci*) (Morin et al., 2002), common housefly (*Musca domestica*) (Tomita and Scott, 1995), and peach-potato aphids (*Myzus persica*) (Foster et al., 1999). However, the use of pyrethroids has led to

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unintentional consequences to invertebrates not directly targeted by their application. Pyrethroid exposure to terrestrial non-target species includes behavioral effects to honeybees (Ingram et al., 2015) and the beneficial beetle *Platynus assimilis* (Tooming et al., 2014). Wild populations of *Drosophila melanogaster* have been shown to have pyrethroid resistance (Vais et al., 2003), despite fruit flies rarely being targeted for control with insecticides.

In 2010, Weston et al. (2013) discovered thriving populations of *H. azteca* at sites with known pyrethroid contamination. The *vgsc* genes of these animals were sequenced and resistant animals had either of two amino acid substitutions associated with pyrethroid resistance in pest species: the M918L or the L925I mutations (Weston et al., 2013). The M918L mutation has been documented in many pest species including cotton aphids (*Aphis gossypii*), diamondback moths (*Plutella xylostella*), and houseflies (*M. domestica*) (Rinkevich et al., 2013). The L925I mutation has also been reported, including in sweet potato whiteflies (*B. tabaci*) and the southern cattle tick (*Rhipicephalus microplus*) (Rinkevich et al., 2013). In addition to populations documented by Weston et al. (2013), a recent study of undeveloped or developed sites (with nearby agriculture or urban development) across California identified many pyrethroid-resistant populations of *H. azteca* (Major et al., in press). Resistance alleles were present at high frequencies only at developed sites—adding to the body of evidence that this is a human-driven phenomenon.

A number of recent studies have documented phenotypic differences (including sensitivity to contaminants and reproductive rate) between populations of *H. azteca* collected from different locations or cultured in different laboratories. These differences have been associated with varied life history characteristics, the experience level of the laboratory conducting the research, and the test protocols and conditions (Major et al., 2013; Pathammavong, 2016; Soucek et al., 2015; Thomas et al., 1997). Organisms morphologically classified as *H. azteca* have been used in toxicity studies since the mid-1980s because of their broad distribution, wide tolerance for natural environmental variables (e.g. salinity), ease of culturing in the laboratory, and relatively short maturation period (U.S. EPA, 2000). While most toxicity testing laboratories are using genetically similar *H. azteca*, wild populations in this species complex are more genetically variable than previously thought (Major et al., 2013).

While the emergence of resistance to pyrethroids may superficially demonstrate the adaptive capacity of *H. azteca* to survive exposure to anthropogenic contaminants, it is important to consider potential fitness costs associated with the developed resistance. Mutations on the *vgsc* that decrease sensitivity to pyrethroids could result in a reduction in overall fitness, because of reduced efficiency of the vgsc and metabolic costs related to the change (Zhao et al., 2000) or through “genetic hitchhiking” of less desirable traits linked to the mutations (Van Straalen and Timmermans, 2002). Fitness costs associated with mutations causing large phenotypic changes (e.g. resistance to xenobiotics) are not a new concept. Fisher (1958) developed a model of adaptation that discusses how independent selection pressures shape current, nearly ideal, phenotypes through gene coevolution. Because of the complex gene interdependence, mutations with large phenotypic effects are likely to induce deleterious effects. Several examples of fitness costs to pyrethroid-resistant populations of pest species exist. Konopka et al. (2012) showed increased developmental time, lower pupal masses, and smaller wing surface areas in the codling moth (*Cydia pomonella*). Peach-potato aphids (*M. persica*) with target-site and metabolic resistance to both pyrethroids and DDT had decreased overwintering survival, reduced alarm pheromone response (Foster et al., 1999), and increased vulnerability to parasitoids (Foster et al., 2005). Finally, potato beetle strains with organophosphate and pyrethroid resistance have decreased fertility and fecundity (Argentine et al., 1989).

The current research project had two major objectives. First, we wanted to confirm that the resistance documented in *H. azteca* populations in the U.S. was a heritable genetic change by monitoring the pyrethroid-resistant trait in long-term culture by exposing non-resistant and resistant populations to permethrin, and genotyping the L925 locus in the resistant population after 16 months of culture with no pyrethroid exposure to monitor for presence of the wild-type non-resistant allele. Second, we wanted to determine potential fitness costs of pyrethroid resistance to *H. azteca* and this was accomplished in two phases. In the first phase, cultured resistant and non-resistant *H. azteca* that belonged to two different clades were compared by measuring the reproductive output and responses to thermal stress, 4, 4′-dichlorodiphenyltrichloroethane (DDT), copper (II) sulfate, and sodium chloride. The second phase consisted of repeating the thermal stress and sodium chloride tests with both resistant and non-resistant field-collected animals from the same clade.

2. Experimental methods

2.1. Chemicals

Permethrin (98% purity; 46% *cis*, 52% *trans*) and DDT (98% purity) were obtained from ChemService (West Chester, PA, USA). Sodium chloride, sodium sulfate, trace metal grade nitric acid, and copper (II) sulfate as well as the pesticide-grade solvents hexane, acetone, and dichloromethane (DCM) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Calibration standards and electrode storage solutions for use with the chloride probe were purchased from Fisher Scientific. The surrogate standard decachlorobiphenyl (DCBP) was obtained from Supelco (Bellefonte, PA, USA).

2.2. Organisms

Four *H. azteca* populations were used in the current study; two non-resistant, and two resistant populations. The first non-resistant population was obtained from the U.S. EPA laboratory in Duluth, Minnesota, and has been cultured at Southern Illinois University Carbondale (SIUC) since 2001 (SIUC Lab). The second non-resistant population was collected from the Mojave River in southern California (35.0380, −116.3814) in November 2016 (Field Mojave). The pyrethroid-resistant populations were collected from Mosher Slough in Stockton, California (38.0325, −121.3654), where Weston et al. (2013) reported sediment pyrethroid concentrations at approximately twice the LC50 (the concentration lethal for 50% of test organisms) for laboratory-cultured *H. azteca*. Subsets of both collections from Mosher Slough were genotyped and found to have the L925I mutation known to provide pyrethroid resistance. The first collection of the resistant Mosher population (SIUC Mosher) has been maintained in pyrethroid-free culture at SIUC since December, 2013 (with one supplement of organisms from the same location in February, 2014). A later collection of the resistant Mosher population was collected in November 2016 and used for the present study within one month of collection (Field Mosher).

The organisms used in the current study have been previously identified as belonging to three species groups, based on maximum likelihood analysis of nucleotide sequences in the mitochondrial gene cytochrome *c* oxidase subunit I (COI) and the 28S nuclear ribosomal large subunit rRNA gene (*28S* rDNA) (Weston et al., 2013). The SIUC Lab belongs to Clade C, animals collected at the collection sites in Mosher Slough were primarily species D with some identified as probable species B, and the Mojave Desert have been

identified as Clade D (Major et al., in press).

2.3. Hyalella culturing

The 40-L *Hyalella* cultures were maintained in reformulated moderately hard reconstituted water (RMHRW) (U.S. EPA, 2000; Smith et al., 1997). Cultures were continuously aerated and maintained at 23 °C ± 2 °C. Aged sugar maple (*Acer saccharum*) leaves were used as substrate (U.S. EPA, 2000). Cultures were fed three times weekly with 10 mL of a 100 g/L TetraMin (Tetra U.S., Blacksburg, VA, USA) slurry of ground flakes suspended in RMHRW (U.S. EPA, 2000). Beginning in March and September 2015, respectively, *Selenastrum capricornutum* originally purchased from Carolina Biological Supply Company (Burlington, North Carolina, USA) and cultured using prepared *Selenastrum* media (U.S. EPA, 2000) and *Thalassiosira weissflogii* purchased from Reed Mariculture Inc. (Campbell, CA, USA) were used as supplemental food for both cultures.

Water quality was checked every other week using a YSI 55 water-quality meter (Yellow Springs Instrument Company, Yellow Springs, OH, USA) and an Oakton conductivity/pH meter (Oakton Instruments, Vernon Hills, IL, USA) to assure that the dissolved oxygen was greater than 5 mg/L, pH was between 7.5 and 8.5, and conductivity was between 300 and 400 μS/cm (U.S. EPA, 2000). An Aquarium Pharmaceuticals Incorporated Freshwater Master Test Kit (Mars Fishcare Inc., Chalfont, PA, USA) was used to monitor ammonia concentrations. If the parameters for *H. azteca* health were not met, daily 30% water changes were conducted until appropriate parameters were met.

2.4. Genotyping

Genomic DNA was extracted from individual *H. azteca* using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). All recommended protocols for animal tissue were followed except the addition of a mechanical maceration step using a stainless steel bead (Tissue Lyser LT, Qiagen; 10–20 min at 1/50 oscillations) as well as an overnight incubation of macerated tissue in Buffer ATL and Proteinase K (Qiagen) at 55 °C before proceeding with recommended extraction steps. A 543 base pair segment of the *vgsc* region was amplified using primers previously developed (Weston et al., 2013) and purchased through Integrated DNA Technologies (Coralville, Iowa, USA) designed to the Domain II Linker S4-S5 region (Lt: AGGGTGTTCAAGCTCGCTAA, Rt: ACATGCTCTCGATCCACTCC). Polymerase Chain Reaction (PCR) was carried out using Thermo Scientific™ Phusion Green Hot Start II High-Fidelity PCR Master Mix in 50 μL reaction volumes and the following PCR program: 98 °C for 30 s; 35 cycles of 98 °C for 10 s, 64.2 °C for 30 s, 72 °C for 30 s; and a final extension step at 72 °C for 10 min. An agarose gel was used to verify PCR products that were purified using the QIAquick PCR Purification Kit (Qiagen). An internal primer (Rt: GGCCGTCTTGAGACCATTT) was used to sequence PCR products at the Massachusetts General Hospital DNA core facility (Cambridge, MA, USA). Sequences were visualized and manually scored for genotype using CLC Main Workbench v.7.7.3 (Qiagen) software. Homozygotes had codons with singular peaks at each position, while heterozygotes were identified by the presence of two subequal peaks at the same position within the codon. Ten animals from the SIUC Lab, Field Mojave, and Field Mosher populations were genotyped, while 20 animals from the SIUC Mosher population were genotyped.

2.5. Reproductive study

Reproduction was measured in the SIUC Lab and SIUC Mosher *H. azteca* populations using 42-d tests based on U.S. EPA protocols (Konopka et al., 2012). To acquire known-age organisms, approximately 300 adults (>3 mm) were removed from the main culture tanks and distributed evenly among five 600 mL beakers containing 500 mL of RMHRW and several pieces of Nitex screen. Immediately after adding organisms, 1 mL of yeast-cerophyll-trout chow (YCT) was added to each beaker (U.S. EPA, 2000). The beakers were held at 23 °C for 48 h to allow gravid females to release juvenile *H. azteca* from their brood pouches. After the 48-h incubation, the contents of each beaker were poured through a 500-μm sieve and rinsed with additional RMHRW. Adults were returned to the main cultures and the <48-h old *H. azteca* were transferred to new 600 mL beakers and fed a mixture of YCT and *Selenastrum* and monitored for an additional 6 d. When the *H. azteca* were 6- to 8- d old, 10 individuals of unknown sex were placed into 300 mL beakers with 100 mL of sediment (4.43% organic carbon) collected from LaRue Pine Hills, Wolf Lake, IL and 175 mL of overlying RMHRW. LaRue Pine Hills sediment was used as a control sediment in previous studies and did not contain detectable levels of pyrethroids (Harwood et al., 2013). Ten replicates were completed for each population. The 300 mL beakers were placed in a flow-through system with three daily changes of overlying water. To assure *H. azteca* did not escape during water changes, a 1.5 cm hole was drilled into the side of each beaker <1 cm from the lip of the beaker and covered with a Nitex screen attached using aquarium-grade silicone. An aliquot of YCT (1 mL) was added to each chamber daily. After 20 d in sediment, when organisms were 26- to 28-d old, the contents of individual test chambers were poured through a 1000 μm sieve and gently rinsed with RMHRW. Adult *H. azteca* that were retained on the screen were transferred to a new chamber containing 275 mL of RMHRW and a Nitex screen and the beakers were returned to the flow-through system under identical water change and feeding conditions as described earlier. The organisms were moved from sediment to water-only conditions for ease of counting newborn *H. azteca*. Reproductive output was measured after 7 and 14 d in the water-only conditions, when the *H. azteca* were 33- to 35-d and 40- to 42-d old. On the first count, offspring were removed and recorded. On the second count, offspring were recorded and the surviving adult *H. azteca* were isolated and their sex distinguished by the enlarged second gnathopods of males (Wen, 1993).

2.6. Thermal tolerance

The upper thermal tolerance was determined for all four populations. Upper thermal tolerance is defined here as the temperature at which an organism was immobilized for 10 s. A 100 mL volume of RMHRW was heated in a 150 mL glass culture dish from 25 °C to 43 °C over a 50 min period using standard heating tape attached to a magnetic base (Kanatec USA Corporation, Bensenville, IL, USA) in conjunction with a Warner Instruments Single Channel Heater Controller (Hamden, CT, USA). Animals ranging in size from 500 to 1000 μm were collected from main culture chambers (held at 23 °C ± 1 °C) and allowed to acclimate to the temperature of the study room (25 °C) for 1 h. Twenty *H. azteca* were transferred to the glass culture dish. The water was heated at an average rate of 0.36 °C/min and *H. azteca* were observed for immobilization. To help monitor immobilized *H. azteca*, the culture dish was placed on a 1 cm × 1 cm gridded paper. When a *H. azteca* was observed to be immobilized for 10 s, it was prodded with a transfer pipette. If the organism did not respond, it was pulled up into the transfer pipette and observed. If movement (swimming or twitching) was noted within 10 s, the *H. azteca* was returned to the culture dish. If no movement was observed, the temperature at which the organism was removed from the culture dish was recorded as immobilized. Five replicates of 20 animals were performed for test conducted

with the SIUC Lab, and SIUC Mosher populations. Five replicates of 20 animals were also conducted with the Mojave population, but due to a limited number of animals, three replicates of 20 animals were conducted for the Field Mosher population.

2.7. Toxicity tests

Toxicity tests were conducted as 96-h static water-only exposures to determine LC50 values for the non-resistant and resistant *H. azteca* populations. The LC50 to permethrin and sodium chloride was determined for all populations, while the LC50 to DDT and copper (II) sulfate were determined for only the SIUC Lab and SIUC Mosher populations. To eliminate risk of food altering toxicant concentrations, organisms were not fed during the 96-h exposure. Tests were conducted in 600 mL beakers containing 500 mL of RMHRW and all tests were conducted in incubators maintained at 23 °C ± 1 °C and using a 16:8 light: dark cycle. Organisms used for toxicity tests were selected by passing water containing *H. azteca* through a 1000 μm and a 500 μm screen. Organisms that passed through the 1000 μm screen, but were retained by the 500 μm screen were used for all tests.

Exposure concentrations were determined based on literature values and screening tests using broad concentration ranges. Five to 10 concentrations with two to four replicates were used for each toxicant (average number of *H. azteca* exposed per concentration in each test is shown in Table 1). Negative (RMHRW only) and solvent controls (acetone at ≤100 μL/L) were used in all toxicity tests. The water used in the toxicity tests was spiked in 4- or 6- L Erlenmeyer flasks, thoroughly mixed for 30 min on a stir plate, and distributed among five 600 mL beakers for each concentration (four beakers for toxicity testing; one for chemical analysis). A 1 cm^2 Nitex mesh screen was added to each beaker to provide a substrate for the *H. azteca* and the beakers were incubated at 23 °C ± 1 °C for approximately 18 h prior to the addition of organisms. The toxicant concentration spiked into the test water was confirmed analytically on water samples after the 18 h of incubation (time zero) and at 96 h.

When selecting *H. azteca*, active individuals that appeared healthy were transferred to the test beakers using a transfer pipette with the tip removed. During transfer, care was taken that each *H. azteca* stayed submerged throughout the process, because air trapped under their carapace could prevent them from being able to submerge and ultimately lead to death (U.S. EPA, 2000). At the end of each test, the number of surviving organisms was recorded. Animals were recorded as alive if any movement was visible when observed in a transfer pipette.

2.8. Analytical testing

Permethrin and DDT were extracted from sample water using a previously developed liquid-liquid extraction method (Wang et al., 2009) slightly modified from U.S. EPA Method 3510 using three 50 mL washes of DCM. Extractions were performed at time zero and at 96 h for all concentrations including negative and solvent controls. For the time zero extractions, 500 mL of water was taken from a single test beaker at each test concentration, placed into a separatory funnel, and spiked with the surrogate DCBP. For the 96 h extractions, 500 mL of water was extracted at each dosing concentration using an equal volume from each replicate for that concentration. Analysis of permethrin, DDT, and surrogate concentrations in the water samples were conducted using an Agilent 6890 series gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a micro-electron capture detector (GC-ECD) and a HP-5ms (30 m × 0.25 mm x 0.25 μm) column and confirmed with a DB-608 (30 m × 0.25 mm x 0.25 μm) column using a previously developed method (You and Lydy, 2007).

Table 1
Toxicity (96-h LC50 and associated 95% confidence intervals) of permethrin, DDT, NaCl, and copper (II) sulfate to SIUC Lab, SIUC Mosher, Field Mojave, and Field Mosher animals.

Population	Clade	Test Date	LC50 (95% Confidence Intervals)	N	X^2	TSK
Permethrin						
SIUC Lab (NR)	C	June 2015	34.59 ng/L (31.28–38.25) [a]	40	73.06	N/A
SIUC Lab (NR)	C	October 2015	31.22 ng/L (26.44–36.87) [a]	40	N/A	17.07%
Field Mojave (NR)	D	November 2016	45.00 ng/L (40.65–49.83)[b]	30	N/A	7.41%
SIUC Mosher (R)	D	July 2015	1144 ng/L (942.5–1389) [c]	40	N/A	9.09%
SIUC Mosher (R)	D	February 2016	1668 ng/L (1381–2014) [c]	40	18.87	N/A
Field Mosher P0 (R)	D	November 2016	3310 ng/L (2680–4087) [d]	21	N/A	23.44%
Field Mosher F1 (R)	D	December 2016	1803 ng/L (1383–2352) [c]	16	N/A	35.92%
DDT						
SIUC Lab (NR)	C	November 2015	340.6 ng/L (303.7–381.9) [a]	40	N/A	27.50%
SIUC Lab (NR)	C	May 2016	306.2 ng/L (276.2–339.6) [a]	40	N/A	0.00%
SIUC Mosher (R)	D	December 2015	265.9 ng/L (218.7–323.3) [a]	40	N/A	30.39%
SIUC Mosher (R)	D	May 2016	124.3 ng/L (76.51–202.0) [b]	40	N/A	47.50%
Sodium chloride						
SIUC Lab (NR)	C	October 2015	6739 mg/L (6550–6933) [a]	40	54.18	N/A
SIUC Lab (NR)	C	April 2016	6345 mg/L (6087–6615) [a]	40	55.11	N/A
Field Mojave (NR)	D	November 2016	6882 mg/L (6510–7275) [a]	30	N/A	29.09%
SIUC Mosher (R)	D	October 2015	5637 mg/L (5370–5917) [b]	40	N/A	5.00%
SIUC Mosher (R)	D	April 2016	5897 mg/L (5605–6205) [a]	40	31.65	N/A
Field Mosher P0 (R)	D	November 2016	5222 mg/L (4792–5690) [b]	30	N/A	7.37%
Copper (II) sulfate						
SIUC Lab (NR)	C	October 2015	69.14 μg/L (60.17–79.45) [a]	40	N/A	37.97%
SIUC Mosher (R)	D	November 2015	44.14 μg/L (39.81–48.94) [b]	40	111.4	N/A

Comparisons for each toxicant were made across species and dates. Superscript letters denote statistically different LC50 values (without overlapping confidence intervals) for each toxicant. N represents the average number of individuals exposed at each test concentration, the Wald chi-squared (X^2) represents goodness of fit calculated for tests in which Probit analysis was appropriate, and the Trimmed Spearman-Karber values (TSK) are provided for tests that required trim. N/A = not applicable.

Copper concentrations were measured using a Varian AA240Z (Agilent Technologies, Santa Clara, CA, USA) graphite furnace (GFAAS) following the method for copper extraction from water in Standard Methods for Examination of Water and Wastewater (APHA, 2005). At time zero and at 96 h, 100 mL samples were preserved with 5 mL of TraceMetal grade nitric acid (APHA, 2005). Because hardness and alkalinity impact metal toxicity, they were measured in the RMHRW used for the copper toxicity tests using standard protocols (APHA, 2005).

Chloride concentrations were measured using an Orion 4-star pH/ISE Meter (Thermo Scientific, Beverly MA, USA) combined with an Accumet Chloride Combination Ion Selective Electrode (Fisher Scientific). The chloride probe was calibrated using standard solutions (Fisher Certified Standard Solutions 0.1 M chloride solution ± 5% chloride and 1000 ppm chloride solution ± 5% chloride).

2.9. Quality assurance/quality control

Samples analyzed on the GC-ECD were spiked with the surrogate DCBP prior to extraction to verify extraction efficiency and evaluate any compound loss. Matrix spikes (MS), matrix spike duplicates (MSD), and lab control blanks (LCB) were also included with every experiment to ensure the quality of each extraction. Acceptable quality assurance results included percent recoveries in the range of 80%–120% for DCBP from the MS, MSD and LCB and the target compound from the MS and MSD (You et al., 2004). When analyzing samples with the GFAAS, the absorbance of each sample was read twice and relative standard deviation between the two measurements needed to be below 25% or the sample did not pass quality assurance. Laboratory control blanks with 18 MΩ deionized water and control water used in the toxicity test were used to measure instrumental background interference and to demonstrate that there was no detectable copper in the control water. Every tenth sample was injected twice to assure reproducibility between injections. In addition to calibration standards, an external quality check of known concentration was conducted near the beginning and at the end each run. Calibration of the chloride electrode following instructions provided with the probe by Fisher Scientific was performed to assure accuracy and precision of measurements.

2.10. Statistical testing

For all toxicity tests, the LC50 and associated 95% confidence interval values for individual toxicity tests was determined by log-probit analysis using SAS software (SAS Institute Cary, NC, USA). If the data were not normally distributed, the Spearman-Karber non-parametric method was used employing the U.S. EPA Trimmed Spearman-Karber (TSK) software (version 1.5). The LC50s of the individual toxicity tests were considered significantly different if the 95% confidence intervals of any two populations did not overlap. For the thermal tolerance and reproduction tests, results were compared using nested ANOVA calculated using SAS software.

3. Results

3.1. Genotyping

Twenty resistant Mosher animals held in pyrethroid-free culture for 16 months and 10 animals taken directly from Mosher Slough were genotyped. The *H. azteca* were screened for the two non-synonymous mutations leading to the M918L, L925I, or L925V substitutions occurring on the *vgsc* previously correlated with pyrethroid resistance in *H. azteca* (Major et al., in press; Weston et al., 2013). All *H. azteca* were homozygous for the wild-type methionine allele at the 918 position. At the 925 position, the wild-type L925 allele was not detected in cultured or field-collected organisms. Of the 20 laboratory-cultured Mosher animals, 17 animals were homozygous for the L925I allele and three were heterozygous with one copy of the L925I and one copy of a L925V mutation. Similarly, in the wild-caught organisms, seven of 10 organisms were homozygous for L925I and three were heterozygotes for the L925I and L925V alleles. Additionally, allele frequencies were compared among populations using Genepop 4.4 (Rousset, 2008). Allele frequencies did not differ between the cultured and field-collected populations (Fixation index (F_{ST}) = −0.003, p = 0.655). Ten *H. azteca* from both the SIUC Lab and Field Mojave populations were also screened for the substitutions. All animals from these populations were homozygous wild-type at both the 918 and 925 positions.

3.2. Reproductive study

Survival from test initiation (known age, 6- to 8-d old *H. azteca*) to 42-d was ≥90% in all replicates for both the non-resistant SIUC Lab and resistant SIUC Mosher populations. Non-resistant *H. azteca* had approximately twice the reproductive rate compared to the resistant animals. Average offspring/female was significantly higher ($F_{1,18} = 13.90$, $p = 0.0015$) for the non-resistant population (5.31 average offspring/female), compared to the resistant population, which had an average of 2.77 offspring/female (Fig. 1).

3.3. Thermal tolerance

The average immobilization temperature of the SIUC Lab population was 41.96 °C (±0.07 °C standard error (SE)) and greater than the immobilization temperature for SIUC Mosher animals (41.10 °C) (±0.07 °C SE) (Fig. 2). As shown by a two-tailed *t*-test, the two populations had highly significant different average immobilization temperatures ($F_{1,8} = 53.60$, $p < 0.0001$). The Field Mojave population had an immobilization temperature of 41.60 °C (±0.05 °C SE) and the Field-collected Mosher Slough animals had an immobilization temperature of 39.64 °C (±0.07 °C SE) (Fig. 3). The Field Mojave population had a significantly higher average immobilization temperature ($F_{1,6} = 52.27$, $p < 0.0001$) than the Field Mosher animals, despite both populations belonging to the same clade. No statistical comparisons were made between the two SIUC cultured populations and the two field-collected animals because each pair

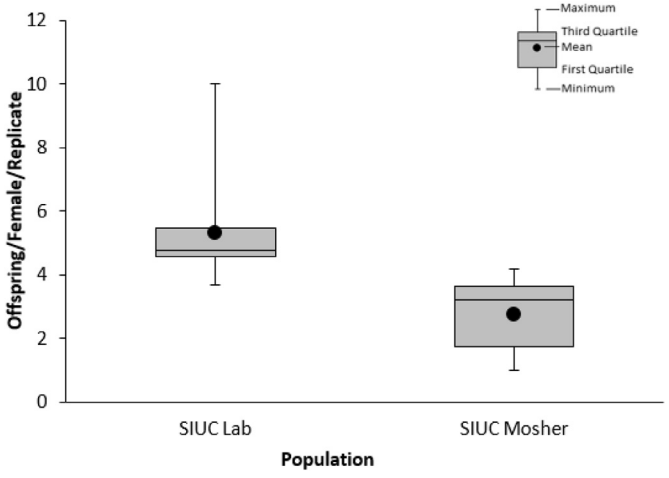

Fig. 1. Box and whisker plot showing the average offspring produced per female in the SIUC Lab and SIUC Mosher populations. Whiskers show range of offspring/female from individual replicates. A nested ANOVA was used to determine significant difference between populations ($F_{1,18} = 13.90$, $p = 0.0015$).

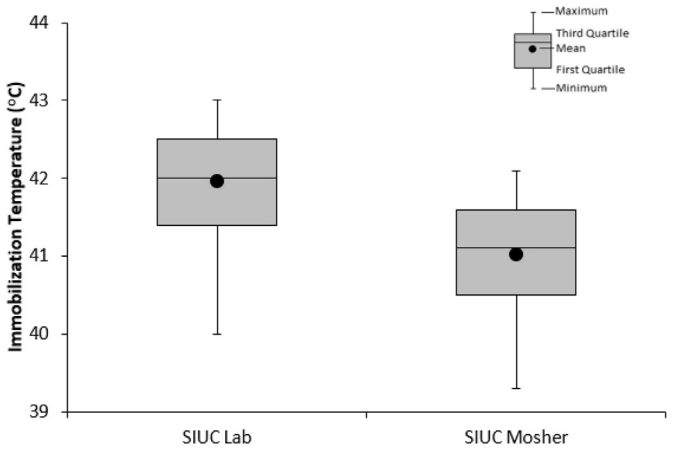

Fig. 2. Box and whisker plot showing immobilization temperatures of SIUC Lab and SIUC Mosher populations, tested concurrently in April, 2016. Whiskers show immobilization temperature range. A nested ANOVA was used to determine significant difference between populations ($F_{1,8} = 53.60$, $p < 0.0001$).

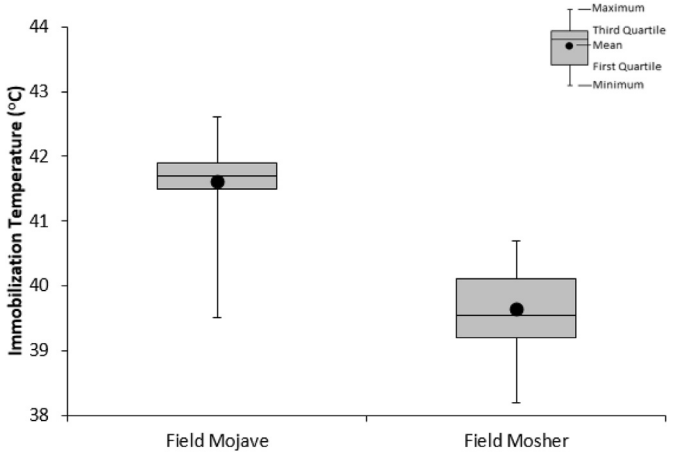

Fig. 3. Box and whisker plot showing immobilization temperatures of field-collected Mojave and Mosher populations, tested concurrently in November, 2016. Whiskers show immobilization temperature range. A nested ANOVA was used to determine significant difference between populations ($F_{1,6} = 52.27$, $p < 0.0001$).

of tests was performed at different times (April 2016 and November 2016, respectively), however on both occasions, the pyrethroid-resistant group was less able to tolerate elevated temperatures.

3.4. Toxicity tests

For tests using permethrin and DDT, the average and range of recoveries of DCBP from all water samples was 103% (81%–119%). The recovery of DCBP from blank and matrix spiked samples was 105% (84%–119%). The average recovery of permethrin and DDT from matrix spike samples was 100% (84%–120%) and 105% (87%–113%), respectively. For tests with copper (II) sulfate, the average concentration of the external quality check was 103% (96%–105%). All target compounds were below detection limits for blank samples. Measured concentrations of target compounds are the average of the time 0 (after 18 h incubation period) and 96 h extractions, while permethrin exposure concentrations averaged 60% (34%–97%) of nominal values. DDT exposure concentrations averaged 59% (40%–78%) of nominal values. Copper (II) sulfate exposure concentrations averaged 78% (72%–92%) of nominal. The NaCl concentrations relative to the nominal averaged 100% (88%–112%).

Survival of *H. azteca* in negative and solvent controls exceeded 95% for all toxicity tests for all populations. For the SIUC Lab and SIUC Mosher populations, toxicity tests were conducted twice for permethrin, DDT, and NaCl, and the tests with copper were conducted once.

The populations referred to herein as non-resistant, and lacking the L925I or L925V mutations, had permethrin LC50s of 31–45 ng/L. In comparison, the resistant populations with the mutations had LC50s of 1144–3310 ng/L (Table 1). The SIUC Lab population was the most sensitive to the pyrethroid, with the Field Mojave population being only slightly less sensitive. The most resistant population was collected from Mosher Slough and tested promptly after collection (3310 ng/L). The LC50 of the F1 generation declined to 1803 ng/L. This LC50 is comparable to the Mosher animals in culture for 22 months (1144–1668 ng/L), which is an indication that some of the resistance was lost in the F1 generation. But even when held in culture, Mosher animals remained approximately 40 times less sensitive than non-resistant populations, including those belonging to the same clade (i.e. Mojave).

DDT sensitivity was tested on two occasions with the SIUC Lab and SIUC Mosher populations. On both occasions, the SIUC Mosher animals were more sensitive (124–266 ng/L versus 306–341 ng/L), but the difference was statistically significant only one time. Similar results were observed for NaCl, with the SIUC Mosher animals seemingly more sensitive than SIUC Lab (5637–5897 mg/L versus 6345–6739 mg/L), but showing a statistically significant difference in only one of the two trials. The Field Mojave population was similar to the SIUC Lab population in NaCl sensitivity, with the Field Mosher population having a statistically lower LC50, and comparable to the SIUC Mosher animals. Again, sensitivity differences were better explained by resistance genotype rather than clade association.

Copper (II) sulfate tests were performed only once for the SIUC Lab and SIUC Mosher populations. The Mosher animals had statistically significant greater sensitivity (44 versus 69 μg/L). Thus, for all non-pyrethroid toxicants tested, the consistent pattern across six tests was for the pyrethroid resistant Mosher organisms to be more sensitive to toxicants than either of the non-resistant populations, both in comparisons between and within clades. However, this conclusion was not definitive, since statistically significant differences were lacking in two of the six tests (a DDT and NaCl trial).

4. Discussion

In 2010, populations of pyrethroid-resistant *H. azteca* were discovered at five sites in central California (Weston et al., 2013). These *H. azteca* had LC50s to cyfluthrin and bifenthrin up to 550 times higher than the standard laboratory-cultured *H. azteca* widely used for toxicity testing. The *vgsc* of these animals was sequenced revealing that resistant animals had nucleotide substitutions leading to either of two amino acid changes associated with pyrethroid resistance in pest species: the M918L or L925I mutations. One resistant population from a site other than those used in the present study was cultured in the laboratory and there was no loss of resistance in juveniles removed from the culture after three months (Weston et al., 2013). Our study confirmed these results at the Mosher site as well, but over nearly two years of culture. Resistance did decline approximately two-fold between freshly collected Mosher animals and the F1 generation, but then remained approximately 40-fold higher than non-resistant animals for nearly two years. We believe this suggests that acclimation may play a minor role, but the presence of mutations in *H. azteca* that have previously been well-documented to confer pyrethroid

resistance, and persistence of resistance over multiple generations in pyrethroid-free culture both indicate genetic adaptation is the dominant factor in the pyrethroid resistance seen in Mosher Slough organisms.

The Mosher Slough organisms from the present study were sequenced after nearly a year and a half in culture without exposure to pyrethroids. They had retained the L925I mutation previously reported (Weston et al., 2013), and no wild-type alleles were found. Interestingly, sequencing 20 animals from the long-term culture and 10 animals collected directly from Mosher Slough in 2016 revealed two mutations at the same locus: the wild-type leucine was most commonly replaced with an isoleucine (L925I), but in a small number of individuals it had been replaced by valine (L925V). The L925V mutation was also noted in animals collected from Mosher Slough in a recent collection (Major et al., in press).

Because the *H. azteca* collected from Mosher Slough contain a resistance mutation, genetically distinct from the wild type, and maintained that mutation in culture without continued pyrethroid exposure, we asked: does this change have a cost to the organisms in their ability to reproduce and survive in a given environment? Population fitness could be impacted by this change if the selection event (exposure to pyrethroids) substantially reduced the genetic variation in the population by means of a population bottleneck (Van Straalen and Timmermans, 2002), "genetic hitchhiking" of less fit traits that may be linked to the mutation (Van Straalen and Timmermans, 2002), or a reduction in overall fitness because of a potential decrease in efficiency of the Vgsc and related metabolic costs (Zhao et al., 2000). The present study aimed to assess fitness of *H. azteca* populations in a variety of laboratory tests. Initially, two laboratory-cultured populations were compared (one with documented resistance to pyrethroids, one wild-type with no previous pyrethroid exposure). To remove possible clade effects, two field-collected *H. azteca* populations were compared in similar tests.

Our finding of the reproductive rate in the resistant *H. azteca* to be only half that of non-resistant animals (Fig. 1) is of interest because high population density is important for success in species like *H. azteca* that may have high mortality rates due to predation or exposure to stressors. *H. azteca* have been identified as an important food source for many fish and waterfowl, for example, providing up to 97% of the diet of female white-winged scoters (*Melanitta fusca*) nesting in Saskatchewan, Canada (Brown and Fredrickson, 1986), or a major diet component in the gut contents of many fish species (Toft et al., 2003). With high predation rates, high fecundity is necessary in order for some individuals to reach sexual maturity. A reduced reproductive rate linked to pyrethroid resistance in wild populations could impair the long-term sustainability of that population, even if it avoids extinction due to the immediate stressor of pyrethroids. It is important to note, however, that the decreased reproductive output of the resistant strain could be due to differences between the high-performing laboratory-cultured strain and a more recently collected population that is not as well adapted to the laboratory culture conditions. Soucek et al. (2015) noted that the U.S. Lab Strain of *H. azteca* benefitted from increased chloride concentration when compared to other strains. It is also important to note that reproductive differences across clades have been measured in tests performed by the same laboratory (Major et al., 2013). We recognize that our comparison was not ideal (and that the reproductive test may not be conclusive on its own). However, the results are consistent with the results from the other tests that indicate that the resistant animals may be less fit. It is difficult and time-consuming to "domesticate" new populations to a point where they are surviving, growing, and reproducing in the lab and the two populations used for the reproductive study were cultured long-term. Additionally, it was not feasible (or environmentally-responsible, in the case of the Mojave Desert animals, where the native population was limited) to collect the number of organisms needed to "domesticate" a population to laboratory conditions.

Water temperature is an important abiotic variable in aquatic ecosystems that can influence physiological mechanisms at the enzymatic and cellular levels and alter metabolic rates (Ward and Stanford, 1982). Because *H. azteca* often inhabit shallow freshwater lakes and ponds (Cooper, 1965), their natural habitats may be sensitive to changing temperatures from both natural causes and those attributable to human activities including thermal pollution from factories, power plants, and agricultural irrigation return flow as well as changes in land use, removal of riparian vegetation, and river regulation (Olden and Naiman, 2010). The resistant animals were immobilized at a lower temperature than the non-resistant animals, both in comparisons between clades (SIUC Lab versus SIUC Mosher) and within a given clade (Field Mojave versus Field Mosher), and thus resistant animals appear more susceptible to thermal stress.

When exposed to other chemical substances, the resistant animals showed a frequent tendency to be more sensitive to these stressors, though results were not definitive because of limited testing for some of the contaminants (copper (II) sulfate), or differences consistent in direction but not statistical significance for others when tested multiple times (DDT, sodium chloride). Elevated copper concentrations are exceptionally toxic to aquatic invertebrates and are known to cause gill damage in aquatic invertebrates, like *H. azteca* (Eisler, 1998), so increased copper sensitivity of a pyrethroid-resistant population could prove detrimental in some environments. *H. azteca* is generally considered to have a broad salinity tolerance, and is used for toxicity testing of both fresh and estuarine waters with salinities up to 15 ppt (Nebeker and Miller, 1988), thus a reduced tolerance to sodium chloride could restrict exploitable habitats. However, the concerns raised by the present study's results are not necessarily specific to the particular chemicals tested, but rather the potential that selection for pyrethroid resistance could reduce the ability of the individual to cope with other chemical stressors in general. Our findings suggest this may be the case, and we recognize that a more rigorous study is needed to fully assess fitness costs of pyrethroid resistance to wild populations of *H. azteca.* While our results demonstrated increased sensitivity to stressors and lower reproductive rates, we were not able to conduct all tests with all populations because of limited funding and numbers of animals available at collection sites. The repeated tests (sodium chloride and thermal stress) were selected as the most relevant to climate change implications on all populations including resistant and wild-type populations from the same clade.

H. azteca are important participants in aquatic food webs, assisting in nutrient cycling and providing a food source for predatory fish, waterfowl, amphibians, and larger invertebrates. If, as suggested by our findings, *H. azteca* populations can be negatively affected by the development of resistance to pyrethroids and have decreased ability to tolerate potential natural and anthropogenic stressors, they and organisms that rely on them may face adverse consequences. Additionally, it has been shown that pyrethroid-resistant *H. azteca* are able to survive exposure to higher pyrethroid concentrations, and accumulate far higher pesticide body burdens, than non-resistant individuals that simply die at low exposure levels. The greater tissue concentrations of pyrethroids and their metabolites that are attained provide a route of trophic transfer of these residues to their predators that would be lacking where only non-resistant individuals are present (Muggelberg et al., 2016). Thus, while the genetic mutations associated with pyrethroid exposure provide the obvious benefit of allowing survival of a population in an area from which it would otherwise be

eliminated, it would be a mistake to view these changes as solely a positive development. The greater potential for trophic transfer of pesticides to predators, as previously documented, or the greater sensitivity to other environmental stressors, as shown by the present study, have fitness costs for the resistant species and risks for other species that depend upon it. Given current trends in global pyrethroid use, and the potential that resistance may have emerged elsewhere beyond where it has already been confirmed in California (Pathammavong, 2016), these costs of resistance merit assessment elsewhere.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2017.12.043.

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