SUBLETHAL EFFECTS OF REPEATED PESTICIDE EXPOSURE ON BLUE CRABS (*Callinectes sapidus*) IN THE GULF OF MEXICO

Prepared by

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ABSTRACT

Insecticides targeting terrestrial arthropod pests may enter aquatic habitats via overspray and runoff, affecting populations and behaviors of nontarget species. Blue crabs (*Callinectes sapidus*) play a significant role in estuarine food webs as both predators and prey, and alterations in their abundance or behavior can significantly affect community structure and function. Like insects, blue crabs are arthropods, and are susceptible to insecticides applied for pest control, which may increase their mortality. Although not as thoroughly investigated, sublethal effects of insecticide exposure can alter blue crab foraging and predator avoidance ability, affecting estuarine food webs. Repeated exposure to insecticides may increase negative effects, but few studies have assessed cumulative effects on nontarget organisms after multiple exposures. I examined how two consecutive exposures of 50 ppb of malathion, an insecticide often used for mosquito abatement, affected blue crab behavior, and examined crab recovery following pesticide removal. Malathion can occur in concentrations as high as 800 ppb in coastal waters and increases blue crab mortality at 100 ppb. To focus on sublethal effects of malathion, I used a concentration of 50 ppb because this concentration interfers with blue crab neuromuscular function but does not significantly increase mortality. I then assessed changes in neuromuscular function, foraging ability, and responses to predation risk cues in blue crabs exposed to malathion in three static non-renewal treatments: control, repeated exposure, and recovery (following single exposure). Sublethal concentrations of malathion altered the behavior of both adult and juvenile blue crabs. Malathion exposure led to a 40% decrease in the blue crabs' ability to right itself when placed on their backs, but, crab righting time behavior returned to levels observed in the control treatment after crabs were placed in malathion-free water for 96 hours. Malathion exposure also affected eyestalk reflexes, causing normal responses to decline by 50%

in adults and 75% in juveniles, with partial recovery taking place in both life stages following pesticide removal. Malathion exposure affected foraging ability, causing blue crabs to seek food more frequently, even in the presence of alarm cues from injured blue crabs. Despite being more willing foragers, adult blue crabs were less able to locate food after pesticide exposure. Malathion, at environmentally-occurring concentrations, interfered with blue crabs' ability to forage and avoid predators. However, two exposures to malathion at 50 ppb did not increase mortality nor further impair behavior, and crab behavior resumed to levels measured before exposure after 96 hours in seawater without insecticides.

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INTRODUCTION

Estuarine ecosystems are dynamic, highly productive, and support a diverse assemblage of plants and animals (Day et al. 1989, McLusky 1989, Scowcroft et al. 2001, NOAA 2008, Grove 2001, Rosen 2014, SeaWeb 2014). Blue crabs are an important species in many estuarine communities (Post et al. 2008). They are both economically and ecologically important (Kennedy and Cronin 2007), both as a commercial fishery and an important component of estuarine food webs (Zinski 2006). Although blue crabs play an integral part in food web dynamics in many estuaries around the United States, their populations are declining (VanderKooy 2013). This decline has been largely attributed to overfishing and disease, with the potential role of pollution largely overlooked.

Pesticides are used worldwide to improve crop yield by reducing insect and weed pests and have been in use for centuries (Sujatha et al. 1999). Pesticides often travel beyond the area in which they are applied and harm nontarget organisms. For example, with aerial application, only 10% of the pesticide reaches the intended crop area, with only 0.1%-5% reaching the actual target organism (NOAA 2008). The remaining pesticide is dispersed to unintended locations and often enters the water via overspray or runoff, affecting nontarget organisms such as fish and invertebrates (Bergamaschi et al. 2001). Some pesticides are also sprayed directly into wetlands to kill mosquitos or other pests, but ultimately harm other organisms such as amphibians (Relyea and Hoverman 2006, Relyea and Diecks 2008).

Malathion is a broad spectrum, non-systemic organophosphorus insecticide used in both crop and non-crop applications (Coppage and Matthews 1974, HSDB 1993, Camp 2011). It was introduced in the 1950s and is commonly used for crop pest management and mosquito abatement (Camp 2011). In 2001, 9.1 million kilograms of malathion were applied in the United States, seventy-five percent of which were applied in Texas (National Agricultural Statistics Service, www.pestmanagement.info/nass/, U.S. Environmental Protection Agency [U.S. EPA] http://www.epa.gov/oppsrrd1/op/malathion/efedrra.pdf, Camp 2011). Malathion inhibits the function

of acetylcholinesterase (AChE), an enzyme that functions in removing acetylcholine (Ach) (Matsumura 1985). Inhibition of AChE results in an increase in Ach in the postsynaptic cleft, overstimulating muscular target cells (Matsumura 1985), often leading to a decreased functionality of organisms within their environment. This overstimulation of cells in the body can lead to uncontrolled muscle movements, slow reflexes, breathing problems, paralysis, and even death (Matsumura 1985, Dell'omo et al. 1996, Key et al. 1998, Lundebye et al. 1997, Scholz et al. 2000, De Guise et al. 2004, Ferner et al. 2005, Newhart 2006, McCarthy and Fuiman 2008, Wendel and Smee 2009). Malathion and other AChE inhibitors are present in many estuarine environments and can cause significant reductions in fish and invertebrate populations (Coppage and Matthews 1974, De Guise et al. 2004). Malathion occurs in the United States at concentrations from 1-800 ppb (Guerrant et al. 1970, Bradley et al. 1997, Newhart 2006) and has been shown to negatively impact aquatic arthropods (De Guise et al. 2004, Wendel and Smee 2009). Within the bays of Hale County, Texas, malathion concentrations were reported as high as 500 ppb shortly after aerial application (Guerrant et al. 1970) but were often present at levels less than 100 ppb (Bradley et al. 1997). In California, coastal malathion concentrations were heavily influenced by runoff, with local bay concentrations increasing from 11.2 ppb following aerial application to about 44.2 ppb following a rainfall event (Bradley et al. 1997). These concentrations are known to alter blue crab behavior (Wendel and Smee 2009). Malathion is toxic to most freshwater fish species, including sheepshead minnows (*Cyprinodon variegatus*) and striped bass (*Morone saxatilis*) (Martinez and Leyhe 2004). American lobsters (*Homarus americanus*) exhibited a 50% mortality rate following malathion exposure at concentrations as low as 38 ppb, with lower concentrations having substantial sublethal effects. Malathion at low concentrations increased lobster susceptibility to the parasite *Paramoeba spp*., significantly increasing mortality (De Guise et al. 2004). Malathion was also linked to 64% of the pesticide related fish kills in US waters between 1980 and 1989 (Camp 2011).

Malathion, like other organophosphate pesticides, has a relatively short half-life of approximately one week and was not thought to persist long enough to adversely affect nontarget organisms. However, frequent applications and toxic intermediate chemicals formed during malathion breakdown have caused unexpected, harmful side effects (Coppage and Mathews 1974, Bradley et al. 1997, De Guise et al. 2004, Martinez and Leyhe 2004, Relyea 2004, Wendel and Smee 2009).

This study determined if repeated exposure of blue crabs to malathion caused additive, negative effects, as well as if blue crabs recovered following exposure. Sublethal concentrations of 50 ppb were used because this concentration can impair neuromuscular function but is not usually lethal, permitting studies of multi-exposure effects on mortality and behavior. All experiments were done in a controlled setting, under optimal conditions, leading to a conservative estimate of how malathion would negatively affect blue crab behavior.

MATERIALS AND METHODS

The effects of malathion on blue crabs were studied at Texas A&M University - Corpus Christi and the CCA Marine Development Center in Corpus Christi, TX. Static, non-renewal tests were used to mimic low water exchange rates typical of estuaries in the Western Gulf of Mexico. This approach allowed pesticides that enter estuaries to accumulate until they breakdown. To test the effects of repeated malathion exposure on blue crabs and recovery after exposure, 3 exposure regimes or treatments were used including: crabs exposed to malathion for 2 consecutive 96 hour periods (henceforth repeated exposure), crabs exposed for 96 hours followed by 96 hours in pesticide-free water (henceforth recovery), and crabs that were never exposed (control- see Table 1).

Seawater was prepared using dechlorinated tap water and Instant Ocean ™ at a salinity of 20 ppt. Prior to the experiment, all glassware were cleaned three times using a 1% liquinox solution, rinsed with DI water, soaked in 10% nitric acid for a minimum of 2 hours, rinsed three more times with DI water, and sprayed with acetone. The glassware was then set aside to dry for a minimum of 48 hours prior to use. Malathion treatments were prepared by making a 10 parts per million (ppm) stock solution composed of 8.13µL of malathion (Sigma-Aldrich®, molar mass: 330.36 g/Mole), 10 mL of ethanol (improves malathion solubility), and 990 mL of deionized water. The stock solution was heated to 25˚C and mixed for 30 minutes and 260 mL of stock solution was then added to 52 L of seawater to make a 50 parts per billion (ppb) solution of malathion. The water was then distributed to appropriate 10 L glass bowls. Control solutions were prepared in the same manner, including ethanol, but without the malathion addition. The second exposure treatment was prepared in the same manner and crabs were moved into new bowls of freshly made water that either contained a second addition of malathion or control water. Throughout the experimental process, 38 adult and 100 juvenile crabs were collected and randomly assigned to the various treatment groups with no mortality occurring. Controls

and both pesticide treatments were performed simultaneously throughout the experiment to make sure results were consistent throughout the entire experiment.

To measure sublethal effects of malathion, behavioral endpoints known to reflect neuromuscular function in blue crabs were selected and measured including: righting time, eyestalk retraction responses, and foraging activity (Abramson and Feinman 1988, Abramson et al. 1988, Blakeslee et al. 2015, Ferner et al. 2005). These behaviors reflect the crabs' coordination, protective reflexes, ability to forage, and reactions to alarm cues from injured conspecific cues, which may have important ecological ramifications for blue crabs (Abramson and Feinman 1988).

Experiment 1

Experimental Design

To test the effects of the multiple pesticide exposures on blue crabs, I measured sublethal effects of malathion on juvenile (8-59 mm carapace width) and adult (60+ mm carapace width) blue crabs. Blue crabs were collected from estuaries near Corpus Christi, TX and acclimated in aerated tanks with salinity 20 ppt for a minimum of 48 hours prior to each experiment. Crabs were exposed to either control or malathion treatments (described in Table 1) in individual, aerated 10-Liter glass bowls. Treatment crabs were exposed to 50 ppb of malathion in a static test for 96 hours. The crabs were then transferred to a second bowl, containing either freshly prepared malathion-treated water or control water without malathion for an additional 96 hours. Transferring the crabs exposed them to malathion in a second static test or to pesticide-free water to determine if recovery was possible. Bowls were surrounded by cardboard to minimize external stimuli such as neighboring crabs in other bowls.

Table 1. Summary of the various treatments that were completed in this experiment

Treatment 1 (Day 1-4)	Treatment 2 (Day 5-8)	Purpose
Control	Control	Baseline data that is used to compare to other treatment categories
Exposed to 50 ppb malathion	Exposed to 50 ppb malathion	Multiple treatments to look at impacts of multiple exposers to the same pesticide
Exposed to 50 ppb malathion	Control	Recovery of organism

Testing Crab Responses to Repeated Malathion Exposure

Sublethal effects were quantified by measuring changes in several behaviors that reflect neuromuscular function at 0, 1, 96 and 192 hours post exposure and comparing them between treatment and control animals (Abramson and Feinman 1988, Abramson et al. 1988, Blakeslee et al. 2015). Behavioral assays were video recorded, and measurements were made from the video to minimize errors. Behaviors were recorded at time zero, one hour after exposure, and at 96 hours increments and changes in behavior were analyzed by taking the percent change between behaviors before and after exposure, to account for any inherent differences among crabs.

Righting time, the time needed for a crab to correct itself after being placed on its back, was chosen as a measure of coordination (Wendel and Smee 2009, Blakeslee et al. 2015). The crab was flipped onto its carapace, and the time needed to return to its normal, upright position on its walking legs was recorded. The change in righting time was calculated by subtracting the time needed to right itself at time zero from the value after being exposed and accounted for any inherent differences among individual crabs. The percent change from time zero was then calculated.

In addition to righting time, crab eyestalk responses were measured by passing a small spatula alongside each crabs' eyestalk. Normally this causes the crab to retract its eyestalk and immediately return to the extended position following the cessation of the stimulus. An abnormal response was for the crab to retract the eyestalk, and for the eyestalk to remain retracted for more than 2 seconds after

cessation of the stimulus (Abramson et al. 1988). Data were recorded binomially as either normal or abnormal.

Statistical Analysis

SAS© (version 9.1; SAS Institute) was used for all statistical analyses. Preliminary analysis was performed using General Linear Models with life stage (adult, juvenile) and toxicant exposure (repeated, recovery, control) as fixed factors with time as a repeated measurement (Sokal and Rohlf 1995). Comparisons were then performed using procedure GENMOD where link function gamma was used to analyze continuous variables and link function binomial was used to analyze dichotomous responses. The percent change in righting time was log transformed to manage skewness within the dataset. Previous studies and preliminary work did not reveal significant differences between male and female crabs, and thus, differences between sexes were not reported (Wendel and Smee 2009, unpublished data). More than 90% of the crabs used were male, and females were evenly dispersed among treatments.

Experiment 2

Experimental Design

A laboratory flume (Figure 1) was used to examine the effects of malathion exposure on crab responses to food cues. Crabs were maintained in glass bowls for 8 days, without food, where they were exposed to the three treatments described above (Table 1), as well as a fourth treatment in which crabs were examined after 48 hours of 50 ppb malathion exposure (henceforth acute exposure). Following exposure, crabs were transported to the CCA Marine Development Center, and their foraging behaviors were examined in a y-flume (Figure 1). The crab was placed at the terminal end of the flume inside a vexar cage (1.0 cm^2 openings), and a food source consisting of 2 grams of cooked,

thawed, Penaid shrimp was placed on one specific side of the flume upstream of the crab. Water flow was ~4.6 cm sec^{-1}, and food cues were placed on alternating sides of the flume between each assay (Smee and Weissburg 2006). Crabs were allowed to acclimate in the flume for 5 minutes. Then, the vexar cage was lifted, allowing the crabs to traverse the flume, and their behavior recorded until they located the food source or searched unsuccessfully for 5 minutes. A cover was placed over the top of the flume during the experimental process in order to minimize external stimuli.

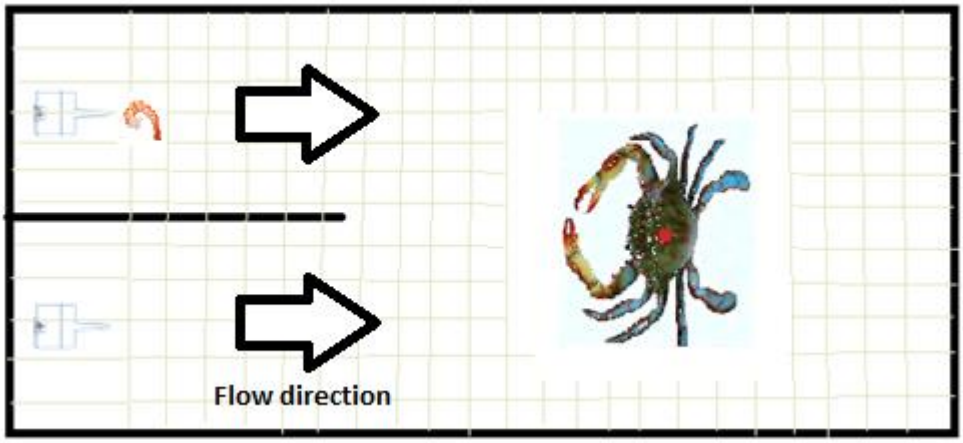

Figure 1. Schematic of flume experiment (top view).

Testing Crab Responses to a Prey Cue after Exposure

Sublethal effects of malathion exposure on foraging was quantified by measuring the ability of the crab to locate the food source. Data was recorded binomially as either normal or abnormal, with normal meaning that the crab was able to successfully locate the food cue by selecting the side of the flume on which the food was present. During each 5 minute assay, I also recorded binomially whether crabs moved directly upstream without downstream movement and without stopping or if they tended move with repeated stops and directional changes.

Statistical Analysis

The number of crabs able to successfully locate the food and the number moving upstream during the location process was calculated and compared among treatments using procedure GLM, with life stage (adult, juvenile) and toxicant exposure (acute, repeated, recovery, control) as fixed factors (Sokal and Rohlf 1995). Contrasts were performed using procedure GENMOD with link function binomial to analyze dichotomous responses.

Table 2. Summary of the various treatments that were completed in this experiment

Treatment	Explanation	Purpose
Control	Placed in water containing 1 ppm of pure acetone for 8 day period	Baseline data that is used to compare to other treatment categories
Prolonged Exposure	Exposed to 50 ppb malathion twice over an 8 day time period	Multiple treatments to look at impacts of multiple exposers to the same pesticide
Crabs able to recover	Exposed to 50 ppb of malathion for 4 days, followed by a control period	Recovery of organism
Acute exposure of the organism	Exposed to 50 ppb of malathion for 48 hours	Interested in seeing if there is a difference between acute and prolong exposure on the organisms behavior

Experiment 2b

Testing Crab Responses to a Negative Cue after Exposure

I also tested whether malathion would affect crab responses to alarm cues when paired with a food cue. Malathion-exposed crabs often moved upstream without stopping or changing direction. I hypothesized that malathion-exposed crabs might move in this manner because pesticide exposure increased their energetic needs, making them more motivated foragers, or because the pesticide inhibited their fear response(s). To test these hypotheses, I repeated the flume experiments described in Experiment 2a, but I also placed an injured blue crab alongside the food cue because blue crabs move

more slowly with frequent directional changes in the presence of injured conspecific cues (Ferner et al. 2005, Moir and Weissburg 2009). Injured blue crabs were 50-74 mm carapace width and were injured by inserting a thin metal rod into the crab's carapace (Ferner et al. 2005). I repeated the flume experiments as previously described and recorded the crabs' movement upstream and downstream, as well as their decision on whether or not to select the side of the flume with the cue. I also measured 2 additional behaviors: percent time that the crab spent moving during the acclimation period (inside the cage) and percent time moving after the crab was released and allowed to move about the flume.

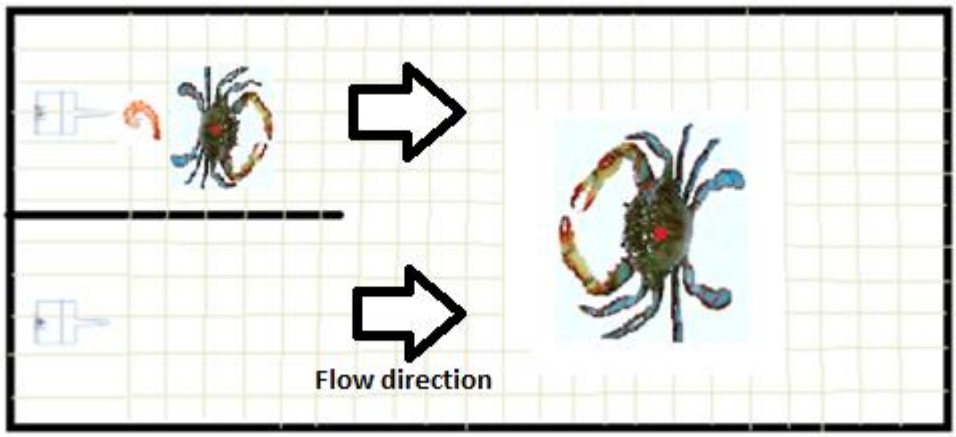

Figure 2. Schematic of flume experiment (top view)

Statistical Analysis

The number of crabs selecting the side of the flume with the cue was recorded binomially as was their directional response. The percent time spent moving during and after the acclimation was also analyzed using procedure GLM, with toxicant exposure (acute, control) (Sokal and Rohlf 1995) as a fixed factors since this procedure can be used for both dichotomous and continuous data.

RESULTS

Experiment 1, Righting time

Malathion had a negative effect on adult blue crab righting time, with a significant improvement in the righting ability following pesticide removal and placement in seawater without malathion (Table 3). A significant interaction between exposure treatment and hour was found (Table 3), indicating that changes in righting time varied by treatment. Adult crabs exposed to malathion took significantly longer to right themselves at hour 96 ($P<0.001$, Table 4, Figure 3) and hour 192 ($P=0.02$, Table 4, Figure 3) when compared to unexposed crabs. Righting time changed in blue crabs 1 hour post-exposure and was significantly different from the controls at $\alpha = 0.1$ ($P=0.07$, Table 4, Figure 3), with no significant difference between those crabs in the repeated exposure and recovery group at that time point ($P=0.37$, Table 4, Figure 3). Repeated exposure to malathion did not significantly amplify the behavioral effects of malathion, as evidenced by nonsignificant pairwise differences in righting time among repeated exposure crabs at hour 96 and 192 ($P=0.92$, Table 4, Figure 3). However, those crabs that were placed in pesticide-free water following 96 hours of exposure exhibited almost complete recovery in their behavior by hour 192, and were not significantly different from unexposed, control crabs ($P=0.69$, Table 4, Figure 3).

Like adults, malathion exposure led to increased righting time in juvenile crabs 96 and 192 hours post-exposure ($P<0.001$, Table 4, Figure 4). Righting time among juvenile crabs was highly variable following 1 hour of exposure (Table 4, Figure 4). Crab responses to pesticide exposure became more uniform by hour 96, indicating that some crabs were affected by the pesticide sooner than others. A second exposure to malathion significantly amplified the behavioral effects of juvenile blue crabs of at $\alpha = 0.1$ ($P=0.08$, Table 4, Figure 4). However, those crabs that were placed in pesticide-free water after being exposed for 96 hours exhibited almost complete recovery in righting time by hour 192, as evidenced by nonsignificant pairwise differences between unexposed, control

crabs and those allowed to recover (P=0.45, Table 4, Figure 4). Overall, malathion also had a negative effect on juvenile blue crab righting time, with a significant improvement in the righting ability following pesticide removal.

Table 3. PROC GLM results for percent change in righting time and eyestalk retraction response

	SS	*df*	*F* ratio	Prob>F
GLM results for percent change in righting time				
Treatment (Control, Repeated Exposure, Recovery)	71253.52	2	7.12	**<0.001**
Life stage (juvenile, adult)	1706.42	1	0.34	0.56
Hour (0, 1, 96, 192 hours)	31851.27	3	2.12	**0.01**
Treatment * Life stage	869.49	2	0.09	0.92
Treatment * Hour	62395.41	6	2.08	**0.06**
Life stage * Hour	12027.27	3	0.80	0.49
GLM results for eyestalk retraction time				
Treatment (Control, Repeated Exposure, Recovery)	0.38	2	80.85	**<0.001**
Life stage (juvenile, adult)	0.12	1	50.02	**<0.001**
Hour (0, 1, 96, 192 hours)	1.60	3	228.92	**<0.001**
Treatment * Life stage	0.002	2	0.55	0.60
Treatment * Hour	0.53	6	37.94	**<0.001**
Life stage * Hour	0.033	3	4.73	**0.05**

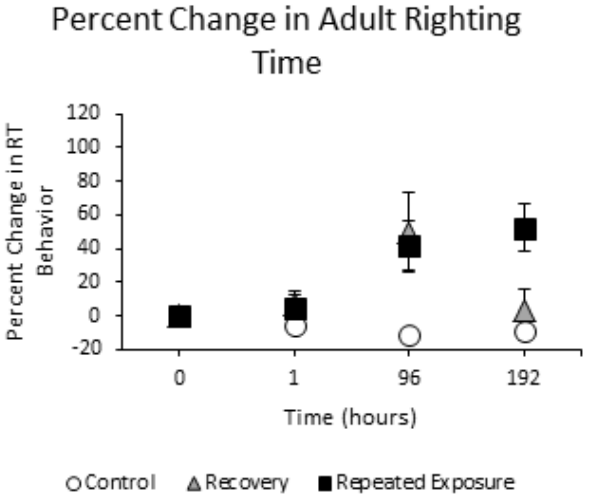

Figure 3. Mean + SE percent change in the righting time behavior of adult blue crabs. Control: never exposed; T*T: exposed twice; T*R: exposed and then allowed to recover; * indicates values significantly different from the control (α=0.05).

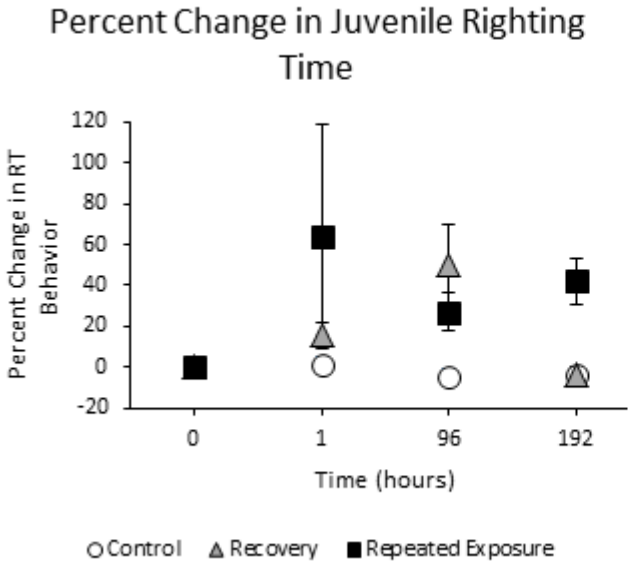

Figure 4. Mean + SE percent change in the right time behavior of juvenile blue crabs. Control: never exposed; T*T: exposed twice; T*R: exposed and then allowed to recover; * indicates that those values are significantly different from the control (α=0.05)

Table 4. Pairwise comparison results for adult and juvenile righting time data using PROC GENMOD

	Mean	Chi-Sq	Pr>ChiSq
(Adult) Hour 1 Control X Treatment	8.9034	3.39	**0.07**
(Adult) Hour 1 Treatment X Recovery	37.4136	0.80	0.37
(Adult) Hour 96 Control X Treatment	4.8875	8.54	**0.003**
(Adult) Hour 96 Treatment X Recovery	-124.745	0.04	0.84
(Adult) Hour 192 Control X Treatment	10.1705	5.16	**0.02**
(Adult) Hour 192 Control X Recovery	-28.0642	0.16	0.69
(Adult) Hour 96 vs 192 Treatment	337.3891	0.01	0.92
(Juvenile) Hour 1 Control X Treatment	7.5208	11.87	**<0.001**
(Juvenile) Hour 1 Control X Recovery	27.7970	0.59	0.44
(Juvenile) Hour 1 Treatment X Recovery	10.3104	5.47	**0.02**
(Juvenile) Hour 96 Control X Treatment	9.0572	13.10	**<0.001**
(Juvenile) Hour 96 Treatment X Recovery	76.1906	0.20	0.66
(Juvenile) Hour 192 Control X Treatment	6.7147	22.50	**<0.001**
(Juvenile) Hour 192 Control X Recovery	-28.3679	0.57	0.45
(Juvenile) Hour 96 vs 192 Treatment	40.6443	3.15	**0.08**

Experiment 1, Eyestalk Reflex Response

There was a significant difference in eye responses within life stage, hour, and treatment, with a significant interaction between hour and treatment (Table 3). The interactions between life stage and

hour was also significantly different at $\alpha = 0.1$, indicating that responses varied between life stage and hour. Therefore, life stages (adults and juveniles) were analyzed in separate GLM models.

Adult crabs exposed to malathion showed an overall reduction in normal eye response at hour 96 and abnormal eye responses decreased 96 hours after being placed in pesticide-free water at hour 192 (Figure 5). However, there were no significant pairwise comparisons detected. Juvenile crabs also showed an overall reduction in normal eye response after malathion exposure at hour 96 ($P<0.001$, Table 5, Figure 6) and hour 192 (<0.001, Table 5, Figure 6), relative to the unexposed crabs. Eyestalk reflex changes in blue crabs 1 hour post-exposure were not significantly different from the controls ($P= 0.99$, Table 5, Figure 6), with no significant differences between those crabs in the repeated exposure treatment and recovery group at that time point ($P=0.99$, Table 5, Figure 6). Additional exposure to malathion did not significantly amplify the effects of malathion, as evidenced by nonsignificant pairwise differences in righting time among crabs at hour 96 and 192 ($P=1.0$, Table 5, Figure 6). Eye reflexes of juvenile crabs in recovery treatments were still significantly different from the controls ($P=0.02$, Table 5, Figure 6) and from crabs in the repeated exposure treatment ($P=0.03$, Table 5, Figure 6), indicating that partial recovery occurred. The response of adult and juvenile crabs following exposure were similar, as depicted in Figure 5 and 6. Overall, malathion significantly altered the crabs reflexes, with a significant improvement in their eye reflexes following pesticide removal.

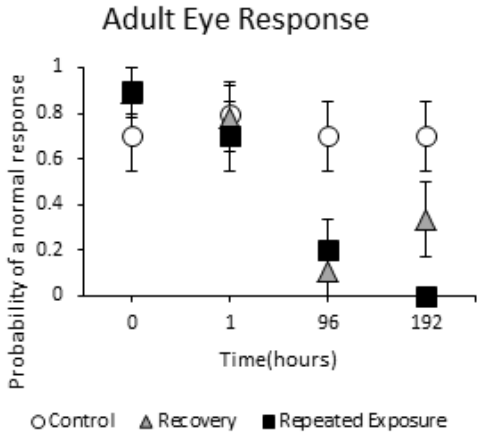

Figure 5. Probability + SE of a normal eye response in the adult blue crab (1=normal, 0=abnormal). Control: never exposed; T*T: exposed twice; T*R: exposed and then allowed to recover; * indicates that those values are significantly different from the control ($\alpha=0.05$).

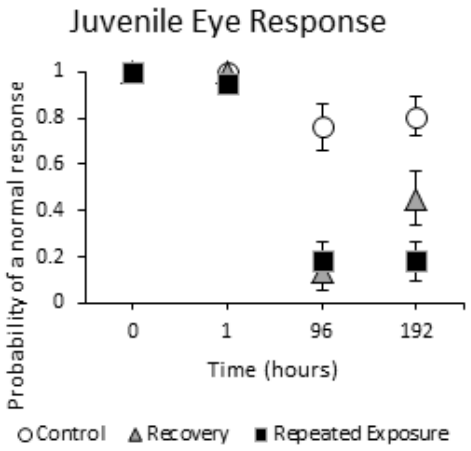

Figure 6. Probability + SE of a normal eye response in the juvenile blue crab (1=normal, 0=abnormal). Control: never exposed; T*T: exposed twice; T*R: exposed and then allowed to recover; * indicates that those values are significantly different from the control (α=0.05).

Table 5. Pairwise comparison results for adult and juvenile eyestalk reflex response using PROC GENMOD

	Mean	Chi-Sq	Pr>ChiSq
(Juvenile) Control Hour 0 X 1	0.5	0.00	1.00
(Juvenile) Control Hour 0 X 96	1.0	0.00	1.00
(Juvenile) Control Hour 0 X 192	1.0	0.00	1.00
(Juvenile) Hour 1 Control X Treatment	1.0	0.00	1.00
(Juvenile) Hour 1 Control X Recovery	0.5	0.00	1.00
(Juvenile) Hour 1 Treatment X Recovery	1.0	0.00	1.00
(Juvenile) Hour 96 Control X Treatment	0.94	12.52	**<0.001**
(Juvenile) Hour 96 Treatment X Recovery	0.44	0.08	0.78
(Juvenile) Hour 192 Control X Treatment	0.96	15.03	<0.001
(Juvenile) Hour 192 Control X Recovery	0.85	5.08	**0.02**
(Juvenile) Hour 96 vs 192 Treatment	0.50	0.00	1.0

Experiment 2a, Directional Response vs Food Cue

There was a significant difference in the directional response of crabs between treatments (P<0.001, Table 6), with no significant difference between life stages or an interaction between life stage and exposure treatment (P=0.84, 0.73, Table 6). The data were combined and analyzed based only on exposure regime. When pooled and analyzed, all treated individuals exhibited a more direct movement toward the food cue as compared to the controls (P<0.001, Table 6), with no significant differences between repeated exposure, acute, and recovery crabs (P>0.05, Table 6).

Table 6. PROC GLM results for directional response and foraging ability when presented with a food cue

	SS	*df*	*F* ratio	Prob>F
GLM results for directional response to food cue				
Treatment (Control, Acute, Repeated Exposure, Recovery)	7.99	3	21.39	**<0.001**
Life stage (Adult, Juvenile)	0.01	1	0.06	0.84
Treatment * Life stage	0.16	3	0.43	0.74
GLM results for Foraging Ability (the ability of crab to locate food)				
Treatment (Control, Acute, Repeated Exposure, Recovery)	0.20	3	0.28	0.84
Life stage (Adult, Juvenile)	1.42	1	6.04	**0.02**
Treatment * Life stage	1.67	3	2.97	**0.07**

Table 7. Pairwise comparison results for directional response when presented with a food cue

	Mean	Chi-Sq	Pr>ChiSq
Control vs Acute	0.9742	18.07	**<0.001**
Control vs Recovery	0.9517	18.59	**<0.001**
Control vs Repeated Exposure	0.9684	21.10	**<0.001**
Recovery vs Repeated Exposure	0.6087	0.30	0.59
Acute vs Repeated Exposure	0.4480	0.05	0.83
Acute vs Recovery	0.3429	0.51	0.48

Experiment 2a, Foraging Ability vs Food Cue

Malathion exposure significantly affected crabs ability to locate food, and there was an interaction between life stage and treatment at $\alpha = 0.1$ (P=0.07, Table 6). Therefore, life stages were analyzed separately. Adult crabs exposed to malathion exhibited a significant reduction in their ability to locate the food cue. Unexposed crabs were significantly more likely to find the food cue relative to the crabs that were exposed to malathion for 48 hours (P=0.05, Table 8, Figure 7), indicating negative effects following short term exposure. After 8 days of exposure, there was a significance difference at $\alpha = 0.1$ (P=0.08, Table 8, Figure 7), indicating negative effects associated with prolonged exposure. Removal of the pesticide resulted in ~20% improvement in the crab's ability to locate food. The ability of crabs in the recovery treatment to locate the food was not significantly different from controls or from those crabs in the repeated exposure treatment (Table 8, Figure 7). Unlike adult crabs, pesticide

exposure did not significantly influence juvenile crabs' ability to locate food (P>0.10, Table 8, Figure 8).

Table 8. Pairwise comparison results for foraging ability when presented with a food cue

	Mean	Chi-Sq	Pr>ChiSq
Adult Control vs Acute Exposure	0.8889	3.84	**0.05**
Adult Control vs Repeated Exposure	0.8571	3.08	**0.08**
Adult Control vs Recovery	0.7619	1.26	0.26
Adult Repeated Exposure vs Recovery	0.3478	0.46	0.50
Juvenile Control vs Acute Exposure	0.6538	0.56	0.46
Juvenile Control vs Repeated Exposure	0.7771	2.63	0.11
Juvenile Control vs Recovery	0.6693	0.7	0.39
Juvenile Repeated Exposure vs Recovery	0.3672	0.63	0.43

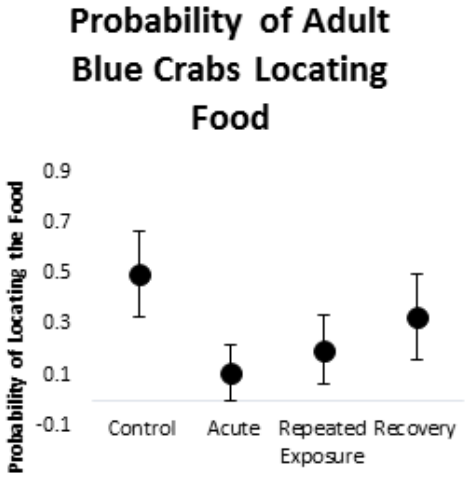

Figure 7. Probability + SE of adult blue crab successfully locating the food source. (1=success, 0=failure/chose the wrong side). Control: never exposed; Acute: exposed 48 hours prior; T*T: exposed twice; T*R: exposed and then allowed to recover; * indicates that those values are significantly different from the control (α=0.05).

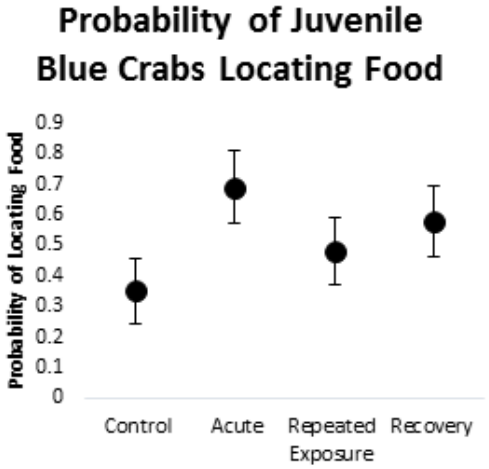

Figure 8. Probability + SE of juvenile blue crab successfully locating the food source. (1=success, 0=failure/chose the wrong side). Control: never exposed; Acute: exposed 48 hours prior; T*T: exposed twice; T*R: exposed and then allowed to recover; * indicates that those values are significantly different from the control (α=0.05).

Experiment 2b, Directional Response vs Injured Crab Cue

Juvenile crabs exposed to malathion for 48 hours moved upstream without stopping or changing directions when presented with a simultaneous positive and negative cue (i.e. food and injured crab, P<0.001, Table 9). In contrast, unexposed crabs retreated to the terminal end of the flume, the maximum possible distance from the injured crab cue, while exposed crabs moved upstream towards the combined food and injured crab cues.

Experiment 2b, Foraging ability and Activity Level vs Injured Crab Cue

Exposure to malathion did not influence blue crabs' ability to select one side of the flume over the other (P=0.42, Table 9). However, unexposed individuals spent significantly less time moving both during (P<0.001, Table 9) and after (P<0.001, Table 8) the acclimation period. Thus, crabs exposed to malathion were active in the presence of injured crab cues while control crabs were inactive.

Table 9. GLM results for predator avoidance following acute exposure

	SS	***R²***	***df***	**F ratio**	**Prob > F**
Directional Movement	3.84	0.81	1	72.47	**<0.001**
Locate food cue alongside negative cue	0.178	0.042	1	0.69	0.42
Percent movement during acclimation	14288.10	0.65	1	30.93	**<0.001**
Percent movement following acclimation	8160.03	0.66	1	30.80	**<0.001**

DISCUSSION

Malathion inhibits the normal function of muscle cells by blocking Acetylcholinesterase (AChE), an enzyme that is typically purpose with removing excess Acetylcholine (Ach) from the body. Without AChE, the Ach levels buildup, leading to the overstimulation of cells, which can often reflect seizure-like behaviors in the organism (Matsumura 1985). This Ach buildup can lead to uncontrolled muscle movements, slow reflexes, breathing problems, paralysis, and even death. These changes are highly dependent on pesticide concentrations and exposure duration (Dutta and Arends 2003). Pesticide exposure also has species specific effects, with some species more affected than others. Sublethal concentrations of malathion used in this experiment had significant effects on neuromuscular function and crab behavior, consistent with previous findings (Abramson et al. 1988, Abramson and Feinman 1988, De Guise et al. 2004, Ferner et al. 2005, Wendel and Smee 2009, Blakeslee et al. 2015).

Following malathion exposure, crabs took longer to right themselves, had abnormal eyestalk reflexes, altered their ability to locate food, and were active in the presence of injured crabs. Malathion exposure altered the adult and juveniles' ability to locate food cues differently. Adults were less likely to locate food following exposure, with a slight improvement following the pesticide removal (Figure 7). Exposed juvenile blue crabs exhibited a focused search pattern and were more willing to move throughout the flume in search of food (Figure 8), with minimal change in behavior following the pesticide removal. Placing the crab in pesticide free water led to an improvement in righting time and eyestalk reflexes of both adults and juveniles. Yet, due to long residence times of some estuaries, crabs are likely exposed to pesticides in the water until the pesticides breakdown, and may face repeated exposure that maintains pesticide levels long term. Environmental conditions also play a role in the persistence and impacts of malathion and

its degradation products, with a longer half-life expected when: pH is low, there is more organic matter in the water, the water is more turbid, and there is less light available (Newhart 2006). While anaerobic conditions are not conducive to malathion persistence, the degradation products have a higher solubility in this environment, amplifying the negative effects on organisms able to survive in these systems (Newhart 2006).

With a solubility of 145 mg/L (at 20 °C) and a K_{OW} of 2.7, malathion dissolves and can be transported in stormwater runoff (Tomlin 1997). Its presence in surface waters along with its more toxic degradates indicate that the results of this study may represent a conservative estimate of the effects of malathion on blue crab behavior, (Williams et al. 1972, Eto 1974, Smith 1993, Solis and Powell 1999) as field conditions may decrease compound degradation resulting in longer exposure periods causing pseudo-persistent behavioral effects.

Behavioral changes following malathion exposure may be caused by loss of neuromuscular function and/or a reduced ability to acquire oxygen. Malathion causes rigid paralysis, inhibiting muscle contraction and relaxation, thereby inhibiting righting behavior and altering eyestalk reflexes. Exposure may also leave the crab malnourished relative to the crabs that were not exposed, potentially leading to the difference in behavior in the flume experiments. Although not seen in this experiment, these altered behaviors make crabs more susceptible to predation, disease, and other threats that could lead to increased mortality in the environment (De Guise et al. 2004).

Toxic exposure has been associated with hypometabolic and hypermetabolic states in many species, depending on pesticide identity and concentration (Holmberg et al. 1972, Cranmer et al. 1978, De Boeck et al. 1997). Pollutants, including copper and carbolfuran can decrease metabolic activity (Cranmer et al. 1978, De Boeck et al. 1997), while Acetylcholinesterase

inhibitors, such as pentachlorophenol, diazinon and malathion may increase metabolic rate (Holmberg et al. 1972, Sastry and Sharma 1981). Hypermetabolic rates increase energy use, requiring crabs to increase foraging rates, and possibly exposing them to greater predation rates (Holmberg et al. 1972, Sastry and Sharma 1981). In this study, crabs exposed to malathion were more active even in the presence of risk cues, suggesting that exposure either alters their ability to detect a predator cue or increases their foraging need, which overrides this predator avoidance behavior.

Nontarget organisms experience both lethal and sublethal effects from a variety of pesticides (Rand 1995). Diazinon, an organophosphate pesticide similar to malathion, decreased the responses of Chinook salmon (*Oncorhynchus tshawytscha)* to predators following acute exposure to concentrations of 1 ppb (Scholz et al. 2000). Similarly, guppies (*Poecilia reticula*) exposed to pentachlorophenol, an organochlorine pesticide, exhibited a reduction in their reflexes when predators were present, increasing the risk of predation (Baldwin et al. 2003). Pesticides altered the ability of Pacific salmon (*Oncorhynchus spp.)* to swim, feed, defend territories, maintain position in the water column, changed their schooling behavior, and increased their risk of disease and predation (Ewing 1999). Exposure to carbaryl, chlordane, 2,4-dichlorophenoxyacetic acid, tributyl phosphorotrithioate, methyl parathion, and pentachlorophenol was shown to alter rainbow trout (*Oncorhynchus mykiss*) swimming activity, swimming capacity, feeding behavior, and vulnerability to predation, but the extent of these effects varied among different toxins (Little et al. 1990). Low concentrations (5 ppb) of atrazine and diuron decreased grouping behavior, increased surfacing activity, and decreased sheltering of goldfish (*Carassius auratus*) (Saglio and Trijasse 1998).

Crabs and other aquatic arthropods are often susceptible to pesticides that target terrestrial arthropods that both increase their mortality directly or alter their behavior in ways that make it harder for them to forage and avoid predators. Wendel and Smee (2009) found that a single exposure to 1ppb and 11.2 ppb of malathion increased blue crab righting time within 36 hours of exposure, and concentrations of 100 ppb significantly increased crab mortality. The organophosphate pesticide dimethoate (2 ppm) caused reductions in heart rate and AChE activity in *C. maenus* (Lundebye et al. 1997). Lobsters (*Homarus americanus*) are susceptible to lethal and sublethal concentrations of malathion in sea water. In 1999, the Long Island Sound experienced a 99% reduction in American lobster populations following malathion Although death was ultimately caused by the parasite *Paramoeba spp*., malathion exposure as low as 5 ppb increased lobster susceptibility to this parasite (De Guise et al. 2004), indicating that sublethal changes can indirectly cause death. Pesticides in the water column negatively impact phytoplankton and zooplankton communities as well, with effects cascading throughout the food web. One study found that organochlorine and organophosphate insecticides tended to be 2 to 10 times higher in algae relative to water, with even higher concentrations found in zooplankton (10-25 times higher) and fish (8-140 times higher) communities. This bioaccumulation of pesticides within upper level organisms could have drastic consequences on top predators, including humans (Favari et al. 2000).

By affecting nontarget organisms, pesticides can indirectly alter food webs and affect entire communities by removing trophic levels and influencing the outcome of predatory interactions. Both detamethrin and atrazine increased populations of autotrophic organisms by decreasing their zooplankton and herbivore consumer populations (Graymore et al. 2001, Knapp et al. 2005). Malathion concentrations of 10 ppb decreased zooplankton, which had a cascading

effect, leading to increased phytoplankton concentrations, decreased competing periphyton communities, and ultimately decreased local tadpole frogs numbers (Relyea and Diecks 2008). In some cases, pesticide exposure is beneficial to individual species. For example, malathion exposure led to a 5-fold increase in tadpole survival in mesocosm experiments by removing insect predators from the food web (Relyea 2005).

Alterations in blue crab populations can often have a cascading negative effect on estuarine communities. Plant biomass and production in marsh habitats are largely controlled by grazers and their predators. Periwinkle snails (*Littorina littorea*), common in estuarine habitats, can destroy *Spartina alterniflora* communities within months when predators cannot regulate their abundance. Blue crabs are one predator of the periwinkle snail (Hamilton 1976) and reductions in crab populations have contributed to the mass die-off of *Spartina* habitats (Silliman and Bertness 2002), ultimately having a negative effect on local fisheries.

Pesticide exposure on nontarget organisms is an ongoing problem. The effects experienced by blue crabs are based on the chemical nature of the compound, the concentration, and exposure duration. Repeated exposure to pesticides at environmentally-relevant concentrations negatively affected blue crab behavior by increasing righting time and delaying eyestalk retraction reflexes, as well as reducing their ability to locate food and react to alarm cues from injured conspecifics. These changes in behavior could lead to increased mortality rates and indirectly affect estuarine food webs. Blue crabs are economically valuable as a commercial fishery, but they may be more important ecologically as they are a vital link between lower and higher trophic levels in estuarine communities and help maintain salt marsh communities by preying on marsh periwinkle snails. Sublethal effects caused by pollution are often difficult to

detect and quantify, but my results reveal that sublethal effects of pesticide exposure are potentially important and should be carefully studied when studying pesticide effects.

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