DAZED AND CONFUSED: PESTICIDES ALTER PHYSIOLOGY, BEHAVIOR, AND PREDATOR-PREY INTERACTIONS OF JUVENILE AND ADULT BLUE CRABS (*CALLINECTES SAPIDUS*)

A Dissertation

by

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This dissertation meets the standards for scope and quality of
Texas A&M University-Corpus Christi and is hereby approved.

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ABSTRACT

Toxicants (i.e., pesticides) and predators may have large and interacting effects on natural communities by removing species (lethal effect) or by altering organismal physiology or behavior (sublethal effect). Studies evaluating the effects of sublethal concentrations of pesticide mixtures are limited, especially in coastal systems [1]. The purpose of this dissertation research was to investigate both lethal and sublethal effects of realistic pesticide exposure scenarios on two life-stages (juvenile and adult) of an important invertebrate estuarine predator, prey, and fishery species, the blue crab *(Callinectes sapidus)*. Importantly, blue crab populations are declining throughout the U.S., but the potential role of pesticides in declines remains largely unexplored.

In a series of laboratory experiments, I investigated: (1) lethal and sublethal effects of a single exposure to carbaryl (carbamate), malathion (organophosphate) and resmethrin (pyrethroid) + PBO (synergist), individually and in mixtures, on juvenile and adult blue crab survival and neuromuscular functioning by measuring changes in mortality, righting time (RT), and eyestalk reflexes. These responses serve as a proxy for the direct effects of exposure on survival and indirect effects on coordinated behaviors critical to blue crab survival (e.g., predator escape or foraging). Pesticides were selected because they are three of the mostly commonly used throughout the U.S. and have different modes of action. Effects observed at the organismal level were subsequently evaluated and linked with changes in (2) predator-prey interactions (mesocosms), and (3) physiological responses (enzyme assays). Lastly, (4) differences in susceptibility between juvenile (post-planktonic) and adult life-stages were also evaluated in behavioral and predator-prey experiments.

Sublethal, legally allowable concentrations of individual pesticides and pesticide mixtures negatively affected juvenile and adult blue crabs by (1) reducing survivorship and locomotor functioning, (2) altering predator-prey interactions via changes on foraging rates and increased vulnerability to predators, and (3) increasing metabolic costs (e.g., AChE synthesis). These findings underscore the importance of studying pesticide effects in an ecological context, as juvenile life-stages were not always the most vulnerable, some effects varied non-linearly with concentration, and interactions between individual pesticides in mixtures were not necessarily predicable based on individual exposures. Notably, blue crabs were most sensitive to exposures including pyrethroid (resmethrin) + PBO, which are representative of common co-components of vector control products.

Pyrethroid use for mosquito abatement and disease control is expected to increase, and the application of such products near aquatic systems should be carefully evaluated. In blue crabs, behavioral changes (e.g., RT) provided a reliable and sensitive endpoint, indicating altered physiological (i.e., increased AChE activity) and predator-prey interactions (i.e., reduced foraging, increased vulnerability to predators) in the pesticide exposures studied. Results also highlight the importance of studying individual responses with increasing levels of biological organization, e.g., changes in species interactions, as increases in RT unexpectedly corresponded with increased consumption rates in juvenile crabs (e.g., hyperactivity, Chapter 2). In the context of fisheries management and environmental regulations, RT may be a useful endpoint when measured in combination with other responses to indicate chances of survival or altered trophic relationships [2, 3].

DEDICATION

This dissertation is dedicated to …

… all my mentors,

… my husband Kyle,

…and blue crabs.

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INTRODUCTION

A fundamental goal of ecology is to understand how natural and anthropogenic stressors affect food webs and species interactions that structure and influence ecosystem dynamics. Predators can have significant top-down effects on community structure in terrestrial, freshwater, and marine systems, but these effects can be altered by pesticides that diminish predator foraging rates and/or increase prey vulnerability to consumers [1]. Most knowledge of pesticide toxicity relies on classic toxicological laboratory experiment designed primarily for regulatory purposes [4-6]. But, recent studies highlight the importance of studying toxicant-induced stress in more relevant contexts [6-8], including investigating lethal and sublethal effects of realistic toxicant concentrations, individually and in mixture, on organismal survival and organismal traits (physiology, behavior) that may mediate critical life history functions [9-11].

Conceptually, the effects of contaminants have been compared to that of predators [6, 7]. Predators exert direct (lethal) and indirect (non-lethal) effects on prey species that can propagate down and across food chains via density mediated indirect interactions (DMII) and trait-mediated indirect interactions (TMII) [6, 12, 13]. Similarly, contaminants can have direct (toxic) and indirect (sublethal) effects on non-target species that may result in the removal of key species, or alteration of ecologically relevant traits including foraging and antipredator behaviors [1]. Toxicant-induced changes in predator-prey interactions may be the result of lethal and sublethal effects on predators, prey, or both [1]. In systems where predators are more vulnerable, prey species may benefit from predator release [14, 15]. Contaminants may also increase or decrease prey vulnerability to predators [1, 16]. For example, contaminants may increase predation risk by reducing antipredator behaviors critical to avoiding detection (i.e.,

hyperactivity may increases conspicuousness) [17, 18], escape ability (i.e., locomotion) or predator detection ("info-disruptors") [9, 16, 19, 20]. In contrast, reduced activity may decrease predator encounters and therefore temporarily decrease prey vulnerability to predation short-term [1, 21].

In estuarine systems, blue crabs (*Callinectes sapidus*) are an important predator, prey, and commercial fishery species that can be affected by anthropogenic stressors. Blue crabs are important omnivorous scavengers that provide a nexus between benthic and water column food webs across several habitat types including salt marshes, sea grass beds, and oyster reefs [22-24]. They also structure benthic habitats via their locomotor and burying behaviors (i.e., bioturbation) [24, 25]. Blue crabs are a valuable commercial fishery and serve as food for other species of sport, commercial and conservation importance including red drum (*Sciaenops ocellatus*), black drum (*Pogonias cromis)*, sheepshead (*Archosargus probatocephalus*), sea turtles (*Caretta caretta, Lepidochelys kempii*), and whooping cranes (*Grus americana*) (reviewed by [22]). Stressors that reduce juvenile or adult blue crab locomotor ability or alter predator-prey relationships may have subtle but significant effects on crab populations and estuarine food webs in general. Currently, blue crab populations throughout the eastern U.S. and Gulf of Mexico have been declining, despite fisheries management efforts [26-28]. This suggests that factors other than fishing pressure (e.g., disease, pollution, habitat loss) may contribute to blue crab population status [29, 30]. In particular, insecticide exposure may interact with other stressors and requires further investigation [31, 32].

Three neurotoxic insecticides, carbaryl (carbamate), malathion (organophosphate), resmethrin (pyrethroid type 1) and a synergist (i.e., piperonyl butoxide "PBO") were selected for study based on their wide usage in mosquito/vector control, agriculture, and by private

landowners, and they have all been measured in the environment (see Table 3.1). These chemicals target the nervous system, resulting in convulsions, muscle spasms, respiratory failure, and death in many non-target taxa [33-36]. Both malathion and carbaryl inhibit acetylcholinesterase (AChE) [33, 36], and resmethrin (a Type I pyrethroid) targets Na^+ channels associated with the nervous system [37]. Piperonyl Butoxide (PBO) is a "synergist" that potentiates the effects of pyrethroids by inhibiting metabolic pathways involved with toxicant elimination (i.e., cytochrome P450s). PBO is commonly sprayed with pyrethroids for vector disease control (e.g., West Nile, malaria, dengue, yellow fever) and agricultural pest management (e.g. cotton pests) [38, 39]. For example, in 2012, Dallas County, Texas, USA aerially sprayed Duet© in an effort to combat West Nile Virus, which contains two pyrethroid insecticides (prallethrin and sumithrin) + PBO.

Given the wide application of these pesticides in terrestrial systems and presence in freshwater systems, pesticides likely enter coastal aquatic systems. Survey data in coastal systems remain limited, however, it follows that pesticides found in tributaries are likely present in coastal systems and may therefore affect important estuarine non-target species [40]. For example, a decade-long survey of U.S. streams conducted by the U.S. Geological Survey (USGS) revealed that insecticides were present in 90% of streams throughout the U.S. [41, 42], which may eventually find their way into coastal system. Even more alarming is that 33% of deep-water wells that collect water from aquifers had more than one pesticide present, indicating that insecticides are often present as mixtures in the environment, potentially synergistically interacting with each other and with other biotic and abiotic stressors such as predation, competition, temperature and salinity [7, 32, 43, 44]. Indeed, all of these pesticide families have been found in tributaries or estuarine systems both in Texas and elsewhere (Table 3.1),

suggesting blue crabs are commonly exposed to pesticide mixtures at sublethal concentrations similar to the levels tested in this study (e.g., ≤10 μg/L, Table 3.1).

Legally allowable, sublethal concentrations of pesticides and other toxicants may become lethal when combined with natural stressors like predation and disease. In tadpoles, sublethal concentrations of carbaryl became lethal when combined with predator exudates [45]. Similarly, lobsters were more vulnerable to disease and faced increased mortality following large-scale pesticide applications [46, 47]. Like lobsters, blue crabs exposed to pesticides may be unable to adapt to other stressors [27]. As all organisms experience multiple stressors simultaneously, including pesticide mixtures, it is likely that non-target species are susceptible to pesticides in ways not predicted by classic regulatory toxicological studies [8, 48].

Just as pesticides affect behavior, they may also affect physiological responses of crabs [7]. Acetylcholinesterase (AChE) is a common physiological biomarker used to evaluate AChE inhibitor exposure (e.g., carbaryl and malathion) [49-51], that may be linked with changes in organismal behaviors related to fitness (e.g., locomotion, growth, reproduction, foraging) [52, 53]. Reduced AChE activity in fish has been linked with decreased stamina (e.g., swimming) [50], which, in-turn may indicate a reduced ability to forage or escape from predators [54]. A clear relationship between AChE and other toxicant induced changes at the organismal level remain unknown for most species, however [50, 55, 56], e.g., AChE inhibition ranging from 30-90% has been linked with mortality [49, 50]. Thus, the relationship between AChE activity and pesticide exposure is complex. Studies of pesticide effects at the physiological, organismal, and population level may be linked to provide a more holistic understanding of how ecosystems and ecosystem components may be affected by common toxicants like those used in these studies.

CHAPTER I. Uncoordinated: Effects of malathion and carbaryl exposure on juvenile and adult blue crabs

ABSTRACT

Blue crabs are an ecologically and economically important estuarine species that may be inadvertently exposed to pesticides commonly used for pest and vector control. I investigated the lethal and sub-lethal effects of two such pesticides, malathion and carbaryl (1 µg/L, 10 µg/L, 100 µg/L), on juvenile and adult blue crab survival and muscular functioning by measuring changes in mortality, righting time (RT), and eyestalk reflexes following a single exposure. These responses serve as a proxy for understanding the direct effects of exposure on survival and indirect effects on behaviors critical to blue crab survival (e.g., predator escape or foraging). Effects on blue crab responses varied with pesticide-type, concentration, life-stage, and exposure-time. In short, all malathion and carbaryl exposures significantly increased juvenile and adult crab RT and abnormal eyestalk responses within 1-12 hours of exposure. Significant lethal effects were only observed in adult crabs exposed to 100 µg/L malathion. Thus, a single exposure to low and legally allowable concentrations (i.e., 1-10 µg/L) of either insecticide negatively affected blue crab behaviors critical to survival (i.e., coordination). Reduced survival and coordination at the individual level may eventually affect higher trophic levels and lead to negative effects on blue crab populations, estuarine food webs, and commercial fisheries.

INTRODUCTION

Pesticides sprayed in terrestrial systems enter coastal systems via several mechanisms including riverine input, storm run-off, wind-borne drift, and accidental spills [41, 57]. Pesticides

are also directly applied in aquatic systems for aquaculture, and vector-control (e.g., mosquito abatement). Although beneficial for controlling agricultural pests and disease-carrying insects, pesticides are not species specific and can harm non-target organisms. For example, two of the most heavily used insecticides in the U.S., carbaryl (carbamate) and malathion (organophosphate), cause convulsions, muscle spasms, respiratory failure, and death in many non-target taxa [33-36]. These insecticides inhibit cholinesterase enzymes, e.g., acetylcholinesterase (AChE), which are present in both invertebrates and vertebrates. Carbamates are less toxic than organophosphates, but both are commonly used in coastal areas and may therefore affect estuarine systems by affecting behaviors and survivorship of non-target organisms [41, 58].

To manage public health risks, every state in the continental U.S. has developed a vector control program tasked with monitoring mosquitoes to reduce the spread diseases like West Nile, malaria, and zika. While chemical control is only one aspect of abatement programs, high environmental concentrations of pesticides have been documented in association with vector-control and other eradication programs [40]. For example, from 1980-1989, malathion used for mosquito abatement was linked to more than half of the recorded fish kills in the U.S. [59]. In the 1990's, boll weevil (cotton pest) and medfly eradication programs in California, Texas, and Florida increased environmentally-occurring malathion concentrations [60]. More often, environmental insecticide concentrations are measured at/below aquatic wildlife benchmarks and drinking water standards [41]. Yet, recent studies indicate that legally allowable, sublethal concentrations of pesticides and other toxicants may become lethal when combined with natural stressors like predation and disease. For example, in several tadpoles species, sublethal concentrations of carbaryl became lethal when combined with predator exudates [45]. As all

organisms experience multiple stressors in their natural environments, it is likely that non-target species may be more susceptible to pesticides than is predicted by traditional or regulatory toxicological studies.

Pesticides and other toxicants can affect natural communities directly by removing organisms (i.e., increased mortality), or indirectly, by altering individual behaviors that mediate species interactions including mating, competition, and predator-prey interactions [1, 11, 61]. Predators have an important role in natural systems, and like pesticides, affect lower trophic levels via direct and indirect interactions [1, 6]. Importantly, predator efficacy can decline when environmental conditions, including toxicant stress, interfere with foraging activities. For example, exposure to diazinon (organophosphate) reduced mosquitofish predation of tadpoles [15]. Similarly, prey species have an important role as primary consumers and as a food resource, but may become more vulnerable to predation when exposed to pollution [16].
In estuarine systems, the blue crab (*Callinectes sapidus*) is both an important predator and prey species that can be negatively affected by pollutants [28, 62]. For example, heavy metal (Hg) exposure decreased adult crab coordination, making them less able to catch and consume active prey, including juvenile blue crabs [11, 30]. Trophic interactions were further affected, as exposed crabs targeted and consumed less active prey (e.g., mussels instead of other crabs). Like heavy metals, pesticides can also disrupt trophic relationships in aquatic systems via effects on both consumers and producers [1, 15, 63]. For example, exposure to 11.2 µg/L malathion reduced adult blue crab coordination and increased righting time after one hour of exposure [64], suggesting that toxicant exposure may compromise blue crab foraging, predator avoidance, and ultimately survival. Blue crab populations have been declining throughout the Eastern U.S. and Gulf of Mexico despite management efforts to increase populations [26, 28]. This suggests that

factors other than fishing pressure (e.g., disease, pollution, habitat loss) may contribute to blue crab population declines. In particular, insecticide exposure may interact with other stressors and requires further investigation [31, 43, 65].

The purpose of this study was to compare the lethal and sublethal effects of two commonly used insecticides, malathion and carbaryl, on juvenile (post-planktonic) and adult life-stages of blue crabs. Carbaryl and malathion were selected because of their widespread usage throughout the U.S.; both represent the top two at-home/garden insecticides, and malathion is commonly used in agriculture and mosquito abatement programs [58]. I focused on behavioral changes indicative of decreased muscular and neurological functioning that may be affected by AChE inhibitors like carbaryl and malathion, which suggest increased mortality, increased predation risk, and decreased foraging capabilities under natural conditions. While pesticides are a necessary component of pest and vector-disease control, understanding how pesticides affect different life-stages of important non-target organisms is needed to better assess the risk and environmental cost that may occur as a result of such practices.

MATERIALS AND METHODS

General design

Male and female adult (101 ±19 mm CW) and juvenile (37 mm ± 8.8 mm CW) blue crabs were exposed to malathion or carbaryl at concentrations of: 1 µg/L, 10 µg/L or 100 µg/L, or control-water (0 µg/L), for seven days using a static non-renewal protocol. To quantify effects of pesticide-type, concentration, and life-stage, crab mortality and changes in behaviors were measured [2, 66, 67] (Table 1.1). A static non-renewal design was chosen to mimic exposure scenarios in bays with long residence times common in the Western Gulf of Mexico [68].

Additionally, this approach exposed crabs to intermediate chemicals formed as the insecticides degraded or were metabolized, some of which are more toxic and water-soluble than the insecticides themselves (e.g., malaoxon from malathion) [33, 36]. Blue crabs were assigned to treatments haphazardly, with an effort to balance sex ratios. Because of space limitations and animal availability, replicate carbaryl trials were performed over several weeks during summer 2012 and 2013. Controls and insecticide treatments were always tested concurrently, and mortality and behavior of crabs in control treatments did not differ among trials or between crabs at time zero (i.e., before pesticide exposure).

Table 1.1. Lethal and sublethal response variables of juvenile and adult blue crabs

Response	Definition, rationale, and levels
Survivorship	Number of hours alive. Considered dead if unresponsive and no scaphognathite movement was observed.
Righting time (RT), % RT difference	Standard method to assess overall neurological and muscular functioning of crabs, where an increase in RT indicates decreased functioning [a]. Defined as the time required for a crab to resume an upright position after being inverted onto its dorsal side (180º). Using a stop watch, measurement began the moment a crab was fully inverted until it was upright. Crabs that remained inverted for 300 s were righted and a RT of 300 s (5 min) was recorded. RT data were transformed for analysis.
Eyestalk retraction speed	Each eyestalk is controlled by 9 muscles [b], and thus, may be affected by Acetylcholinesterase (AChE) inhibitors. A similar reflex response has been used to assess crab health in the field [c]. Retraction was tested by quickly moving a cooking spatula towards the eyestalks and were classified as either: (0) Normal: crab fully retracted both eyestalks, rapidly. (1) Abnormal: delayed, partial, or no retraction of one or both eyestalks.
Eyestalk touch-response	Indicates if the spatula came into contact with either eyestalk while retraction speed was measured. Contact almost exclusively occurred when eyestalk retraction was extremely delayed or absent. Response was recorded as either: (0) Normal: no contact with either eyestalk. (1) Abnormal: one or both eyestalks were touched.

[a] [28]
[b] [29]
[c] [27]

Animal collection and housing

Adult and juvenile blue crabs were collected from estuaries near Corpus Christi, Texas, USA. Crabs were acclimated in tanks at salinity 20 and water temperature of 22-24º C for a minimum of 48 hours before use in experiments. All seawater was made using dechlorinated tap water and Instant Ocean™. Crabs were fed shrimp daily but not in the 24 hours prior to the start of each experiment, and were kept at a 12:12-14:10 hour light:dark schedule during the acclimation period and experiments.

Solutions and exposure set-up

Crabs were exposed to control or treatment water in individual glass bowls (salinity 20): adults in 10-L (with aerators), and juveniles in 0.5-L (without aerators) of solution. Crabs were shielded from each other during experiments to minimize external stimuli using cardboard barriers between bowls. Ethanol was used to increase toxicant solubility in stock solutions, resulting in 100 µL/L (v/v) of ethanol in the highest treatment. All control treatments included 100 µg/L ethanol to discern any effects possibly caused by ethanol, and none were observed. Experimental conditions were also tested before experiments began. Crabs were able to survive in 100 µg/L ethanol solutions for 2-3 weeks, which is more than twice the duration of the experiments. Because ethanol was present in all treatments and did not produce detectable changes in crab mortality or behavior in controls, all changes in mortality and behavior were attributed to pesticide exposure. A stock solution was made in a glass volumetric flask, roughly 3-5 hours before each experiment. To ensure consistency between individual exposure containers, stock solutions were first diluted and mixed in large containers and then immediately poured into individual glass bowls. Chemicals used were malathion (Pestanol©), carbaryl (1-

Naphthyl-*N*-methylcarbamate, Pestanol©), and ethanol (absolute); all were ordered from Sigma-Aldrich ™.

Response variables (Table 1.1)

Crab survivorship, righting time (RT), and eyestalk reflexes were measured immediately prior to exposure (T_0), 1 hour after exposure (T_1), and every subsequent 12 hours (T_{12n}) for seven days. T_0 responses were used to verify crabs were healthy and performing similarly among treatments prior to pesticide exposure and to identify normal response-ranges.

Statistical analysis

Experimental replicates were individual crabs; total replicates are listed in Table 1.2. The effects of pesticide concentration on adult crab survival time (hours alive) were tested with a time-to-event analysis, which is appropriate for right-censored data (i.e., accounts for crabs with survival time beyond 168-hours). First, two separate analyses were completed for adult malathion and adult carbaryl experiments, which compared treatment survival curves against controls using a Log-rank test. Then, a third analysis was completed to determine pesticide-type differences, which compared malathion versus carbaryl survival curves at the same concentration level. All post hoc contrasts were adjusted using the Holm Method to account for multiple comparisons [69-71]. Survival analysis did not include juvenile data because zero mortality was observed in juvenile experiments (Table 1.2). Survival analysis was completed using the Proc Lifetest procedure in SAS 9.4©.

Table 1.2. Mortality, molt frequency, and replicate information of blue crabs exposed to malathion, carbaryl, or control treatments

Pesticide-Type, Concentration	Adult Deaths (dead/total n)	Juvenile Deaths (dead/total n)	% Molt (n) Juveniles [a]
Malathion			
0 µg/L	0/9	0/10	50 (5)
1 µg/L	0/9	0/10	50 (5)
10 µg/L	1/9	0/10	60 (6)
100 µg/L	3/9 *	0/10	30 (3)
Carbaryl			
0 µg/L	0/18	0/14	21 (3)
1 µg/L	1/16	0/14	0.0 (0)
10 µg/L	1/16	0/15	7.0 (1)
100 µg/L	0/15	0/19	21 (4)

[a] - Only juvenile crabs molted during exposures.

* $p < 0.05$; significant difference from controls (Lifetest, Log-Rank $\chi^2 = 6.74$, $p = 0.08$)

Effects of insecticide concentration, insecticide-type, life-stage, and time on RT and eyestalk responses were investigated using Repeated Measures Generalized Estimating Equations (RM-GEEs). This approach was selected because GEEs are appropriate for modeling different response variable-types (e.g., continuous, binomial) with non-normal distributions, and for modeling correlated responses within-subjects (i.e., crabs) and groups (e.g., treatment levels), over time [70, 134-137]. Briefly, GEEs are an extension of generalized linear models (GLM), for correlated data. β-estimates are estimated via quasi-likelihood estimation, instead of maximum likelihood, but are conceptually similar to GLM regression coefficients. RT analysis was completed using the log10 transformed percent change (difference) from T0. This transformation and use of percent-change data were necessary to account for scale differences between life-stages. For example, healthy adult blue crabs often require more than 1 second to right themselves, whereas healthy juvenile crabs often require much less than 1 second. The final equation was: Log10[(Tn - T0 / T0)*100 + x]. where x = largest RT difference rounded to nearest tenth and was added to ensure positive values (gamma distribution).

Separate GEE analyses (A and B) were completed to investigate main and interacting effects on each response variable because models including all explanatory variables and interactions (e.g., concentration, life-stage, pesticide, time, molt) were too complex. GEE models included: (GEE-A) concentration, life-stage, and time; and (GEE-B) pesticide-type, concentration, and time. Separate GEE-A models were completed for malathion and carbaryl experiments, which included both life-stages. In addition, separate GEE-B models were completed for each life-stage to compare pesticide-type effects. Molt was included as a binominal covariate in RM-GEE post-hoc analysis of juvenile responses (see molt sub-section).

Final RM-GEE parameter selection was determined by Type III generalized score tests, and are reported as raw p-values, $\chi 2$, and degrees of freedom (Table 1.3 and 1.4) [68]. Also, see Supplemental Data (Supplemental Data Table S1.1-S1.3). In cases where $p>0.05$ for explanatory parameters, final model selection was based on parsimony and comparison of Quasi-likelihood based Information Criterion (QIC) values [69, 70]. Control treatments were never significantly different for the duration of experiments ($p > 0.75$), so replicate experiments performed on different dates were pooled for analyses. Similarly, no significant differences were found between sexes ($p > 0.50$).

GEE-A planned contrasts were completed to test for significant differences between treatments vs. controls and between life-stages. Specifically, I compared (1) control versus treatment concentrations within the same life-stage, e.g., juvenile RT in control versus each treatment level, and (2) juvenile versus adult responses at the same concentration, e.g., juvenile versus adult RT exposed to 100 µg/L malathion. GEE-B contrasts compared effects of malathion versus carbaryl at the same concentration and life-stage, e.g., juvenile RT in 100 µg/L malathion versus 100 µg/L carbaryl. To balance Type-1 and Type-2 errors associated with multiple comparisons, I set a False Discovery Rate of 5%, per model [71, 72]. Significance was determined by ranking raw p-values (low to high) and then comparing those p-values against a pre-calculated threshold determined by the number of planned hypothesis tests. Thus, 3 of the 62 significant contrasts I report may be false positives. Analyses of behavioral responses (GEE models) were completed using the Proc Genmod procedure in SAS 9.4©.

Juvenile molting (covariate)

Molting was included as a binominal covariate in RM-GEE analyses of juvenile responses. Molting was only observed in juvenile crabs, and occurred in all controls and nearly all pesticide treatments (Table 1.2). Importantly, molt and non-molt crab responses were not significantly different within control treatments, suggesting that molting alone did not significantly affect responses in the absence of pesticides. An exploratory RM-GEE analysis was completed to compare molt and non-molt responses and is discussed. RT data for molt vs. non-molt crabs exposed to malathion are provided in Supplemental Data (Figure S1.1), as an example. Discussion and extrapolation is limited, however, because of small replicate size.

Table 1.3. Concentration and life-stage comparison RM-GEE model results (p-value, generalized score X^2, and DF)

	Malathion exposures only (juveniles and adults)						Carbaryl exposures only (juveniles and adults)					
	Righting time		Retraction speed		Touch-response		Righting time		Retraction speed		Touch-response	
Model parameter	p-value	X^2, DF	p-value	X^2, DF	p-value	X^2, DF	p-value	X^2, DF	p-value	X^2, DF	p-value	X^2, DF
Concentration	<0.001	23.5, 3	<0.001	30.8, 3	<0.001	21.3, 3	<0.001	23.3, 3	<0.001	49.4, 3	<0.001	33.9, 3
Time (Day)	<0.001	29.6, 7	<0.001	31.5, 7	0.19 [a]	9.95, 7	0.07 [a]	13.1, 7	<0.001	30.9, 7	<0.01	20.0, 7
Life-stage	0.03	4.73, 1	0.06 [a]	3.5, 1	0.93 [b]	0.01, 1	0.27 [b]	1.21, 1	0.12 [b]	2.43, 1	0.32 [b]	0.99, 1
Concentration *Time	0.10 [a]	29.8, 21	-	-	-	-	0.02	35.8, 21	-	-	-	-
Concentration* Life-stage	0.04 [a]	8.05, 3	0.63 [a]	1.72, 3	0.46 [a]	3.62,4	0.87 [a]	0.70, 3	0.03	11.0, 4	<0.001	19.8, 4
Molt (covariate)	0.15 [a]	2.03, 1	<0.01	8.73, 1	0.02	5.21, 1	<0.01	7.1,1	0.05	3.7, 1	0.06 [a]	3.62, 1

[a] $p > 0.05$, but parameter included in final model (increased model fit)

[b] Parameter excluded from final model (reduced or had no effect on model fit

Table 1.4. Pesticide-type comparisons: RM-GEE model results (p-value, generalized score X^2, and DF)

	Juveniles: malathion vs. carbaryl						Adults: malathion vs. carbaryl					
	Righting time		Retract speed		Touch-response		Righting time		Retract speed		Touch-response	
Model parameter	p-value	X^2, DF	p-value	X^2, DF	p-value	X^2, DF	p-value	X^2, DF	p-value	X^2, DF	p-value	X^2, DF
Concentration	<0.001	24.3, 3	<0.001	38.2, 3	<0.001	26.9, 3	<0.001	18.6, 3	<0.001	35.9, 3	<0.001	22.1, 3
Time (Day)	0.04	14.6, 7	<0.001	43.6, 7	0.02	16.2, 7	<0.001	26.6, 7	<0.001	22.5, 7	0.06 [a]	13.6, 7
Pesticide-type	0.10 [a]	2.65,1	<0.001	12.3, 1	0.02	5.85, 1	0.13 [a]	2.33, 1	0.54 [b]	0.37, 1	0.40 [b]	0.7, 1
Concentration *Pesticide-type	0.19 [a]	4.72, 3	0.93 [b]	0.43, 3	0.98 [b]	0.19, 3	0.10 [a]	6.36, 3	0.35 [a]	4.43, 4	0.04	9.92, 4
Molt (covariate)	<0.01	8.1, 1	<0.001	12.2, 1	<0.01	9.04, 1	-	-	-	-	-	-

[a] $p > 0.05$, but parameter included in final model (increased model fit)

[b] Parameter excluded from final model (reduced or had no effect on model fit).

RESULTS

All malathion and carbaryl treatments (1 µg/L, 10 µg/L, and 100 µg/L) significantly increased RT and abnormal eyestalk responses in both juvenile and adult blue crabs, and malathion decreased survival time of adult blue crabs only (Table 1.2, 1.3, 1.4). The magnitude of effects varied with concentration, life-stage, pesticide-type, and time. Comprehensive RM-GEE results with concentration and life-stage specific contrasts are reported in Supplemental Data for malathion exposure (Table S1.1), carbaryl exposure (Table S1.2), and malathion vs. carbaryl (Table S1.3) models.

Survival time, malathion & carbaryl

Significant lethal effects were only observed in adult malathion exposures (Life-test, Log-Rank $\chi^2 = 6.74$, $p = 0.08$) (Table 1.2). Specifically, adult crabs exposed to 100 µg/L malathion had significantly shorter survival times than controls, with 33% ($n = 3$) of crabs dying after an average exposure-time of 56 hours ($p = 0.03$). Carbaryl did not significantly affect adult blue crab survival time (Life-test, Log-Rank $\chi^2 = 2.09$, $p = 0.55$), and neither malathion or carbaryl affected juvenile survival.

RT, malathion

RT was significantly affected by malathion concentration ($p < 0.001$), time ($p < 0.001$), and life-stage ($p < 0.03$) (Table 1.3). A significant concentration*life-stage ($p = 0.04$) and a marginally significant concentration*time ($p = 0.10$) interaction were also found, indicating effects over time and life-stage differences varied with concentration. All malathion exposures significantly increased juvenile and adult RT compared to controls ($p \leq 0.02$, Figure 1.1A and 1.1B) (see Supplemental Data, Table S1.1, for all contrasts). Interestingly, juvenile RT did not

increase monotonically with concentration as expected; RT was significantly *highest* in the two *lower* treatments (1,10 µg/L) than in 100 µg/L exposures ($p < 0.001$, Figure 1.1A). Juvenile RT increased significantly within 1-12 hours of exposure ($p < 0.02$) and remained significantly higher than controls through 7 days ($p < 0.01$). As expected, adult RT increased monotonically with concentration, with the greatest effects observed in 100 µg/L malathion ($p<0.01$, Figure 1.1B). Adult RT increased significantly within 1-36 hours ($p < 0.01$), and like juveniles, remained significantly higher than controls in the two highest treatments through 7 days ($p < 0.01$). Effects on adult RT in 1 µg/L varied with time, and increases were only statistically significant from controls on days 2 and 5 ($p \leq 0.10$). RT was consistently higher than controls for the full 7 days, however. Life-stage differences (juvenile vs. adult) varied significantly with malathion concentration ($p = 0.04$, Concentration*Life-stage interaction, Table 1.3). As expected, juvenile RT was significantly more affected by malathion exposures than adult RT ($p \leq 0.001$, Figure 1.1A vs. 1.1B).

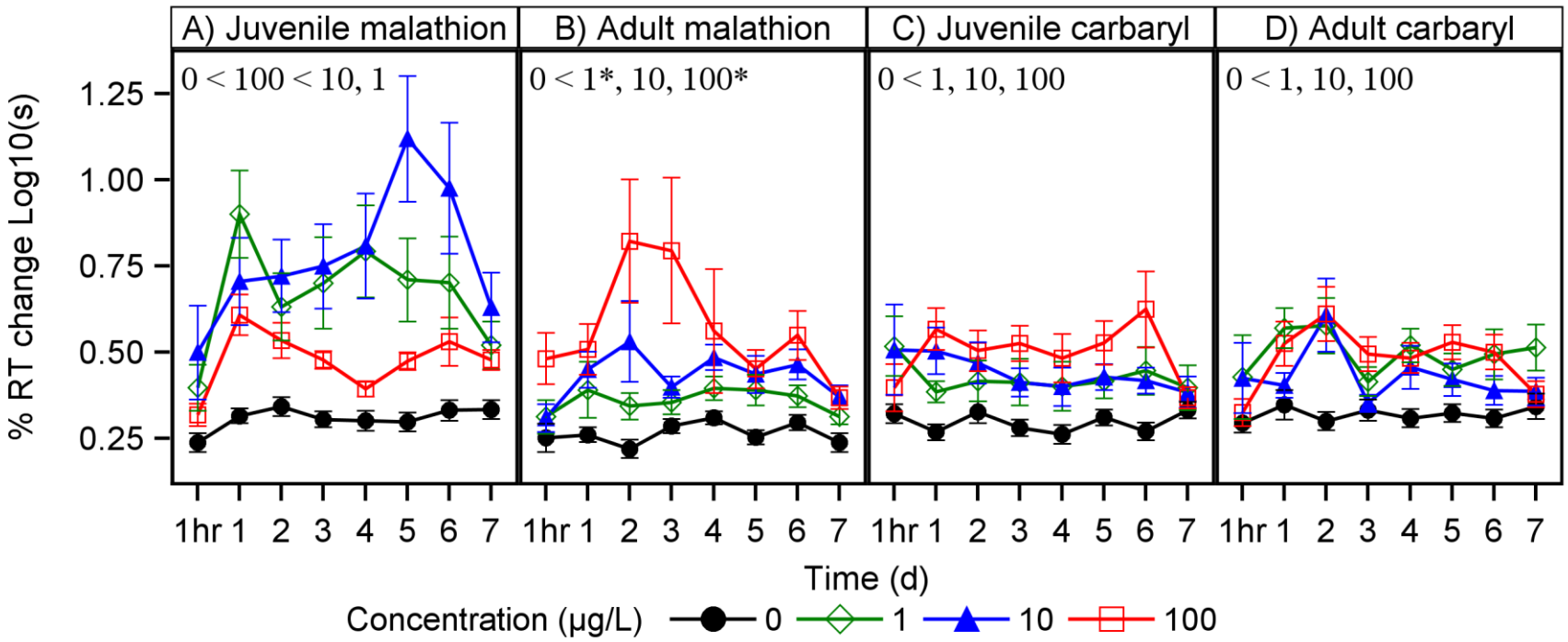

Figure 1.1. Mean (± SE) RT response of juvenile and adult blue crabs during a single exposure to malathion (A, B) or carbaryl (C, D) over seven days. Values are log-transformed mean (± SE) % change in RT from Time-0 (pre-exposure); positive values indicate an increase in RT. Angle brackets (<) and stars (*) within each panel indicate direction and significant concentration differences as determined by RM-GEE post-hoc contrasts ($p \leq 0.02$), e.g., (A) All malathion treatments significantly increased juvenile RT compared to controls; 1 and 10 µg/L exposures significantly increased RT more than 100 µg/L ($p < 0.001$). Life-stage differences: (A vs. B) Juvenile RT was significantly higher than adult RT in 1 and 10 µg/L malathion treatments ($p \leq 0.001$, post-hoc contrasts). (C vs. D) Carbaryl effects were similar for both life-stages at all treatment levels ($p = 0.27$, RM-GEE Type 3 score).

RT, *carbaryl*

Like malathion, carbaryl concentration significantly affected crab RT ($p < 0.001$), and a significant concentration*time interaction was found ($p = 0.02$) (Table 1.3). Juvenile RT significantly increased in all carbaryl treatments as compared to controls ($p < 0.001$), but carbaryl concentration differences were non-significant ($p > 0.43$, Figure 1.1C, Supplemental Data Table S1.2). Juvenile RT increased within 1-12 hours ($p < 0.01$, all treatments), and remained significantly higher than controls through 6 days (144 hours) in 10 µg/L and 100 µg/L exposures ($p < 0.01$), suggesting recovery by day 7. Like juveniles, adult RT significantly increased in all carbaryl treatments compared to controls ($p \leq 0.02$), and concentration differences were non-significant (Figure 1.1D, Supplemental Data Table S1.2). Adult RT increased within 36-48 hours of exposure ($p < 0.01$), and remained significantly higher than controls in 1 µg/L and 100 µg/L through 6-7 days ($p < 0.01$). In 10 µg/L, however, significance from controls varied more over time. That is, even though adult RT was consistently higher than controls after 36-hours, effects were only statistically significant for 4 out of 7 days (i.e., day 2, 4, 5 and 6, but not day 3). This is likely due to high variability within the treatment group rather than no effect. Lastly, life-stage differences were non-significant for all carbaryl treatments ($p = 0.27$, Figure 1.1C vs. 1.1D, Supplemental Data Table S1.3).

In summary, all malathion and carbaryl concentrations increased juvenile and adult crab RT when compared to controls. Malathion effects on RT varied non-monotonically with treatment concentration, and differed between life-stages. In contrast, carbaryl effects did not differ significantly between treatment levels or life-stages.

Eyestalk responses, malathion

Malathion effects on eyestalk responses were similar to RT. Malathion concentration ($p < 0.001$), time ($p < 0.001$), and life-stage ($p = 0.06$) all had significant effects on eyestalk retraction speed (Table 1.3). Similarly, for the eyestalk touch-response, there were significant effects of concentration ($p < 0.001$), but not time ($p < 0.19$) or life-stage ($p = 0.93$) (Table 1.3). A significant time parameter indicated that abnormal responses increased with exposure time, but for simplicity, the overall mean proportion (%) of abnormal responses are graphed without time (Figure 1.2A and 1.2B). The proportion (%) of juvenile crabs with abnormal eyestalk retraction speed and touch-responses was significantly higher in all malathion treatments than in controls ($p < 0.01$, Figure 1.2A), and malathion concentration differences were non-significant ($p > 0.50$) (see Supplemental Data Table S1.1 for contrast details). Similarly, the proportion (%) of both abnormal eyestalk responses in adults significantly increased in all but the highest malathion treatment ($p < 0.01$, Figure 1.2B), i.e., 100 µg/L malathion did not significantly increase the proportion (%) of adults with an abnormal touch-response compared to controls. Effects on retraction speed varied significantly between life-stages in all treatments ($p \leq 0.03$), with juveniles being 1.8-2.5x more likely than adults to have a delayed retraction speed (Figure 1.2A vs. 1.2B). In contrast, effects on the eyestalk touch-response did not differ between life-stages ($p = 0.93$, Figure 1.2A vs. 1.2B, Supplemental Data Table S1.1).

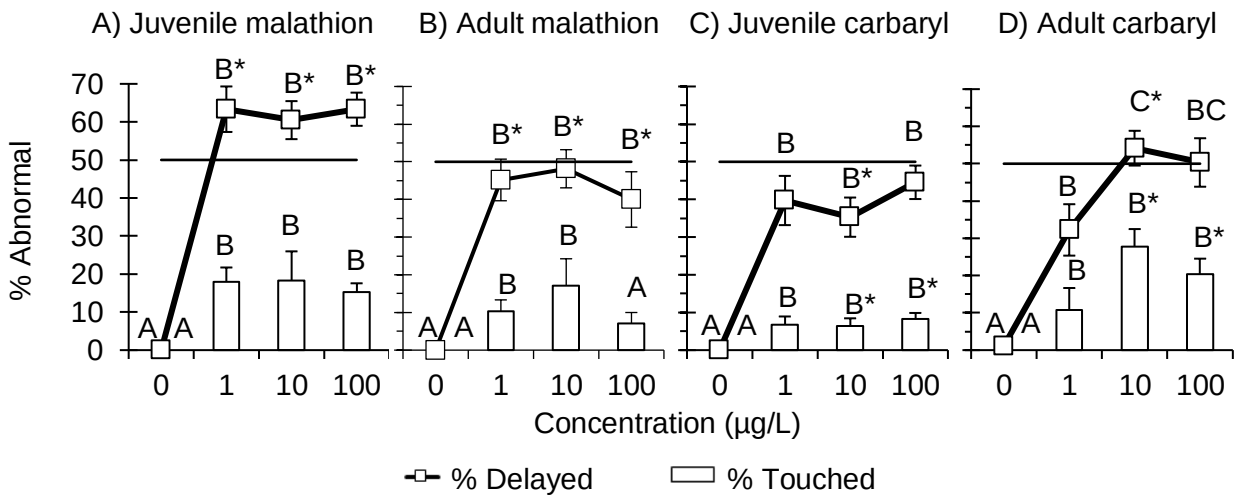

Figure 1.2. Mean (± SE) percent abnormal eyestalk retraction speed (lines) and touch-response (bars) of juvenile and adult blue crabs exposed to malathion (A, B) or carbaryl (C, D). Reference line (solid) at 50% is shown to aid in comparisons across panels. Letters indicate significant differences between concentrations within each panel ($p < 0.01$, RM-GEE contrasts). *Stars indicate significant differences between life-stages (juveniles vs. adults) exposed to the same pesticide ($p \leq 0.03$, RM-GEE contrasts).

Eyestalk responses, carbaryl

Carbaryl concentration ($p < 0.001$) and time ($p \leq 0.01$) significantly affected eyestalk responses and the concentration*life-stage interaction was significant ($p \leq 0.03$) on both eyestalk retraction speed and the eyestalk touch-response (Table 1.3). All carbaryl exposures significantly increased the proportion (%) of juvenile and adult crabs with delayed eyestalk retraction and abnormal touch-responses compared to controls ($p < 0.001$, Figure 1.2C and 1.2D). All treatment comparisons were non-significant ($p > 0.15$), except in adult-10 µg/L treatments ($p < 0.01$, Figure 1.2D, Supplemental Data Table S1.2). Life-stage differences were significant for 10 µg/L and 100 µg/L treatment comparisons, with adults being 2.2-6.5x more likely to have abnormal eyestalk responses than juveniles exposed to the same concentration ($p < 0.01$, Figure 1.2C vs. 1.2D, Supplemental Data Table S1.2).

In summary, malathion and carbaryl exposures increased the proportion (%) of both abnormal eyestalk responses in juvenile and adult crabs (Figure 1.2A-1.2D). Overall, an abnormal (delayed) eyestalk retraction speed was more commonly observed than an abnormal eyestalk touch-response. An abnormal eyestalk touch-response (i.e., eyestalk was touched) co-occurred almost exclusively with a delayed retraction speed, thus, usually indicating an extreme case of a delayed retraction response. Lastly, when responses differed between life-stages, juvenile eyestalk responses tended to be more affected (abnormal) by malathion than adults, whereas carbaryl effects tended to be greater for adult crabs than juveniles.

Pesticide-type: malathion vs. carbaryl

In juveniles, the effect of pesticide-type was marginally significant for RT ($p = 0.10$), and significant for both eyestalk retraction ($p < 0.001$), and eyestalk touch-response ($p = 0.02$), but neither pesticide affected juvenile crab survivorship (Table 1.2 and 1.4, Supplemental Data Table

S1.3). Contrasts revealed malathion effects on RT were 2.3-2.6x higher than carbaryl effects in 1 µg/L and 10 µg/L treatments ($p < 0.01$, Figure 1.3B and 1.3C), but were similar in 100 µg/L ($p > 0.74$, Figure 1.3D). Similarly, juvenile crabs exposed to malathion (all levels) were 2-3x more likely than crabs exposed to carbaryl to have one or both abnormal eyestalk responses ($p \leq 0.01$, Figure 1.4A). In summary, for all juvenile behavioral responses, malathion effects were equal to or 2-3x more severe than carbaryl effects.

Unlike juveniles, adult crab pesticide-type differences were concentration dependent; as indicated by a marginal or significant pesticide-type*concentration interaction term for survivorship-time (Lifetest, Log-rank $\chi 2 = 17.05$, DF = 7, $p = 0.02$), RT ($p = 0.10$), and the eyestalk touch-response ($p = 0.04$) (Table 1.4, Supplemental Data Table S1.3). Post-hoc analysis revealed: (1) significantly lower survival-time in malathion 100 µg/L than in carbaryl exposures ($p < 0.01$, Table 1.2); (2) 1 µg/L carbaryl significantly increased RT more than malathion ($p = 0.01$, Figure 1.3F), and (3) crabs exposed to 10 and 100 µg/L carbaryl were 2.8-4.2x as likely to have an abnormal eyestalk-touch response than crabs exposed to malathion ($p \leq 0.02$, Figure 1.4B). All other pesticide-type*concentration comparisons were non-significant (Supplemental Data Table S1.3). In summary, pesticide-type differences among adult crabs were concentration dependent; malathion decreased survival time in the highest treatment, whereas carbaryl tended to increase RT and abnormal eyestalk responses in lower treatments.

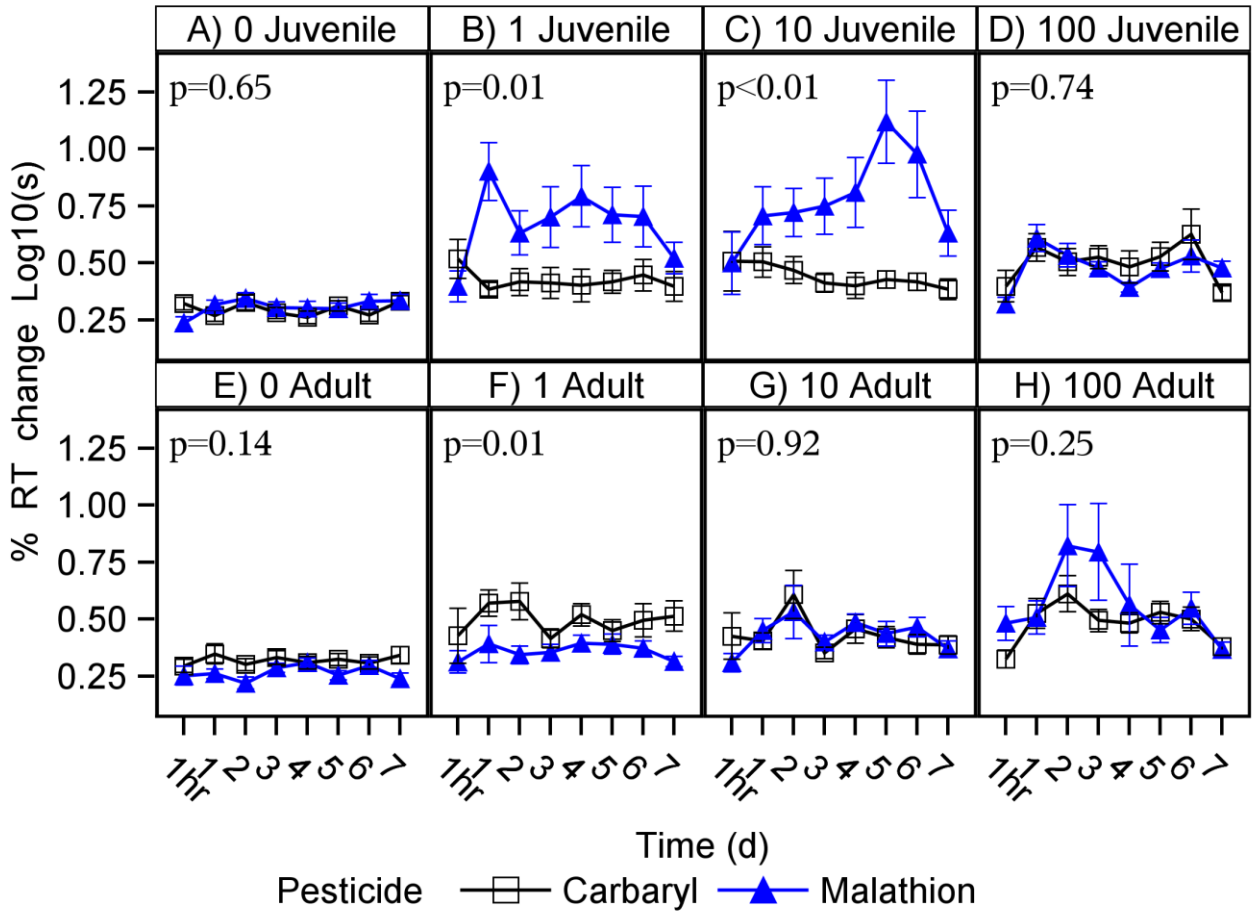

Figure 1.3. Carbaryl (open squares, black) vs. malathion (filled triangles, blue): RT response of juvenile (A-D) and adult (E-H) blue crabs during a single exposure, over seven days. Values are log-transformed mean (± SE) % change in RT from T_0 (pre-exposure). Positive values indicate an increase in RT. p-values: indicate statistical outcome of RM-GEE post-hoc contrast between malathion and carbaryl, per treatment level. (A-D) Juvenile comparisons: pesticide-type was marginally significant in the juvenile model (p=0.10, Type 3 Score). (E-H) Adult comparisons: pesticide-type (p = 0.13, Type 3 Score) and pesticide-type*concentration (p = 0.10, Type 3 Score) were marginally significant in the adult crab model.

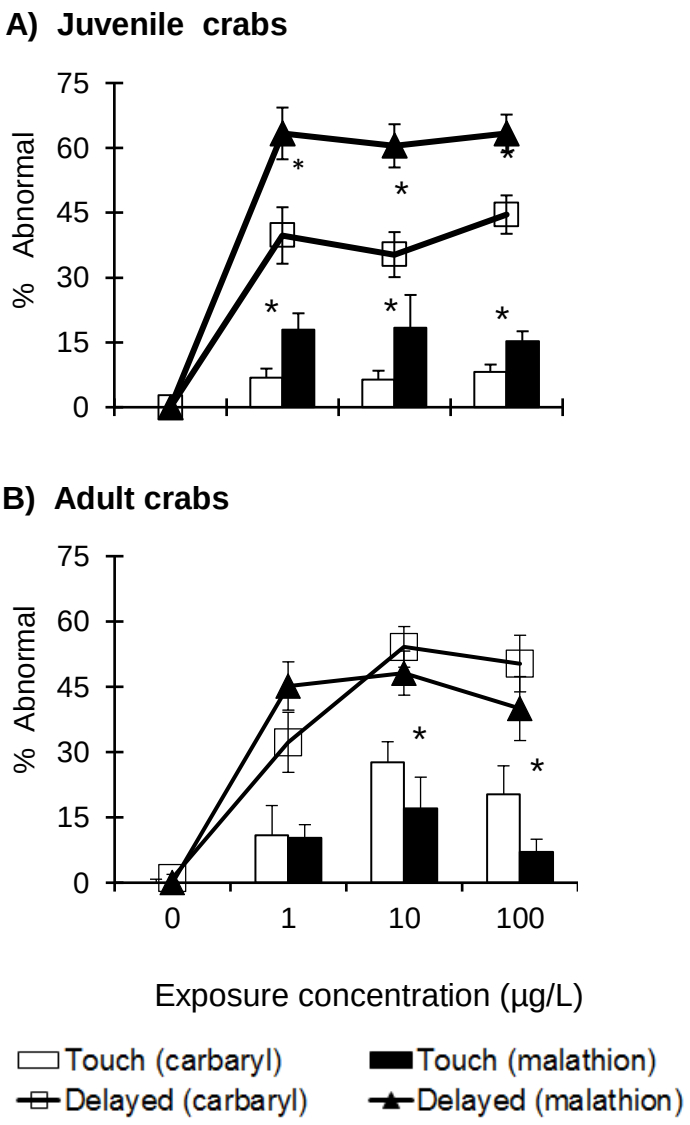

Figure 1.4. Malathion vs. carbaryl effects on eyestalk retraction speed (lines) and touch-response (bars) of juvenile (A) and adult (B) blue crabs. Values are mean (± SE) % abnormal eyestalk response. Stars (*) indicate significant differences between responses in the same concentration ($p \leq 0.03$, RM-GEE post-hoc contrasts).

Molting, juveniles

Molting was observed in juvenile crabs in both pesticide experiments and was included as a covariate to increase RM-GEE model fit (Table 1.2-1.4). Importantly, the overall pattern of effect (i.e., my interpretations) did not change when a molt covariate was added to any models. Additional exploratory analyses revealed molt and non-molt crab responses were not different in any control treatments, suggesting that molting did not affect juvenile responses in the absence of pesticides. In the presence of pesticides, however, differences between molt and non-molt crabs were variable. For example, RT was consistently higher for molt crabs (vs. non-molt crabs) exposed to 10 µg/L malathion, whereas RT was similar between molt and non-molt crabs in other treatment levels (Supplemental Data, Figure S1.1). Molting in the presence of pesticides may increase the adverse effects of toxicant exposure for some individuals, but not for others. This trend requires further study, however, given the limited sample size.

DISCUSSION

A single 7-day exposure to malathion or carbaryl (1µg/L, 10 µg/L and 100 µg/L) negatively affected juvenile and adult blue crab behaviors (e.g., RT and reflexes). Within 1-12 hours after exposure, RT increased significantly and eyestalk reflexes became abnormal, suggesting both pesticides impaired neuromuscular function. Malathion (100 µg/L) increased mortality in adult crabs, but, lower concentrations of malathion, and all tested concentrations of carbaryl, did not increase crab mortality. Thus, a single exposure to malathion or carbaryl at concentrations often reported in aquatic systems (e.g., 1-10 µg/L) can alter blue crab neuromuscular functioning and may ultimately interfere with critical life history functions such as mating, predator avoidance, and foraging.

Although both insecticides affected blue crab behavior, the effects of malathion were often more intense. Further, malathion at 100 µg/L increased adult mortality while carbaryl at this concentration did not. These results are consistent with the mode of action of malathion (organophosphate) and carbaryl (carbamate), which both inhibit acetylcholinesterase (AChE). Organophosphates like malathion bond with AChE irreversibly, forming a more stable bond that occurs for longer periods than bonds formed by carbamates [36]. AChE normally breaks down the neurotransmitter acetylcholine, but inhibition allows acetylcholine to persist and results in uncontrolled movements, spasms, stiff muscles, and sometimes death [36]. Muscle spasms and uncontrolled limb movements in crabs were observed in all pesticide treatments, and seemed to be more pronounced and common in malathion treatments than carbaryl (personal observation). Specifically, all deceased crabs in malathion treatments (n = 4) were found on their backs with expanded mouth-parts and scaphognathite (gill-bailers). This is consistent with other observations [64], and suggests asphyxiation and muscle spasms, possibly from increased metabolic requirements or stress associated with malathion exposure. Furthermore, malathion exposures were likely more toxic because the metabolite and degradate of malathion (i.e., malaooxon) is more water soluble and toxic than either malathion and carbaryl [36].

Differences between adult and juvenile life-stages were mixed, and differed between the pesticides. Often, early life-stages and smaller-bodied individuals are more sensitive to toxicant exposure than are older, larger adult forms [72]. But in some situations, larger, older life stages are more vulnerable. For example, the insecticides fipronil and endosulfan were more toxic to adult grass shrimp (LC_{50} 0.32 µg/L, 1.01 µg/L) than both larval (LC_{50} 0.68 µg/L, 2.56 µg/L) and embryonic life-stages (LC_{50}>512 µg/L, 117 µg/L) [73]. In this study, I found that adult blue crabs were more likely to die than juvenile crabs after exposure to the highest malathion

treatment (100 µg/L), whereas in the lower treatments, juvenile crab RT and eye reflexes were more negatively affected than adults. A possible explanation for increased adult crab mortality is the bio-activation of malathion into the more toxic compound malaoxon. It may be that adult crabs metabolized malathion into malaoxon more quickly than juveniles, and became overwhelmed at the highest concentration.

Juvenile and adult mortality was also not significantly affected by either 1 µg/L or 10 µg/L malathion exposures, though one adult crab died (10 µg/L exposure). This finding contrasts with that of Wendel and Smee [64], who found juvenile crab mortality to be significantly higher than adults exposed to malathion concentrations as low as 11.2 µg/L. A potentially important difference between these studies is the purity of malathion used. Here, technical grade malathion was purchased (Sigma Aldrich), for the purposes of studying the effects of malathion/malaoxon in isolation from other compounds, whereas Wendel and Smee [64] used commercial grade malathion purchased from a local store. Commercial products may only be ~50% malathion and contain other compounds, some of which could directly affect crabs and/or amplify the effects of malathion. Commercial grade products may also contain toxic intermediate forms of malathion as it breaks down, some of which are more toxic than malathion (e.g., malaoxon). Regardless, results from this study and those from Wendel and Smee [64] indicate that exposure to AChE inhibitors can significant increase blue crab mortality and alter crab neuromuscular function and behavior.

The effects of pesticides may also be exacerbated in molting or newly molted crabs. In particular, juvenile life-stage may be impacted more because juveniles molt more frequently than adults [74]. In this study, juvenile crabs molted in control and several pesticide treatments. I found that molting alone did not affect behavioral responses of juvenile control crabs, whereas

molting + pesticide exposure tended to increase effects of some treatments. Molting + pesticide exposure may also affect survivorship, though it did not in the current study. Wendel & Smee [64] noted that all molt juvenile blue crabs exposed to malathion died (n = 8), whereas all molt + control crabs survived (n =3). Furthermore, juvenile blue crabs exposed to insecticides (i.e., acephate, aldicard, and imidaclorprid) were more likely to die after molting than non-poisoned crabs [75]. The mechanism was not investigated, but death may be the result of increased metabolic activity and/or permeability associated with molting, as new tissue must be grown and increased water intake may inadvertently increase toxicant uptake from the environment.

Results underscore the importance of measuring sublethal responses, and indicate crabs do not always respond to malathion and carbaryl exposures linearly. I found that intermediate levels of exposure resulted in the highest effects on juvenile RT (10 µg/L malathion) and the least effects on adult crab RT (10 µg/L carbaryl). This is important, because regulatory decisions often rely heavily on a linear-effect assumption (i.e., effects increase with concentration) and the usage of "no observed effect concentration" (NOEC) and/or "lowest observed effect concentration" (LOEC) values [76-78]. While regulatory toxicological studies focused on identifying EC*x*, NOEC, LOECs in a few select model organisms remain a staple aspect of risk assessment, rarely do they include the ecological context or complexity needed to assess the effects of pollutants in many natural systems.

Malathion and carbaryl are the two most commonly used insecticides in the U.S. and may enter coastal systems via indirect and direct mechanisms, ranging from storm run-off and wind-borne drift, to large-scale applications aimed at controlling mosquitoes or agricultural pests [58]. Both are used because of their relatively short half-life/s and broad efficacy as AChE inhibitors, but results indicate that environmentally occurring and legally allowable concentrations can

negatively affect blue crab neurological functioning, and reflexes, suggesting that crabs may be less able to mate, forage, or escape from predators in the environment**.** Because highly coordinated locomotor skills are critical to blue crab survival, reduced coordination and overall well-being of individual crabs in response to AChE-inhibitor exposure may have propagating negative effects on blue crab populations, and coastal ecosystem functioning (e.g., fisheries and other services) in which blue crabs or other invertebrate predators have an important role.

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CHAPTER II. Dazed, confused, hungry: Resmethrin and PBO alter blue crab predator-prey interactions

ABSTRACT

Contaminant stressors (e.g., pesticides) and predators may have large and interacting effects on natural communities by removing species or by altering behaviors. Few studies in estuarine systems have evaluated the effects of a single, low dose exposure to pesticides on key predators, however. Here, I report the effects of realistic exposures of a commonly applied mosquito abatement pesticide mixture consisting of a pyrethroid insecticide (resmethrin) and synergist (piperonyl butoxide; PBO) on two life-stages (adult and juvenile) of an important invertebrate estuarine predator, prey, and fishery species, the blue crab *(Callinectes sapidus)*. Separate experiments were conducted to evaluate effects of resmethrin + PBO (Res-PBO) exposures: (1) First, in static non-renewal exposures, juvenile and adult crabs exposed to 1:3 µg/L, 10:30 µg/L, 100:300 µg/L Res-PBO or 300 µg/L PBO-alone had increased mortality and reduced locomotor ability. Importantly, in 1:3 µg/L and 10:30 µg/L exposures, lethal and sublethal effects on adult crabs were more severe than on juveniles. (2) In mesocosm experiments, exposure to 1:3 µg/L Res-PBO altered the foraging ability of both adult and juvenile crabs, lowering the ability of adult crabs to cannibalize juvenile crabs but increasing juvenile crab foraging rates on shrimp. Juvenile crabs were also more vulnerable to predation following pesticide exposure. Thus, a single, 12-hour exposure to low, environmentally-occurring concentrations of a pyrethroid + synergist reduced juvenile and adult blue crab survivorship and locomotor functioning, and altered predator-prey interactions by changing foraging rates and increasing vulnerability to predators. Our results indicate that pesticide-

stressors may play an important, but underestimated, role in shaping coastal ecosystems in which invertebrate predators are an important component.

INTRODUCTION

A fundamental goal of ecology is to understand how natural and anthropogenic stressors affect food webs and species interactions that structure and influence ecosystem dynamics [7]. Predators can have significant top-down effects on community structure in terrestrial, freshwater, and marine systems, but these effects can be altered by pesticides that diminish predator foraging rates or increase prey vulnerability to consumers [1, 6]. Most information of pesticide toxicity relies on classic toxicological laboratory experiments designed primarily for regulatory purposes in freshwater systems [4-6, 79, 80]. More recent studies highlight the importance of studying toxicant-induced stress in an ecologically relevant context, including the investigation both lethal and sublethal effects of pesticides on both predators and prey [6, 7].

Conceptually, the effects of contaminants have been compared to that of predators [6, 7]. Predators exert direct (lethal) and indirect (non-lethal) effects on prey species that can propagate down and across food chains via density mediated indirect interactions (DMII) and trait-mediated indirect interactions (TMII) [6, 12, 13]. Similarly, contaminants can have direct (lethal) and indirect (sublethal) effects on non-target species that may result in the removal of key species, or alteration of ecologically relevant traits including foraging and antipredator behaviors [1]. Toxicant-induced changes in predator-prey interactions may be the result of lethal and sublethal effects on predators, prey, or both [1]. Contaminants may benefit prey by interfering with predator foraging or survival [14, 15] or they may increase prey susceptibility to predators by altering their ability to evade potential consumers [1, 16]. For example, contaminants may increase predation risk by reducing antipredator behaviors critical to escape ability (i.e.,

locomotion) or predator detection ("info-disruptors") [9, 16, 19, 20]. In contrast, reduced activity may decrease predator encounters, and therefore decrease prey vulnerability to predation [1, 21].

I studied the effects of realistic exposure scenarios of a commonly applied mosquito abatement pesticide mixture consisting of a pyrethroid insecticide (resmethrin) + synergist (piperonyl butoxide; PBO) on two life-stages (adult and juvenile) of an important invertebrate estuarine predator, prey, and fishery species, the blue crab *(Callinectes sapidus)*. An overarching objective of this study was to link an easily measured response (i.e., righting time) at the organismal level [64] with changes in predator-prey interactions studied in mesocosms [7, 61]. Blue crabs are important omnivorous scavengers that provide a nexus between benthic and water column food webs across several habitats including salt marshes, sea grass beds, and oyster reefs [22-24]. Blue crabs also structure benthic habitats via their locomotor and burying behaviors (i.e., bioturbation) [24, 25]. Blue crabs are a valuable commercial fishery and serve as food for other species of sport, commercial, and conservation importance including red drum (*Sciaenops ocellatus*), black drum (*Pogonias cromis),* sheepshead (*Archosargus probatocephalus*), sea turtles (*Caretta caretta, Lepidochelys kempii*), and whooping cranes (*Grus americana*) (reviewed by [22]). Stressors that reduce juvenile or adult blue crab locomotor ability or alter predator-prey relationships may have subtle but significant effects on crab populations and estuarine food webs in general. Currently, blue crab populations throughout the eastern U.S. and Gulf of Mexico have been declining, despite fisheries management efforts [26-28]. This suggests that factors other than fishing pressure (e.g., disease, pollution, habitat loss) may contribute to blue crab population status [29, 30]. In particular, insecticide exposure may interact with other stressors and requires further investigation [31, 32].

Pesticide Background: Resmethrin + PBO

Pesticides and other toxicants may enter estuarine systems unintendedly via several sources including vector control, aquaculture, storm run-off, windborne drift, and tributaries. Of interest are pesticides sprayed for mosquito abatement and vector control programs (e.g., Anvil©, Scourge©, Duet©), which may be directly applied near aquatic systems and affect non-target species [81, 82]. These products are commonly mixtures consisting of pyrethroid + synergist + inert compounds [39, 83]. In the present study, I tested the effects of resmethrin (pyrethroid) + PBO (a synergist) mixtures, which are the active ingredients of the vector-control product Scourge© [46, 47]. In freshwater systems, such pesticides and pesticide mixtures have been recorded throughout the U.S. [41, 42]. Long-term environmental monitoring of pyrethroids is fairly limited, however, but concentrations up to 15 µg/L PBO were measured in Long Island surface waters 3 days after spraying Scourge© and a heavy rainfall event [47]. In California, 20 µg/L PBO was recorded in surface waters [38, 82] and pyrethroids have also been measured in Texas aquatic sediments [84]. Thus, pyrethroids are likely present in many estuarine systems [41, 42], but our understanding of their effects on estuarine systems remains limited [1, 85].

Pyrethroids target sodium channels of nerve cells, causing prolonged depolarization and excitation [86, 87]. Because of their short half-lives (<1d via photolysis), pyrethroids serve as an alternative to organochlorines and organophosphates, but for this reason they are often sprayed with synergists (e.g., PBO, MGK-264). Synergists are often mixed function oxidase (MFOs) inhibitors that inhibit the metabolic breakdown of contaminants like pyrethroids [86]. The half-life of PBO and pyrethroids are highly dependent on environmental conditions, however, and may persist for >>1 d. For example, both can persist in soils >> 30 d [83, 84, 88, 89]. Knowledge of synergist toxicity in mixtures is fairly limited [8, 85], though concentrations as low as 2-4

µg/L PBO in sediments doubled pyrethroid toxicity to amphipods (*Hyalella azteca*) [82]. PBO is also a human carcinogen [39] and has been investigated in combination with pyrethroids, organophosphates and carbamates in freshwater and terrestrial model systems [90-92].

MATERIALS & METHODS

General approach

Three experiments were conducted: (1) individual, static non-renewal assays to determine lethal and sublethal range of resmethrin + PBO (Res-PBO) exposures and to assess behavioral changes after a single exposure, (2) adult + juvenile crab mesocosm experiments, to assess how cannibalistic predator-prey interactions between adult and juvenile blue crabs are affected by a 12 hour sublethal exposure to Res-PBO, and (3) juvenile crab + shrimp mesocosm experiments, to assess changes in juvenile blue crab consumption rates of brown shrimp (*Farfantepenaeus aztecus*) after a 12 hour sublethal exposure to Res-PBO.

Exposure scenarios and concentrations were selected to reflect resmethrin + PBO product ratios, which range from 1:3 to 1:20 (w/w, resmethrin-to-PBO), as well as other pyrethroid + PBO products [38]. I elected to test resmethrin and PBO at ratio of 1:3 because this ratio was approved for mosquito abatement applications in Texas. A static non-renewal design was chosen to mimic a single exposure, which may occur after a storm or a mosquito spray event. This approach also mimics exposure scenarios in bays with long residence times, which common in the western Gulf of Mexico [68].

Animal collection and housing

Adult and juvenile blue crabs and brown shrimp were caught locally. Experiments 1, 2, and 3 were completed during the summer months of 2012, 2013, and 2014 (respectively), in the

Marine Ecology Lab at Texas A&M University-Corpus Christi, TX, USA. Animals were acclimated to experimental conditions in indoor tanks at salinity 20 and temp 22-24^{o} C for a minimum of 48 hours. Crabs were kept at a 12:12-14:10 hour light:dark schedule during the acclimation period and experiments. Crabs were fed commercially purchased shrimp daily, and starved 24 hours prior to the start of an experiment. All seawater was made using dechlorinated tap water and Instant Ocean™.

Solutions and exposure set-up

In all experiments, animals were exposed to a single dose of Res-PBO mixtures or control water using the same protocol. Chemicals used were resmethrin, PBO (90% purity), and ethanol (absolute) from Sigma-Aldrich. Ethanol was used to increase toxicant solubility in stock solutions, resulting in 100 µL/L (v/v) of ethanol in the highest treatment. All control treatments included 100 µg/L ethanol to discern any effects possibly caused by ethanol and none were observed. Thus, changes in mortality and behavior were attributed to pesticide exposure. Stock solutions were made roughly 2-3 hours before each experiment. Sea water, resmethrin, PBO, and ethanol were added to a volumetric flask, manually mixed, and then placed on a stir plate and mixed for an additional 1-1.5 hours (25 ˚C). Animals were exposed to control or pesticides in individual glass bowls or jars (salinity 20), as follows: adults in 10-L (with aerators), and juveniles and shrimp in 0.5-L (without aerators) of solution. Animals were shielded from each other during experiments to minimize external stimuli.

Experiment 1: Individual assays, survival, RT

Male and female juvenile (avg. 34 ± 8 mm CW) and adult (avg. 96 ± 12 mm CW) blue crabs were exposed to control water or Res-PBO treatments for 7 days (168 hr), using a static non-renewal protocol. Treatments are listed in ratios of resmethrin-to-PBO and included

concentrations of: 1:3 µg/L, 10:30 µg/L, 100:300 µg/L, and 300 µg/L PBO-alone ("PBO-300"). Ten crabs (n =10) were haphazardly assigned to each treatment with an effort to balance sex ratios. Due to space limitations and animal availability, replicate trials were performed over several weeks during summer 2012. Controls and insecticide treatments were always tested concurrently, and mortality and behavior of crabs did not differ among trials or between crabs at time zero in any treatments (i.e. before pesticide exposure) for any experiments.

To quantify individual effects, survivorship and righting time (RT) were measured immediately prior to exposure (T_0), 1 hour after exposure (T_1), and every subsequent 12 hours (T_{12n}) for 7 days. RT is defined as the amount of time it takes a crab to resume its normal position after being placed on its back (180°); and is a proxy to assess the overall neurological and muscular functioning of crabs [64, 66, 93]. Survival data are reported for the full 7 days, but RT data are presented for 0 to 48 hours because of high mortality in nearly all treatments after 36-48 hours. Pre-exposure (T_0) responses were used to verify crabs were healthy and performing similarly and to identify normal response-ranges.

Experiment 2: Adult crab predator + juvenile crab prey (cannibalistic) mesocosm

Blue crabs are cannibalistic and adults routinely prey on juvenile crabs [94]. Therefore, I assessed the effects of sublethal Res-PBO exposures on both life-stages using adults as predators and juveniles as prey (Table 2.1). Mesocosms were 20-L indoor tubs filled with pesticide-free sea water (salinity 20); each had 1 filter to circulate water, 1 layer of clean/dead oyster shell to provide refuge, and a lid to prevent crabs from escaping. First, juvenile (avg. 18 ± 0.5 mm CW) and adult male blue crabs (avg. 100 ± 9 mm CW) were individually exposed in glass containers to control (0 µg/L), 1:3 µg/L Res-PBO, or 10:30 µg/L Res-PBO for 12 hours (same exposure protocol as used in Experiment 1). Adult female crabs were difficult to find, and thus only male

crabs were used as predators, but both female and male juvenile crabs were used as prey. Immediately following 12-hour exposures, 8 juveniles (prey) + 1 adult male crab (predator) were added to mesocosms in five different treatment combinations, or scenarios, including: 1:3 predator-only exposure, 1:3 prey-only exposure, 10:30 prey-only exposure, 1:3-both exposure, and control without either being exposed (Table 2.1). A 10:30 exposure scenario on the adult blue crabs used as predators was not completed because of high adult blue crab mortality at this concentration (experiment 1), thus, only juveniles were exposed to 10:30 µg/L Res-PBO. Juvenile prey crabs were allowed 5 minutes to acclimate before 1 adult predator crab was added. Juvenile deaths were attributed to adult crab predation only, because no juvenile cannibalism was observed throughout experiments. No adult crabs died after being placed in mesocosms. Prey survival was recorded at hour 1, 3, 8, 12, and 24.

Table 2.1. Summary of Res-PBO mesocosm treatment scenarios [a]

Mesocosm treatment	Prey [Expo. µg/L]	Predator [Expo. µg/L]	n replicates
Exp. 2 Adult + juvenile crabs			
Control-both	0	0	24
Both Expo 1:3	1:3	1:3	13
Adult Expo 1:3	0	1:3	12
Juv. Expo 1:3	1:3	0	19
Juv. Expo 10:30	10:30	0	10
Exp. 3 Juvenile crabs + shrimp			
Control-both	0	0	6
Shrimp Expo 1:3	0	1:3	5
Juv. Expo 1:3	1:3	0	6
Both Expo 1:3	1:3	1:3	5

[a] Following 12-h exposures, animals were placed in mesocosms with pesticide-free water

Experiment 3: Juvenile crab predator + shrimp-prey mesocosm

Following the same exposure protocol (Experiment 1) and mesocosm set-up (Experiment 2), I investigated the effects of pesticide exposure on juvenile crab predation of brown shrimp under four exposure scenarios. Male juvenile crabs (avg. 47 ± 6 mm CW) and brown shrimp (avg. 37.7 ± 3 mm CW) were exposed to control or 1:3 µg/L Res-PBO for 12 hours before being placed in mesocosms with pesticide-free water. As before, 8 prey (shrimp) were added to each mesocosm (14-L tubs) and allowed to acclimate for 5 minutes, before 1 predator (juvenile crab) was added. Four mesocosm treatments, or scenarios, were studied including: 1:3 µg/L predator-only exposure, 1:3 µg/L prey-only exposure, 1:3-both exposure, and control-both exposure (Table 2.1). Based on preliminary experiments, I added an additional observation time after 2 hours (i.e., juveniles consumed shrimp prey quickly). Thus, prey survival was recorded at hour 1, 2, 3, 8, 12, and 24.

Statistical analysis

Experimental replicates in static exposure experiments were individual crabs (n = 10 per treatment, per life-stage). The effects of Res-PBO concentration and life-stage on crab survival time (hours alive) were tested with a time-to-event analysis. A single model was completed, using a log-rank test, which compared treatment survival curves against controls within the same life-stage, and also compared juveniles vs. adults exposed to the same concentration. Post hoc contrasts were adjusted using the Holm Method to account for multiple comparisons [69-71]. Detailed post-hoc contrasts are reported in Supplemental Data (Table S2.1).

Effects of Res-PBO concentration, life-stage, and time on RT were investigated using Repeated Measures Generalized Estimating Equations (RM-GEEs), which are appropriate for modeling non-normally distributed data and correlated responses within-subjects (i.e., crabs) and groups (e.g., treatment levels), over time [95-99]. RT analysis was completed using the $\log_{10}$ transformed % change (difference) from T_0. This transformation and use of percent-change data were necessary to account for scale differences between life-stages, e.g., on average, healthy adult crabs require more time to right themselves than healthy juvenile crabs. The final equation was: $\text{Log}_{10}[((T_n - T_0 / T_0)*100) + z]$, where z = the largest RT difference value. Post-hoc contrasts were completed to test for significant differences between pesticide treatments vs. controls within the same life-stage, and between life-stages at the same concentration (e.g., adults vs. juveniles in 1:3 µg/L Res-PBO). Post hoc contrasts were adjusted using the Holm Method to account for multiple comparisons [69-71]. Detailed RM-GEE model and post-hoc contrasts for all responses, including estimates and odds ratios, are reported in Supplemental Data (Table S2.1).

For mesocosm experiments, replicates were individual mesocosm tubs, which are listed in Table 2.1. Prey survival in mesocosms (experiment 2 and 3) were analyzed using separate RM-GEE. Final distribution and correlation structures were selected based on QIC/QICu values and final parameter selection (all models) was determined by Type III generalized score tests, which are reported as raw p-values, $\chi 2$, and degrees of freedom [100]. Also see Supplemental Data for full results (Supplemental Data Table S2.2, S2.3). In cases where $0.05 < p \leq 0.15$ for explanatory parameters, final model selection was based on parsimony and comparison of Quasi-likelihood based Information Criterion (QIC) values [98, 101]. Control treatments were never significantly different for the duration of any experiments ($p > 0.75$), nor where differences

observed in responses at time zero, so replicate experiments performed on different dates were pooled for analyses.

Mesocosm analyses were completed using the fraction of prey alive at hours 1, 3, 8, 12 and 24 h (experiment 2) or using count data (n prey alive) at hours 1, 2, 3, 8, 12 and 24 h (experiment 3). Post-hoc contrasts were completed to test overall differences between each pesticide treatment mesocosm vs. controls, while controlling for time. If these contrasts were significant, additional post-hoc tests were completed to compare pesticide treatment mesocosms vs. controls at each time point. To balance Type-1 and Type-2 errors associated with multiple comparisons, I set a False Discovery Rate (FDR) of 5% for survival and RM-GEE models (experiment 1 and 3) [102, 103]. An FDR of 10% was selected for experiment 2 analysis (cannibalism mesocosm), however, to investigate effects of time (also see Supplemental Data Table S2.1-S2.3). Significance of post-hoc contrasts were determined by ranking raw p-values (low to high) and then comparing those p-values against a pre-calculated threshold determined by the number of planned hypothesis tests (i.e., sequential Bonferroni or Holm method) [71]. Survival and RM-GEE analyses were completed in SAS 9.4©, using the Proc Lifetest and Proc Genmod procedures, respectively.

RESULTS

Experiment 1: Survival

Res-PBO and PBO-300 exposures significantly increased mortality of both juvenile and adult blue crabs, with 90-100% mortality observed in all toxicant exposure treatments except for juveniles exposed to 1:3 µg/L ($p < 0.001$, $\chi^2 = 110.12$, Lifetest Log-Rank) (Table 2.2). Compared to controls, adult survival-time was significantly reduced in all Res-PBO and PBO-300 exposures ($p < 0.01$, Supplementary Data Table S2.1). Juvenile survival-time was also

significantly reduced in all exposures ($p < 0.01$), except in 1:3 µg/L treatments ($p = 0.46$). Adult crabs died sooner than juvenile crabs in all Res-PBO treatments, but life-stage differences were significantly different only in the lowest concentration tested ($p < 0.001$).

Table 2.2. Mortality (%) and hours alive (mean [a], min, max) of juvenile and adult crabs exposed to Res-PBO or PBO-300 [b]

	1:3 µg/L			10:30 µg/L			100:300 µg/L			PBO-300 µg/L		
Life Stage	% Mortality	Avg. Hours Alive	Min, Max Hours to Death	% Mortality	Avg. Hours Alive	Min, Max Hours to Death	% Mortality	Avg. Hours Alive	Min, Max Hours to Death	% Mortality	Avg. Hours Alive	Min, Max Hours to Death
Juvenile	33	104 [c]	72,132	100	26.4	12, 60	100	25.2	12, 84	100	31.2	12, 48
Adult	100	22.8	12,48	100	19.2	12,36	100	16.8	12,24	90	67.2 [d]	36,108

[a] averages include deceased crabs only

[b] Zero mortality observed in controls (not included in table); *n = 10* for all treatments and controls

[c] n = 3 crabs that died

[d] n = 9 crabs that died

Experiment 1: RT

Effects of pesticide exposure on RT were similar to those on survival-time. All exposures significantly increased juvenile and adult RT, ($p < 0.001$, Score $\chi^2 = 23.85$, DF = 4) (Figure 2.1), and effects varied significantly with time ($p < 0.001$, score $\chi^2 = 43.94$, DF = 5). RT significantly increased within 1-12 hours in all treatments for both life-stages and remained significantly higher than controls through 48 hours or until death. Life-stage differences were nearly significant ($p = 0.14$, Score $\chi^2 = 2.19$, DF =1), and a life-stage*concentration interaction was included to increase model fit ($p = 0.29$, Score $\chi^2 = 6.98$). Adult RT was significantly higher than juvenile RT in 1:3 µg/L exposures ($p = 0.03$). Other life-stage RT differences were non-significant (Supplemental Data Table S2.1).

Exposure to both Res-PBO and PBO-300 decreased survival-time and significantly increased RT, showing both lethal and sublethal effects on crabs. Effects tended to be more severe and rapid for adults than juvenile crabs, especially in the 1:3 µg/L treatment.

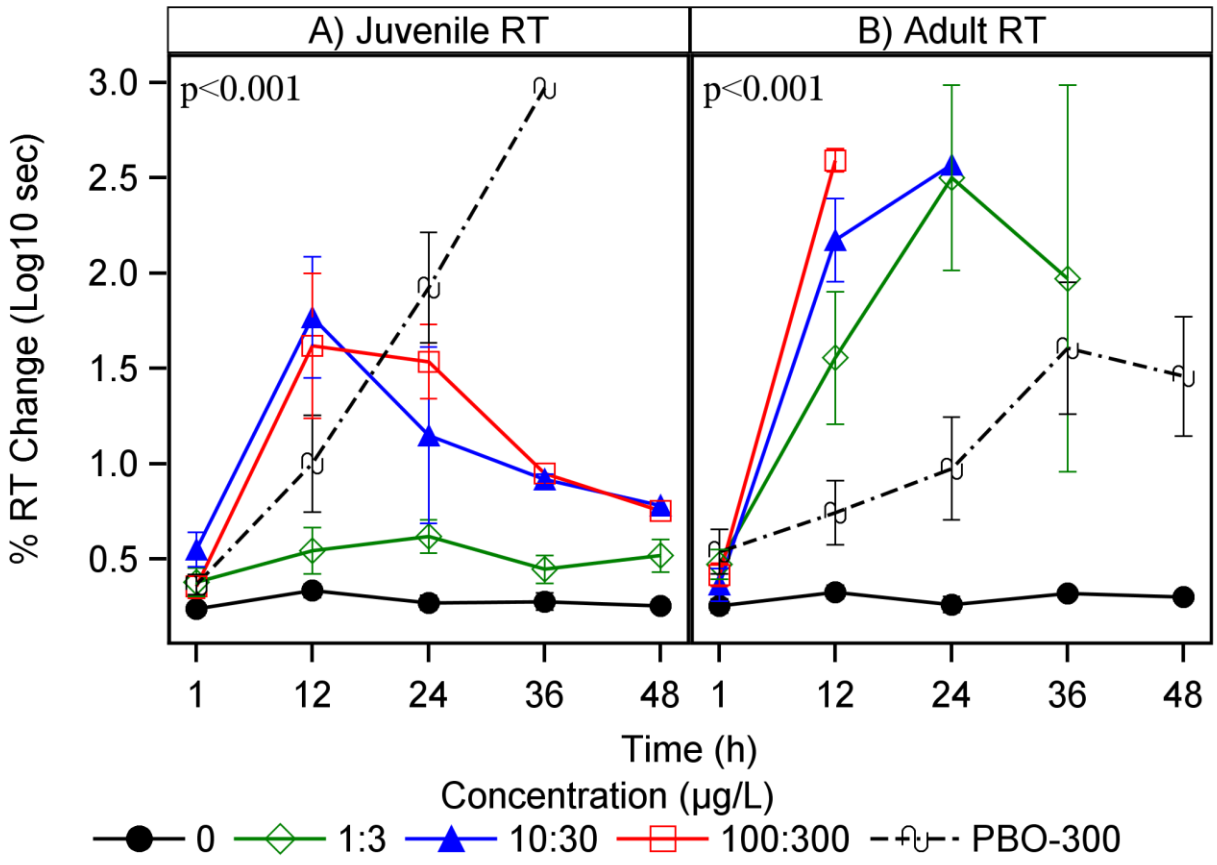

Figure 2.1. RT response: Mean (± SE) percent change of juvenile (A) and adult (B) blue crab RT during a single exposure to Res-PBO for 48 hours. Positive values indicate an increase in RT. (A and B) Compared to controls, all treatments significantly increased juvenile and adult crab RT ($p < 0.001$, RM-GEE post-hoc contrasts). (A vs. B) Life-stage comparisons: In 1:3 µg/L treatments, adult RT was significantly higher than juvenile RT ($p = 0.03$, RM-GEE post-hoc contrasts). Other life-stage RT differences were non-significant (Supplemental Data Table S2.1).

Experiment 2: Adult + juvenile cannibalistic mesocosm

Data were best fit with mesocosm treatment and a mesocosm treatment*time interaction term, with a Poisson distribution and autoregressive correlation (RM-GEE). Juvenile crab (prey) survival varied significantly with mesocosm-treatment scenario ($p < 0.001$, Score $\chi^2 = 75.98$, DF = 5) and mesocosm-treatment*time ($p < 0.001$, Score $\chi^2 = 76.54$, DF = 20) (Figure 2.2). Thus, pesticide effects varied with concentration and time, and differed for predators (adults) and prey (juveniles). Also, see Supplementary Data Table S2.2 for time specific post hoc contrasts. Adult-juvenile predator-prey interactions were primarily driven by predator health (i.e., adult blue crabs), as adult crabs were more vulnerable to 1:3 res-PBO exposures than juveniles. Predator-prey interactions were modified by juvenile-prey health, however, as juveniles exposed to res-PBO were more susceptible to predation.

Adult (predator) exposures. Juvenile crab (i.e., prey) survival significantly increased in mesocosms in which adult crabs were previously exposed to 1:3 µg/L Res-PBO (i.e., adult-only exposure and both-exposure), and was highest when predators were poisoned but prey were not (Figure 2.2). Previous exposure to 1:3 µg/L suppressed 98% of all adult blue predation for the initial 3 hours ($p < 0.01$). Thereafter, consumption increased with time but remained lower than controls (8-12-hrs), indicating recovery from exposure over several hours. By 24 hours, consumption was equal or higher than controls.

Juvenile (prey) exposures. Healthy adult crabs benefited from poisoned prey. Previous exposure to 1:3 and 10:30 µg/L significantly increased juvenile crab (prey) vulnerability to predation, and effects increased with Res-PBO concentration ($p < 0.01$, Figure 2.2). Juveniles exposed to 10:30 Res-PBO were consumed at a significantly higher rate than controls for the first 3 hours ($p \leq 0.02$). Similarly, prey exposed to 1:3 µg/L tended to be eaten more than

controls, but this difference was only marginally significant at 12-24 hours ($p < 0.10$, Figure 2.2).

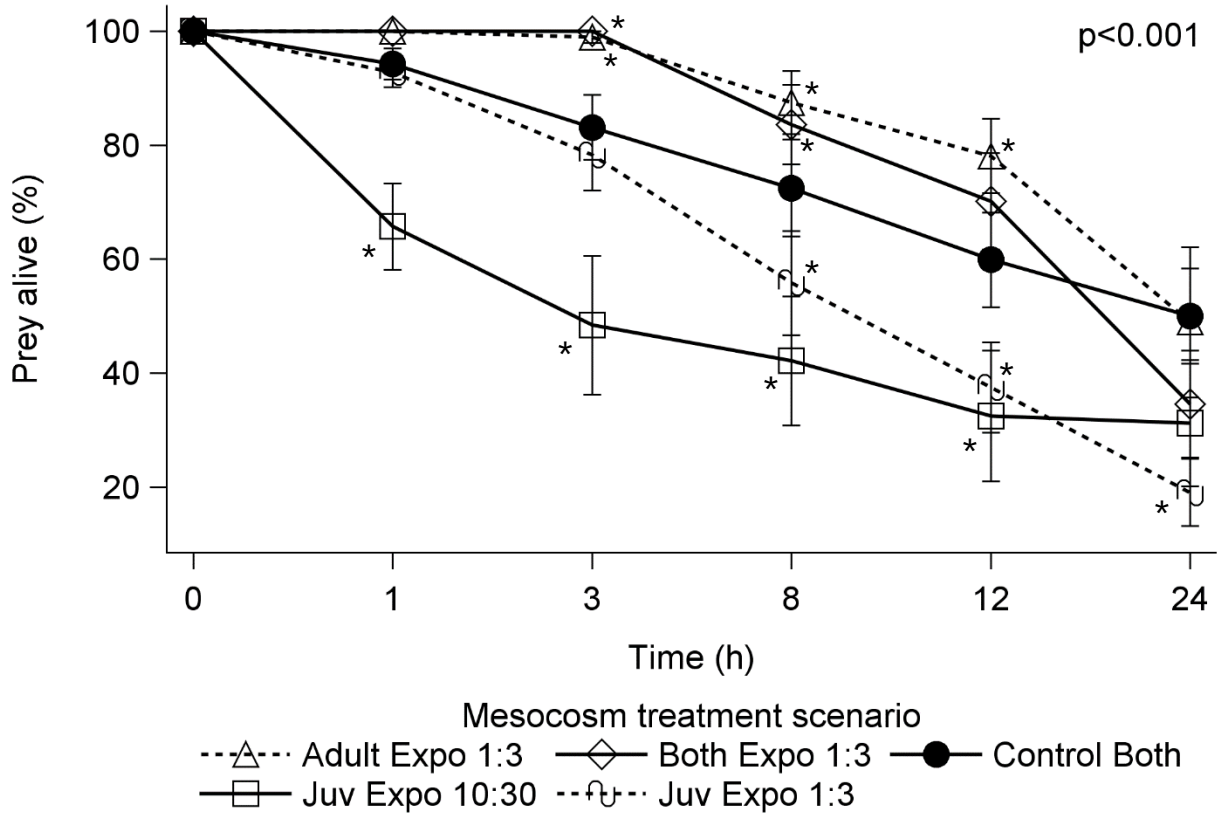

Figure 2.2. Adult + juvenile cannibalistic mesocosm results: Mean (± SE) % survival of prey following 12-h exposures to Res-PBO or control water. Stars (*) indicate significant differences in survival between controls and treatments at each time point. Prey survival varied significantly with mesocosm-treatment scenario ($p < 0.001$, Score $\chi^2 = 75.98$, DF = 5) and mesocosm-treatment*time ($p < 0.001$, Score $\chi^2 = 76.54$, DF = 20). Significant differences between mesocosm treatments vs. controls at each hour ($p \leq 0.03$, RM-GEE contrasts) are as follows: (a) Adult Expo vs. control at 3, 8, and 12 h. (b) Both expo vs. control at 3 h. (c) Juv-Expo 1:3 at 12 and 24 h. (d) Juv-Expo 10:30 at 1, 3, and 12 h. Also see Supplemental Data Table S2.2.

Experiment 3: Juvenile-predator + shrimp-prey mesocosm

Data were best fit with mesocosm treatment and time as independent variables (no interaction), with a Poisson distribution and exchangeable-type correlation (RM-GEE). Prey survival (shrimp) varied significantly with mesocosm treatment scenario ($p = 0.04$, Score $\chi^2 = 8.13$, DF = 3) and time ($p < 0.001$, score $\chi^2 = 21.5$, DF = 5). Also see Supplementary Data Table S2.3.

Similar to adult + juvenile mesocosms (experiment 2), juvenile + shrimp mesocosms were primarily driven by predator health (i.e., juvenile blue crabs). However, unlike adult mesocosms, exposure to 1:3 µg/L Res-PBO tended to stimulate juvenile crab consumption rates of brown shrimp (Figure 2.3).

Predator-juvenile exposures. Juveniles previously exposed to 1:3 µg/L Res-PBO significantly increased consumption rates in the predator-only exposure mesocosms ($p < 0.001$) and both-exposure mesocosms ($p < 0.001$, Figure 2.3). This increase in consumption rate occurred regardless if shrimp were poisoned or not, though after 1-hr, shrimp exposed to 1:3 were consumed at a slightly higher rate than those not exposed.

Prey-shrimp exposure. When juvenile crabs (predator) were unexposed to pesticides, prey (shrimp) previously exposed to 1:3 ppb were consumed at similar rate as controls ($p = 0.07$, Figure 2.3). However, when both predator (juveniles) and prey (shrimp) were previously exposed to 1:3 ppb, shrimp were more vulnerable to predation (i.e., consumed at a higher rate) than controls.

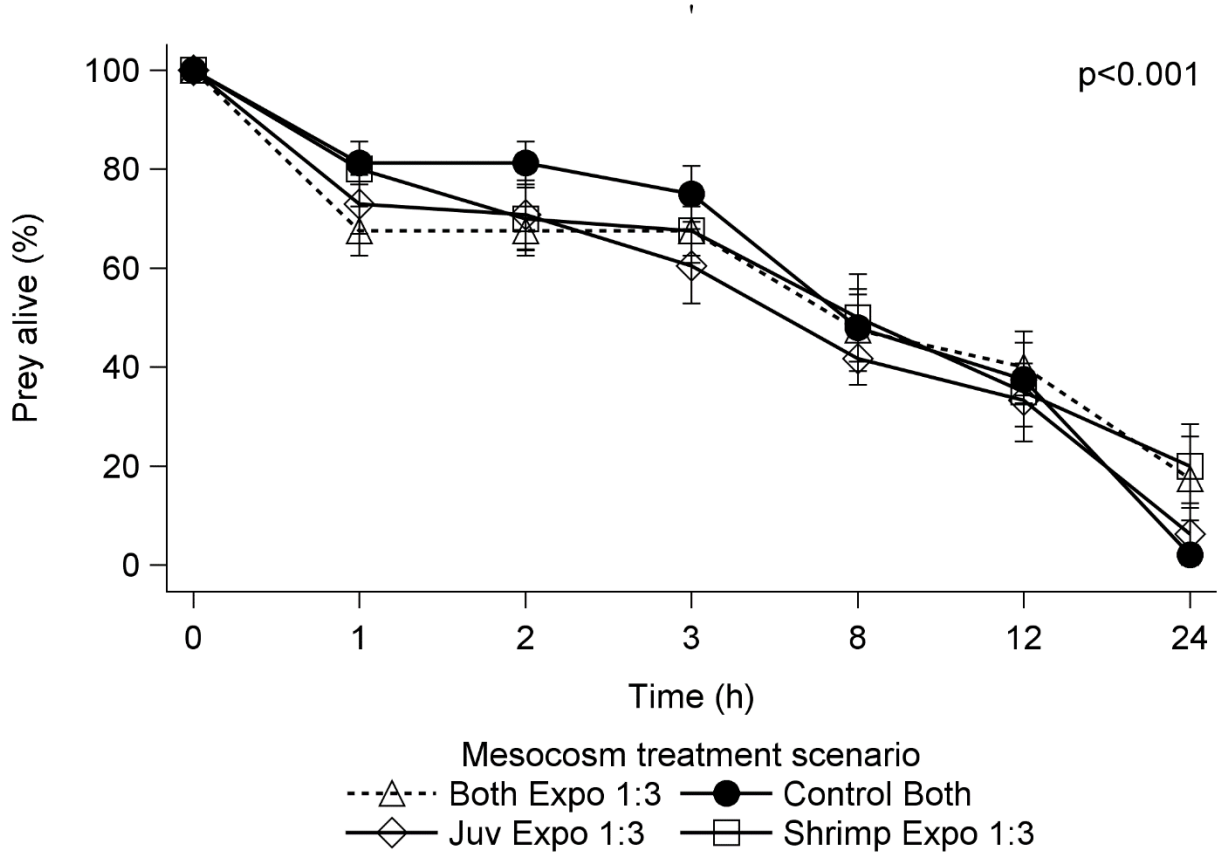

Figure 2.3. Juvenile + shrimp mesocosm results: Mean (± SE) percent survival of brown shrimp following 12-h exposures to Res-PBO or control water. Prey survival (shrimp) varied significantly with mesocosm treatment scenario ($p = 0.04$, Score $\chi^2 = 8.13$, DF = 3) and time ($p < 0.001$, score $\chi^2 = 21.52$, DF = 5). Significant differences between controls vs. pesticide mesocosm are as follows: consumption rates were significantly higher in the (a) Both Expo 1:3 mesocosms ($p < 0.001$, RM-GEE post-hoc contrast) and (b) Juvenile Exposure 1:3 mesocosms ($p < 0.001$, RM-GEE post-hoc contrast). Also, see Supplemental Data Table S2.3.

DISCUSSION

Pyrethroids are the fourth most commonly applied pesticide-family in the U.S. and their usage is expected to increase as more persistent pesticides are phased-out of use [48]. Pyrethroids are commonly sprayed in combination with synergists (e.g., PBO) for vector control in coastal systems [86, 87]. Because of their broad efficacy as Na+ inhibitors (pyrethroids) and a MFO inhibitors (PBO), non-target species, particularly non-insect arthropods like blue crabs, may be affected by exposures. Resmethrin and PBO have been recorded in aquatic environments at similar or higher concentrations than tested in the present study, including: 0.98 µg/L resmethrin [47] and 0.44-20 µg/L PBO [47, 82]. PBO and resmethrin may persist under certain conditions (e.g., >30 days in soil), and may interact with other contaminants in the environment [82]. Additionally, some pyrethroids bioaccumulate in fishes, and are endocrine disruptors [104]. As PBO + pyrethroid mixtures continue to be sprayed near and in aquatic systems to combat vector-borne disease, it will be important to consider the synergistic effects of PBO with other contaminants and stressors that may already be present at low concentrations.

Individual exposures: effects on individuals

Res-PBO (1:3, 10:30, 100:300 µg/L) and PBO-300 exposures were highly toxic to juvenile and adult blue crabs, causing 33-100% mortality < 60 hours. This is consistent with other studies which found the 48-96 hour LC50 of resmethrin for invertebrates and fish ranged from 0.22-15 µg/L [105]. All Res-PBO and PBO-300 exposures increased juvenile and adult RT within 1-12 hours of exposure, indicating rapid declines in neurological functioning and coordination. In natural systems, contaminants like Res-PBO and other stressors may have profound effects by removing key species or by altering traits that in-turn alter species

interactions (e.g., density mediate indirect interactions and trait mediated indirect interactions). For example, reductions in blue crab abundances associated with commercial and recreational fishing has been linked with increased grazer populations and salt marsh die-offs [23, 27]. Pesticides may also interact with other stressors, e.g., fishing pressure, disease, drought, pH [29].

Importantly at low, Res-PBO exposure levels (1:3 and 10:30 µg/L) effects on adult RT and mortality were more severe than effects on juveniles. For example, following 12 hours of exposure to 1:3 µg/L Res-PBO, the average juvenile RT increased to 4.2 seconds vs. an average RT of 82 seconds for adult crabs. Early life-stages and smaller-bodied individuals are frequently more sensitive to toxicant exposure than older, larger adult forms [72, 106], though not always [107]. For example, the insecticides fipronil and endosulfan were more toxic to adult grass shrimp (LC_{50} 0.32 µg/L, 1.01 µg/L) than both larval (LC_{50} 0.68 µg/L, 2.56 µg/L) and embryonic life-stages (LC_{50}>512 µg/L, 117 µg/L) [73]. The mechanism explaining these life-stage differences is unknown, but may be related to several factors including differences in respiration rates, uptake/absorption rates [79], but likely not gill surface area [108, 109]. It may also be that adult crabs have more MFO enzymes that can be inhibited by PBO, which in turn could increase the efficacy of resmethrin. These life-stage differences require further study and were the basis of predator-prey mesocosm experiments (experiment 2 and 3).

Mesocosms: predator effects

Adult crab (predator) foraging ability was greatly reduced by 1:3 Res-PBO exposures, even in mesocosm in which juveniles (prey) were also exposed to 1:3 Res-PBO (experiment 2). Thus, adult blue crab (predator) health was the primary driver of prey survival in mesocosm experiments. Results are similar to those reported by Kerby *et al*. (2012), in which diazinon (organophosphate) reduced predator vigor (defined as predator activity and attack rates) and

indirectly benefited prey by reducing predation rates [15]. An important nuance of Kerby *et al*. (2012) and of the present study, is both predator and prey were negatively affected by pesticide exposure, but because predators (adults) were more vulnerable to pesticides, prey (juveniles) indirectly benefited.

Effects on adult blue crab consumption rates may be the result of reduced coordination, altered sensory ability, or decreased motivation. Initially (<1 hour), several adult crabs (predators) were observed flipping (360˚) in mesocosms; severe muscle spasms and erratic limb movements were observed in mesocosms and similar effects were also observed in RT assays. These observations corresponded with zero consumption rates in mesocosm experiments. Subsequently (>3 hours), reduced consumption rates in adults may have persisted because of an inability to catch prey as a result of incoordination or lethargy, rather than lack of motivation (*personal observation*). Both unexposed and Res-PBO exposed adult crabs increasingly moved, flipped, and piled oyster shells as experiments progressed (*data not presented*), which might suggest that Res-PBO exposed adult crabs were searching for prey. In other studies [9, 30], adult blue crabs exposed to heavy metals were uncoordinated and targeted less active prey species. Adults were not given an alternative prey source in the present study, but pesticide exposure may have similar effects as heavy metals.

In contrast to effects on adult crab predators, previous exposure to 1:3 µg/L Res-PBO tended to initially increase (stimulate) juvenile crab consumption rates of brown shrimp (experiment 3). Increased consumption rates (i.e., hyperactivity) in juveniles may indicate a compensatory stress response to increased energy needs associated with the cost of detoxification or direct toxicity effects of res-PBO exposure [15]. Increased consumption rates may have long term negative effects on growth and reproduction for juvenile blue crabs. For example, juvenile

fish fed a zinc rich diet, increased their food consumption but they also grew more slowly than fish fed a normal diet [110]. Additional investigation of long-term effects of pesticides exposures on juvenile crab growth and energy requirements could provide insight into the mechanism explaining these results. Finally, increased foraging rates by juvenile crabs could decrease prey populations or increase intraspecific juvenile competition and may indirectly impact other trophic interactions.

Mesocosms: prey vulnerability

Previous exposure to 1:3 µg/L and 10:30 µg/L Res-PBO increased juvenile crab (prey) vulnerability to healthy (unexposed) adult blue crab predators (experiment 2). Increased juvenile foraging activity, i.e., hyperactivity, observed in juvenile-predator mesocosms (experiment 3) may help explain increased vulnerability to adult crab predators. Toxicant induced hyperactivity has been linked with increased vulnerability to predation in several predator-prey studies [15, 19, 111-113]. Like juveniles, adult crabs may be more vulnerable to predation after exposure to Res-PBO. Although I did not measure predation on adult crabs directly, increased RT (experiment 1) and an inability to capture prey (experiment 2) corresponded with severe incoordination and crabs flipping/spinning in the water column (*personal observation*), suggesting increased vulnerability to predators.

In summary, a single, short (12 hour) sublethal exposure to environmentally realistic concentration of a common pyrethroid + synergist mixture used for mosquito abatement negatively affected juvenile and adult blue crab survival, locomotor coordination, and predator-prey interactions. For both juvenile and adult life-stages, a reduced ability to avoid predation or acquire resources may result in altered trophic relationships or abundances [23, 27]. Furthermore, adult crabs were more vulnerable to Res-PBO than juvenile crabs. This study

highlights the importance of investigating toxicant-induced behaviors in an ecological context, e.g., effects on ecologically relevant behaviors and interactions that structure communities [7, 12]. This is especially true in estuarine systems, which remain less studied than freshwater systems [1]. Finally, as all organisms experience several sources of natural and anthropogenic stressors in their natural environments simultaneously (e.g., fishing pressure, disease, temperature, salinity, other pollutants, etc.), it is likely that non-target organisms and species interactions may be altered by pesticides and other stressors in complex and interacting ways not measured at the individual level [7, 12, 43, 47]. Given that contaminants may occur in nearly every environment on Earth, studying ecological interactions under different, sublethal toxicant exposure scenarios is also valuable and necessary for a realistic understanding of how ecosystems components (e.g., species, populations, communities) interact and respond to environmental stressors.

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CHAPTER III. Effects of pesticide mixtures on adult and juvenile blue crabs: Malathion, carbaryl, resmethrin, and piperonyl butoxide (PBO)

ABSTRACT

Organisms are often simultaneously exposed to multiple forms of pollution in the environment, yet, experiments are usually designed to measure effects of a single substance at varying concentrations. Importantly, these pesticides may interact synergistically to kill non-target species and to alter critical life history functions such as foraging, predator-avoidance, and mating. Blue crabs (*Callinectes sapidus*) are an important ecological and economic estuarine species that may be inadvertently exposed to pesticide mixtures at concentrations that increase mortality and alter behaviors critical to survival. Here, I investigated the effects of three commonly used insecticides (malathion, carbaryl, resmethrin) and a synergist (PBO) individually and in combination at 5 concentrations (0 µg/L, 1 µg/L, 3.33 µg/L, 5 µg/L and 10 µg/L) on juvenile and adult blue crab survival and neuromuscular functioning. All mixture treatments significantly reduced survival and increased righting time (RT), a proxy for neuromuscular functioning. Effects in mixture treatments peaked within the first 12-24 hours, with most deaths occurring < 36 hours, and persisted for 7 days. Compared to individual exposures, mixture effects were the same as those seen in response to resmethrin + PBO exposures, suggesting mixtures containing all four chemicals are not working synergistically. However, exposures to low, environmentally occurring concentrations of pesticide mixtures reduced blue crab survival and altered their behavior, suggesting pesticides likely influence estuarine food webs and commercial fisheries.

INTRODUCTION

Insecticides are often present as mixtures and can have synergistic interactions with each other and with other biotic and abiotic stressors such as predation, competition, temperature, and salinity [7, 32, 43, 44]. Synergistic interactions between anthropogenic and natural stressors may have large effects on natural communities by removing organisms (lethal effect) and by affecting critical life history functions such as mating, foraging and predator avoidance (sublethal effects). Studies examining the effects of insecticide mixtures are limited, particularly in marine systems [1]. Carbamates, organophosphates (OP), and pyrethroid insecticides represent three of the top four most commonly applied insecticide-families in the U.S., and have been measured in aquatic systems as mixtures (Table 3.1) [41, 42] (also see subsection, *Pesticide Background*). Because of their common usage and broad efficacy as neurotoxic insecticides, these pesticides may have large effects on non-target species and were studied here. In estuaries, blue crabs (*Callinectes sapidus*) are an important ecological and fishery species but are vulnerable to insecticides because they, like the insects targeted by insecticides, are arthropods [22-24, 28, 62].

Adult crabs are a valuable fishery for several coastal states along the southern and eastern coast of the U.S., but their populations are declining [114-117]. Declines have often been attributed to fishing pressure [116, 117]. Other factors (e.g., disease, pollution, habitat loss) may also contribute to blue crab population status, but their effects, particularly of insecticide exposure, have not been well studied [31]. Heavy metal exposure is known to affect blue crabs [10, 11, 30, 118, 119], however, causing decreased crab coordination [11, 30]. In those studies, trophic interactions were also affected by heavy metals, as exposed crabs targeted and consumed less active prey that were easier to catch [30].

Table 3.1. Classification, modes of action, and environmental concentrations: malathion, carbaryl, resmethrin and PBO

Chemical	Class	Mode of Action	Aquatic Half-Life	Aquatic [Observed] (µg/L)	Texas [Observed] (µg/L)	Health Advisory Level [120] (µg/kg/day)	48-h LC_{50}, blue crabs	48-96 h LC_{50} marine invertebrates (µg/L)
Carbaryl	Carbamate	AChE inhibitor	21 d [34]	4800 [121, 122] 0.021-33.5 [41]	3.64 (carbamates) [123]	10	320-550 [124, 125]	1.5-22,700 [125, 126]
Malathion	Organophosphate	AChE inhibitor	7 d [35]	583- 787 [60, 127, 128]	0.32 -11 [40, 129, 130]	70	> 1000 [131]	0.50 – 2960 [35, 128]
Resmethrin	Pyrethroid	Prolonged opening of Na^+ channels	22-90 hr [83, 132]	0.98 [38, 47, 83]	8.9-11.2 (soil) [84], *Present*, Nueces River [b]	20 [a]	--	0.22-10.3 [47, 133]
Piperonyl Butoxide (PBO)	Synergist	Inhibition of cytochrome P450s	30 d [38]	20 [47, 82, 88, 134]	*Present*, Nueces River [b]	--	--	4,900 [39]

[a] HA for permethrin, a pyrethroid similar to resmethrin.

[b] P.V. Zimba, Texas A&M University-CC, Corpus Christi, TX, USA, personal communication

Like heavy metals, pesticides can also disrupt trophic relationships in aquatic systems via effects on both consumers and producers [1, 15, 63]. In blue crabs, exposure to 11.2 µg/L malathion reduced adult blue crab coordination and increased righting time (RT) after one hour of exposure [64]. And carbaryl, malathion, and resmethrin + piperonyl butoxide (PBO) exposures (1 µg/L, 10 µg/L, and 100 µg/L) increased adult and juvenile blue crab RT and reduced coordination (Chapter 1 and 2). RT is a common proxy measurement used to indicate crab health and neurological functioning [66, 93], and is defined as the amount of time it takes a crab to resume its normal position after being placed on its back (180°). Importantly, increased RT caused by sublethal pyrethroid exposure was linked with increased vulnerability to predation in juvenile blue crabs, and decreased foraging abilities in adult blue crabs (Chapter 2). Thus, individual toxicants can compromise blue crab foraging and predator avoidance behaviors and ultimately reduce survival.

Pesticides are often present as mixtures in the environment [8, 41, 42], and understanding how mixtures may affect key species is a necessary step to evaluating potential risk and costs associated with pesticide use. Ultimately, the effects of pesticide mixtures may be more severe than the effects of individual exposures, but these effects have not yet been evaluated in blue crabs. The lethal and sublethal effects of pesticide mixtures comprised of three insecticides (carbaryl, malathion, resmethrin) and a synergist (PBO), on juvenile (post-planktonic) and adult blue crabs were investigated. These pesticides (and pesticides of the same family) are frequently found in non-target environments (Table 3.1) and affected juvenile and adult blue crabs in previous experiments (Chapter 1 and 2; also see [64]). I focused on behavioral changes (i.e., RT). RT is a proxy used to assess the overall neurological and muscular functioning of crabs [64, 66, 93], and was linked with altered blue crab predator-prey interactions previously (Chapter 2).

Pesticide background: carbaryl, malathion, resmethrin & PBO

Three insecticides (carbaryl, malathion, resmethrin) and a pesticide synergist (PBO) were selected for study based on of their wide usage in mosquito/vector control, agriculture, and by private landowners, and have all been recorded in non-target aquatic environments (Table 3.1). They also represent three commonly used insecticide families: organophosphates (OP), carbamates, and pyrethroids. These pesticides target the nervous system, resulting in convulsions, muscle spasms, respiratory failure, and death in many non-target taxa [33-36]. Both malathion and carbaryl inhibit acetylcholinesterase (AChE) [33, 36], and resmethrin (a Type I pyrethroid) targets Na^+ channels associated with the nervous system [37]. PBO is a "synergist" that increases the effects of pyrethroids by inhibiting enzymes involved with toxicant elimination (i.e., cytochrome P450s). PBO is commonly sprayed with pyrethroids for vector disease control (e.g., West Nile, malaria, dengue, yellow fever) and agricultural pest management (e.g. cotton pests) [38, 39]. For example, in 2012, Dallas County Texas, USA aerially sprayed Duet© to combat West Nile Virus. DUET© contains two pyrethroid insecticides (prallethrin and sumithrin) + PBO [135]. A similar product's components, Scourge©, which contains resmethrin + PBO were tested in the current study [46, 47] and were previously approved for spraying by local municipalities in Texas.

Given the wide application of these pesticide families in Texas and throughout the U.S., they likely contaminate coastal aquatic systems. Survey data in coastal systems remain limited. However, pesticides are often present in coastal rivers and may therefore enter estuaries and affect non-target species [40]. For example, the nonregulatory Health Advisory Levels in drinking water for these insecticides (or similar insecticides) range from 10-70 µg/kg/day (Table 3.1), indicating these concentrations may legally exist in nature. Furthermore, a decade-long survey of U.S. streams conducted by the U.S. Geological Survey (USGS) revealed that

insecticides were present in 90% of streams throughout the U.S. [41], which may eventually find their way into coastal system. Long-term environmental monitoring of pyrethroids is especially limited, largely because of their short half-lives. However, concentrations up to 15 µg/L PBO were measured in Long Island surface waters 3 days after a Scourge© application and subsequent rainfall (Table 3.1) [47], suggesting that pyrethroids were likely also present at one time in these estuarine systems because PBO and pyrethroids are commonly sprayed together. Additionally, in the American and Sacramento Rivers near Sacramento, California, 20 µg/L PBO was recorded [38, 82],

In Texas, pyrethroids have been measured in sediments of major cities [84], as well as in the Nueces River, a tributary of Corpus Christi Bay in South Texas (P.V. Zimba, Texas A&M University-CC, Corpus Christi, TX, USA, personal communication). Indeed, all the pesticide families (e.g., carbamates, organophosphates, etc.) tested in this study were recorded in Nueces River (TX) (P.V. Zimba, personal communication,). These pesticide families have been found in other estuarine systems both in Texas and elsewhere (Table 3.1), suggesting blue crabs are commonly exposed to pesticide mixtures at sublethal concentrations similar to the lower levels tested in this study (e.g., ≤10 µg/L, Table 3.1).

MATERIALS & METHODS

General design

Male and female juvenile (27 mm ± 7.8 mm CW, sexually immature) and adult (107 ± 7.8 mm CW, sexually mature) blue crabs were exposed to one of five pesticide mixture concentrations, 0 µg/L (control), 1 µg/L, 3.33 µg/L, 5 µg/L, or 10 µg/L for seven days using a static non-renewal protocol. Mixture concentrations were additive and consisted of: carbaryl + malathion + resmethrin + PBO in a 1:1:1:3 ug/L ratio (w/w/w/w), i.e., 10 µg/L mixtures

included: 10 µg/L malathion + 10 µg/L carbaryl + 10 µg/L resmethrin + 30 µg/L PBO. Malathion, carbaryl, and resmethrin were kept in equal parts, and exposure ratios were also selected to reflect resmethrin + PBO product ratios, which often range from 1:3 to 1:20 (w/w, resmethrin-to-PBO). This ratio (1:3) reflects other pyrethroid + PBO products as well [38]. Mixture concentrations were selected based on previous experiments with individual pesticides and represent a range of realistic environmental concentrations (Table 3.1, Chapter 1 and 2). Specifically, previous experiments with individual exposures investigated the effects of 0 µg/L, 1 µg/L, 10 µg/L and 100 µg/L carbaryl, malathion, resmethrin and resmethrin +PBO. To compare with mixture exposures, however, I investigated the effects of mixture concentrations equal or lower than 10 µg/L, because crabs can survive in these concentrations but experience sublethal effects. Additionally, these concentrations represent a realistic exposure range. Using higher mixture concentrations would likely have resulted in crab death too quickly and at high rates.

One artifact of this design was separating mixture effects with those of overall concentration, as changes in crab mortality or behavior in mixture treatments could result from synergistic/additive pesticide interactions or from a larger amount of pesticide present. For example, treatments using 10 µg/L mixtures were chosen to compare with 10 µg/L individual exposures, but because 10 µg/L mixtures contain a total of 30 µg/L of pesticide I used lower mixture concentrations to account for adding more pesticides overall. Thus, in addition to 10 µg/L of each pesticide (30 µg/L total), I also exposed crabs to 3.33 µg/L (10 µg/L total) and 5 µg/L (15 µg/L total) as an intermediate level. Lastly, a static non-renewal design was chosen to mimic exposure scenarios in bays with long residence times, which is common in the western Gulf of Mexico where residence times ranged from 10 to 360 days [68].

To quantify effects of pesticide concentration and differences between life-stage, crab survival and changes in behaviors were measured for 7 days [2, 66, 67]. Blue crabs were assigned to treatments haphazardly, with an effort to balance sex ratios. Replicates per treatment and life-stage were as follows for (a) juveniles: 0 µg/L (n = 27), 1 µg/L (n = 24), 3.33 µg/L (n = 30), 5 µg/L (n = 19), 10 µg/L (n = 24), and (b) adults: 0 µg/L (n = 23), 1 µg/L (n = 10), 3.33 µg/L (n = 14), 5 µg/L (n = 10), 10 µg/L (n = 10). Because of space limitations and animal availability, replicate trials were performed over several weeks during the summers of 2012 and 2013. Controls and insecticide treatments were always tested concurrently, and mortality and behavior of crabs in control treatments did not differ among trials or between crabs at time zero (i.e., before pesticide exposure).

Animal collection and housing

Adult and juvenile blue crabs were collected from estuaries near Corpus Christi, Texas, USA and taken to Texas A&M University-Corpus Christi, TX, USA for experiments. Crabs were acclimated to experimental conditions in tanks at salinity 20 and water temperature of 22-24^{o} C for a minimum of 48 hours. All seawater was made using dechlorinated tap water and Instant Ocean™. Crabs were fed shrimp daily but not in the 24 hours prior to the start of each experiment, and were kept at a 12:12-14:10 hour light:dark schedule during the acclimation period and experiments.

Exposure set-up and solutions

Crabs were exposed to control or pesticide-water in individual glass bowls (salinity 20): adults in 10-L (with aerators), and juveniles in 0.5-L (without aerators) of solution. Previous experiments found that juveniles did not require aerators, that crabs were able to survive in experimental conditions for 2 weeks, and that juvenile and adult blue crab RT (percent change)

in control treatments were never significantly different using this exposure regime. Crabs were shielded from each other during experiments to minimize external stimuli. Stock solutions were made 2-3 hours before each experiment. Sea water, malathion, carbaryl, resmethrin, PBO, and ethanol were added to a volumetric flask, manually mixed, and then placed on a stir plate and mixed for an additional 60-75 minutes. Ethanol was used to increase toxicant solubility in stock solutions, resulting in 100 µg/L (v/v) of ethanol in the highest treatment. All control treatments included 100 µg/L ethanol to discern any effects possibly caused by ethanol and none were observed. Thus, changes in mortality and behavior were attributed to pesticide exposure. Chemicals used were malathion (Pestanol©), carbaryl (1-Naphthyl-*N*-methylcarbamate, Pestanol©**),** resmethrin, PBO (90% purity), and ethanol (absolute); all from Sigma-Aldrich.

Response variables: description and collection

Crab survival time and RT were measured immediately prior to exposure (T_0), 1 hour after exposure (T_1), and every subsequent 12 hours (T_{12n}) for seven days (168 hours). T_0 responses were used to verify crabs were healthy and not different prior to pesticide exposure. Survival time was recorded as the number of hours alive, and crabs were considered dead if unresponsive and no scaphognathite movement was observed. RT, defined as the time required for a crab to resume an upright position after being inverted onto its dorsal side (180º), is a standard method to assess overall neurological and muscular functioning of crabs; an increase in RT indicates decreased functioning [66, 93]. Using a stop watch, RT measurement began the moment a crab was fully inverted until it was upright. Crabs that remained inverted for 300 s were righted and a RT of 300 s (5 min) was recorded.

Statistical analysis

Mixture exposures only (survival). The effects of mixture concentration and life-stage on crab survival time (hours alive) were tested with a time-to-event analysis [15]. A single model was completed, using a log-rank test, which compared all survival curves (i.e., pairwise) within the same life-stage, as well as juveniles vs. adults exposed to the same concentration. Post hoc contrasts were adjusted using the Holm Method (i.e., sequential Bonferroni) to account for multiple comparisons [69-71]. Detailed model and post-hoc contrasts are reported in Supplemental Data (Table S3.1). Survival analyses were completed in SAS 9.4© using the Proc Lifetest procedure.

Mixtures vs individual exposures (survival). The effects of mixture vs. individual pesticide exposure (i.e., pesticide-type) on crab survival time (hours alive) were also tested with a time-to-event analysis. A separate model was completed, using a log-rank test, for each life-stage which compared survival curves of all treatment levels of each pesticide-type. Post hoc contrasts were completed to compare pairwise contrasts between mixture exposures and individual exposures of the same level (e.g., mixture 1 µg/L vs. all individual exposures of 1 µg/L); 3.33 µg/L mixture exposures were also compared with 10 µg/L individual exposures adjusted using the Holm Method [69-71]. Detailed model and post hoc contrasts are reported in Supplemental Data (Table S3.2).

Mixture exposures only (RT). RT data were analyzed using Repeated Measures Generalized Estimating Equations (RM-GEEs), which are appropriate for modeling non-normally distributed data and correlated responses within-subjects (i.e., crabs) and groups (e.g., treatment levels) over time [95-99]. The RM-GEE model was best fit as follows:

RT were best fit with concentration, time (day), and a concentration*time interaction term as independent variables. The percent change in RT from T_0 (before pesticide exposure) was $\log_{10}$ transformed prior to analysis. This transformation and use of percent-change data were necessary to account for scale differences between life-stages, because adult crabs require more time to right themselves than healthy juvenile crabs. The final equation was: $\text{Log}_{10}[((T_n - T_0 / T_0)*100) + z]$, where z = the largest RT difference value. Post-hoc contrasts were completed to test for significant differences between pesticide treatments vs. controls within the same life-stage, and between life-stages at the same concentration (e.g., adults vs. juveniles in 1 µg/L mixtures). Post hoc contrasts were adjusted using the Holm Method to account for multiple comparisons [69-71]. Detailed RM-GEE model and post-hoc contrasts for all responses, including estimates and odds ratios, are reported in Supplemental Data (Table S3.1).

Final RM-GEE parameter selection was determined by Type III generalized score tests, and are reported as raw p-values, $\chi 2$, and degrees of freedom [100]. Also, see Supplemental Data (Supplemental Data Table S3.1). In cases where $p>0.05$ for explanatory parameters, final model selection was based on parsimony and comparison of Quasi-likelihood based Information Criterion (QIC) values [98, 101]. Control treatments were never significantly different for the duration of experiments ($p > 0.75$), so replicate experiments performed on different dates were pooled for analyses. Similarly, no significant differences were found between sexes ($p > 0.50$). RT analyses were completed in SAS 9.4© using the Proc Genmod procedures.

Mixtures vs individual exposures (RT). To compare effects of mixture exposures vs. individual exposures on juvenile and adult crab RT after 12 hours of exposure, data were analyzed using separate ANOVAs for each life-stage. The initial analyses were 2-way ANOVAs, which examined the effects of all pesticide-types and concentration (control, 1 µg/L, and 3.33

µg/L with 10 µg/L) for each life-stage separately. When a significant interaction term between pesticide-type and concentration occurred, I completed univariate tests [136]. Specifically, the log-log transformed % change in RT (difference) from T_0 was analyzed using separate 1-way ANOVAs for each concentration level and life-stage, with pesticide-type as a fixed factor. Data were transformed to meet normality standards of an ANOVA. Experimental replicates were individual crabs. Post hoc analysis was completed using the Holm Method to complete pair-wise contrasts of each pesticide-type within each concentration [69-71]. RT analyses were completed in SAS 9.4©.

RESULTS

Survival time, mixtures only

All mixture exposures (1 µg/L, 3.33 µg/L, 5 µg/L, and 10 µg/L) significantly increased mortality of both juvenile and adult blue crabs ($p < 0.001$, $\chi^2 = 132.51$, Lifetest Log-Rank) (Figure 3.1 and 3.2). Compared to controls, juvenile and adult survival-time was significantly reduced in all mixture exposures ($p \leq 0.01$). Mixture 10 µg/L significantly reduced juvenile crab survival time more than all lower treatments (1-5 µg/L). And in adult crabs, 5 µg/L and 10 µg/L significantly reduced survival time more than the two lowest treatments (1 and 3.33 µg/L). Life-stage differences were significantly different in 3.33 µg/L and 10 µg/L treatments ($p < 0.001$), with juveniles dying sooner in 3.33 µg/L than adults and adults dying sooner in 10 µg/L than juveniles. Adults also suffered higher total mortality than juveniles in the two highest concentrations, whereas juvenile mortality was higher than adults in the two lowest concentrations (Figure 3.1). See Supplemental Data (Table S3.1, S3.2) for all post hoc contrasts and survival data in tabular format.

Survival time, mixtures vs. individual exposures

Survival time was significantly dependent on pesticide-type for both juvenile ($p < 0.001$, $\chi^2 = 209.52$, Lifetest Log-Rank) and adult ($p < 0.001$, $\chi^2 = 255.02$, Lifetest Log-Rank) blue crabs (Figure 3.2A-E). Post hoc analysis revealed that 10:30 µg/L resmethrin + PBO, and 10 µg/L mixture treatments significantly reduced survival of juvenile crabs as compared to all resmethrin, carbaryl, and malathion exposures, as well as compared to the lower mixtures exposures (1-5 µg/L) (Figure 3.2, top panels). See Supplemental Data (Table S3.3) for all contrast results. In adult crabs, resmethrin + PBO (1:3 µg/L and 10:30 µg/L), resmethrin (10 µg/L), and mixtures

(10 µg/L and 5 µg/L) treatments significantly reduced survival time as compared to all other treatment levels and pesticide-types (Figure 3.2, bottom panels).

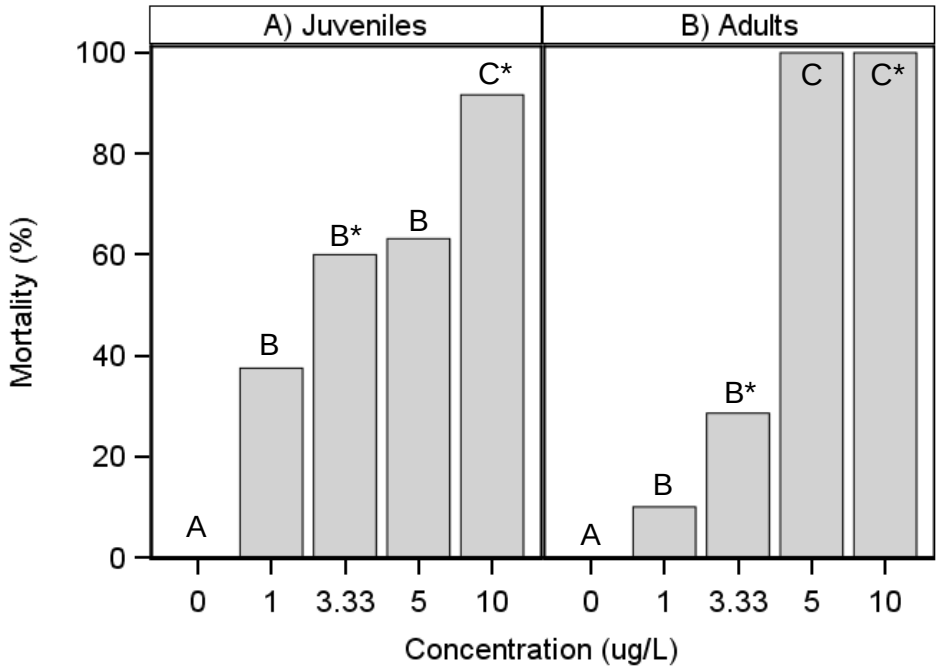

Figure 3.1. Total percent mortality for juvenile (A) and adult (B) blue crabs exposed to mixture or control treatments for 7 days. Letters indicate significant differences in survival within the same life-stage panel. Stars (*) indicate significant life-stage differences at the same concentration (e.g., juvenile 3.33 vs. adult 3.33). Compared to controls, juvenile (A) and adult (B) survival-time was significantly reduced in all mixture exposures ($p \leq 0.01$, log-rank lifetest post-hoc tests). (A vs. B) Life-stage differences were significant in 3.33 µg/L and 10 µg/L treatments ($p < 0.001$, RM-GEE post-hoc contrasts), with juveniles dying sooner in 3.33 µg/L than adults and adults dying sooner in 10 µg/L than juveniles.

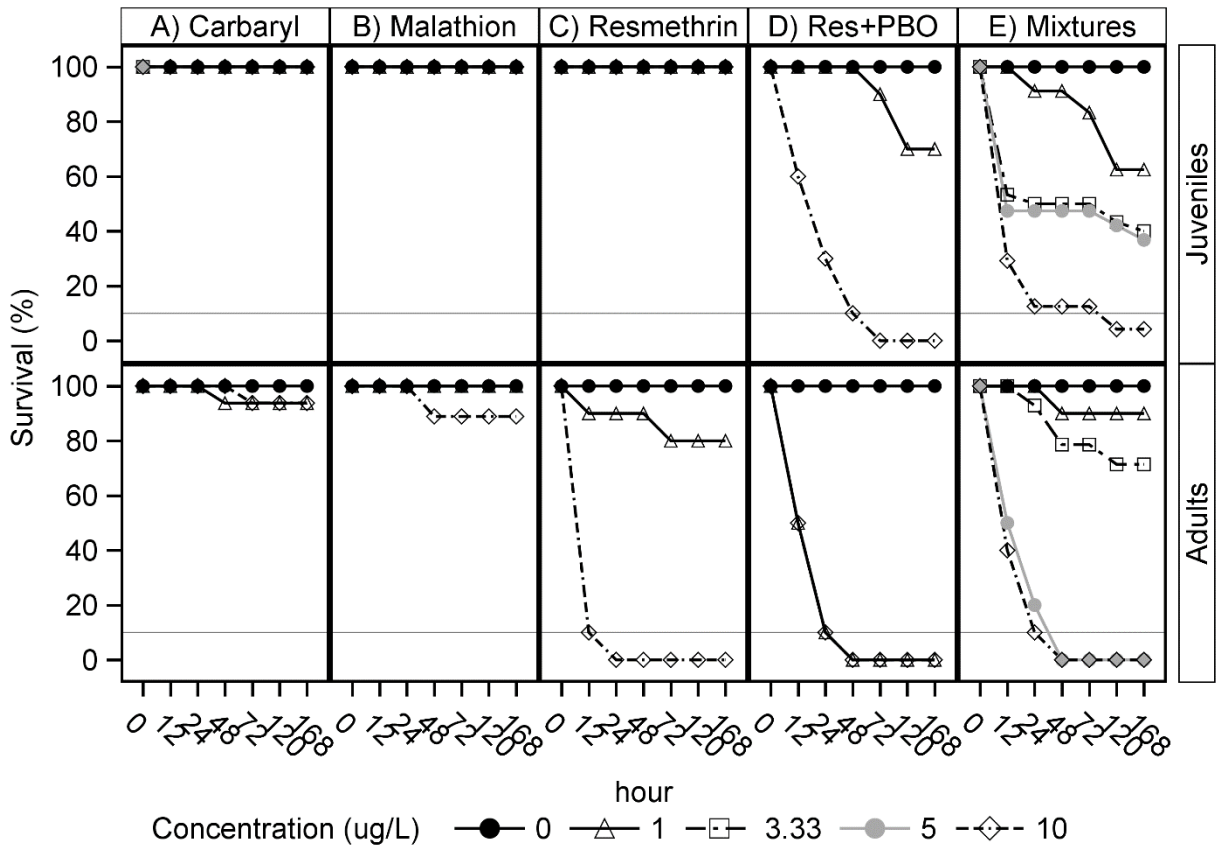

Figure 3.2. Percent survival of juvenile (top) and adult (bottom) blue crabs exposed to carbaryl, malathion, resmethrin, resmethrin + PBO, or mixture treatments for 7 days (168 h). Data are presented at select hours that are representative of the full data set, including 1,12, 24, 48, 72, 120 (day 5), 168 (day 7). For comparisons, reference line (grey) shows 10% survival level.

RT, mixtures only

All mixture exposure concentrations significantly increased juvenile and adult RT ($p < 0.001$, Score $\chi^2 = 64.45$, DF = 4) (Figure 3.3). Effects varied significantly with time ($p < 0.001$, score $\chi^2 = 31.79$, DF = 7, Supplemental Data Figure S3.1). RT increased before death, and crabs that survived tended to recover. For example, all but 3 juveniles exposed to 10 µg/L mixtures died < 24 hours, thus data after 24 h RT are from the individuals that survived past 24 hours and that were less affected by exposure. A Life-stage*Concentration interaction was included to increase the RM-GEE model fit ($p = 0.16$, Score $\chi^2 = 7.87$, DF = 5). Adult RT was significantly higher than juvenile RT in 5 µg/L and 10 µg/L exposures ($p \leq 0.04$). Other life-stage RT differences were non-significant (Supplemental Data Table S3.1).

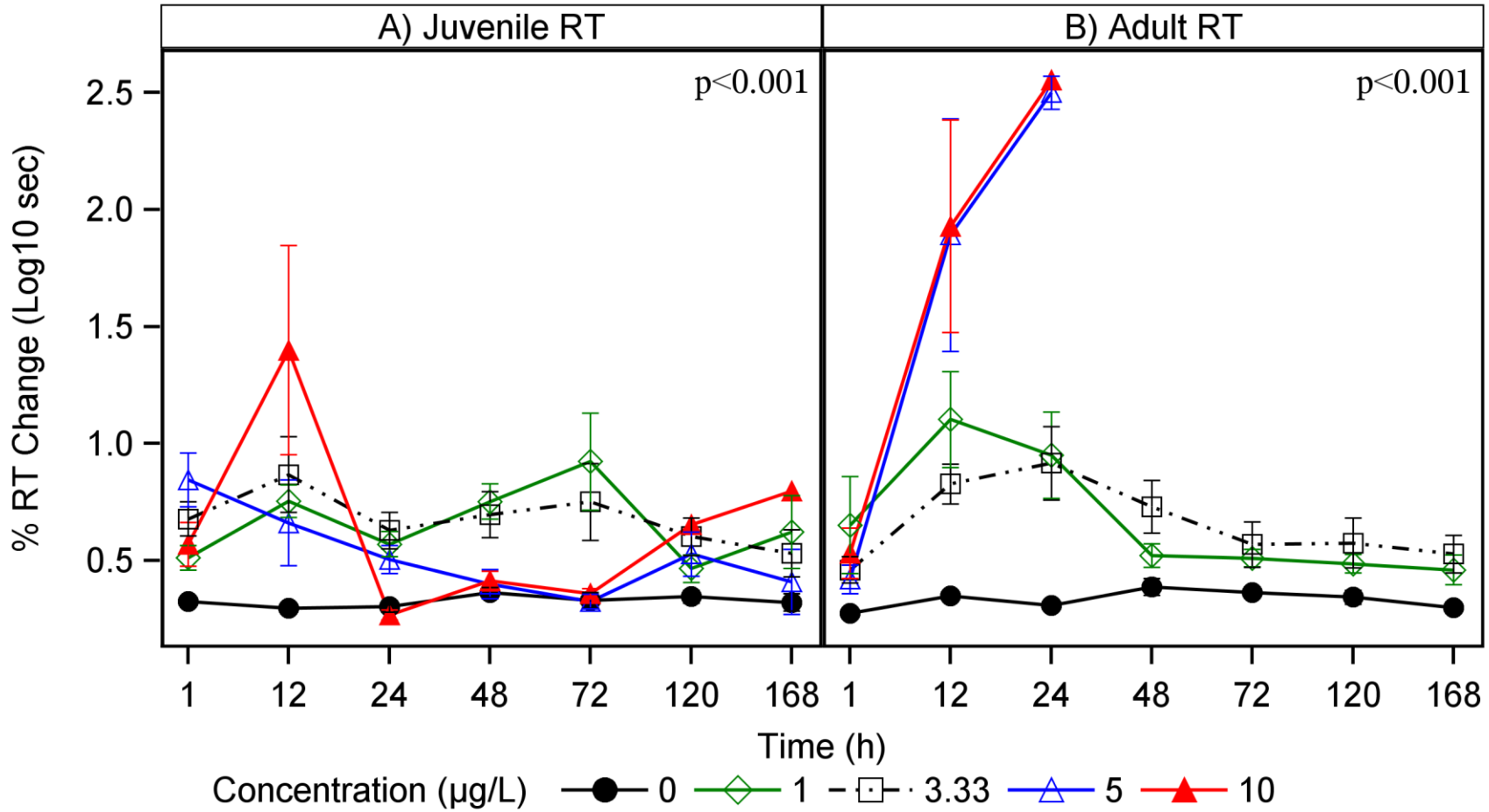

Figure 3.3. RT response: Mean (± SE) percent change of juvenile (A) and adult (B) blue crab RT during a single exposure to mixtures after 1, 12, 24, 48, 72, 120, and 168 h. Positive values indicate an increase in RT. (A and B) Compared to controls, all treatments significantly increased juvenile and adult crab RT ($p \leq 0.01$, RM-GEE post-hoc contrasts). (A) Note that after 24 h, juvenile 10 µg/L n = 3. (B) 100% mortality in 5 µg/L and 10 µg/L occurred within 48 h and 36 h, respectively. (A vs. B) Life-stage comparisons: In 5 µg/L treatments, adult RT was significantly higher than juvenile RT ($p < 0.01$, RM-GEE post-hoc contrasts). Other life-stage RT differences were non-significant (Supplemental Data Table S3.1). For all RT data (7d) see Supplemental Data (Figure S3.1).

RT, mixtures vs individual exposures

No significant differences in juvenile blue crab RT occurred in any control groups ($F_{4,66} = 0.98$, $p = 0.42$), but a significant pesticide-type effect in 1 µg/L ($F_{4,63} = 5.07$, $p = 0.001$) and in 3.33 µg/L and 10 µg/L ($F_{5,58} = 4.66$, $p = 0.001$) exposures was found (Figure 3.4). Similarly, no significant differences in adult blue crab RT occurred in any control groups ($F_{4,62} = 0.46$, $p = 0.77$), but a significant effect in 1 µg/L ($F_{4,44} = 6.15$, $p < 0.001$) and in 3.33 µg/L and 10 µg/L ($F_{5,43} = 16.2$, $p < 0.001$) exposures was found (Figure 3.5). Thus, crabs exposed to control water had similar RT responses, but crabs exposed to different pesticides did not.

Post hoc analysis revealed that 1 µg/L exposures of malathion, Res-PBO and mixture exposures caused the highest RT increases in juvenile crabs (Figure 3.4). Malathion did not cause any juvenile mortality, however, whereas 1µg/L resmethrin + PBO and mixture exposures caused 30-40% mortality (Figure 3.2). At higher exposure levels, 10 µg/L resmethrin + PBO and mixture exposures caused significantly higher increases in juvenile crab RT than 10 µg/L resmethrin, carbaryl and malathion (Figure 3.4). Exposure to 3.33 µg/L mixtures caused an intermediate increase in juvenile crab RT as compared to 10 µg/L mixture and resmethrin + PBO and (versus) resmethrin, carbaryl and malathion exposures.

In adult crabs, resmethrin + PBO, resmethrin, and mixture treatments caused significantly higher increases in adult crab RT than carbaryl and malathion (Figure 3.5). This was true for all exposure levels, 1 µg/L, 3.33 µg/L and 10 µg/L. Similar to juveniles, exposure to 3.33 µg/L mixtures caused an intermediate increase in adult crab RT as compared to 10 µg/L mixture, resmethrin + PBO, and resmethrin and (versus) carbaryl and malathion exposures.

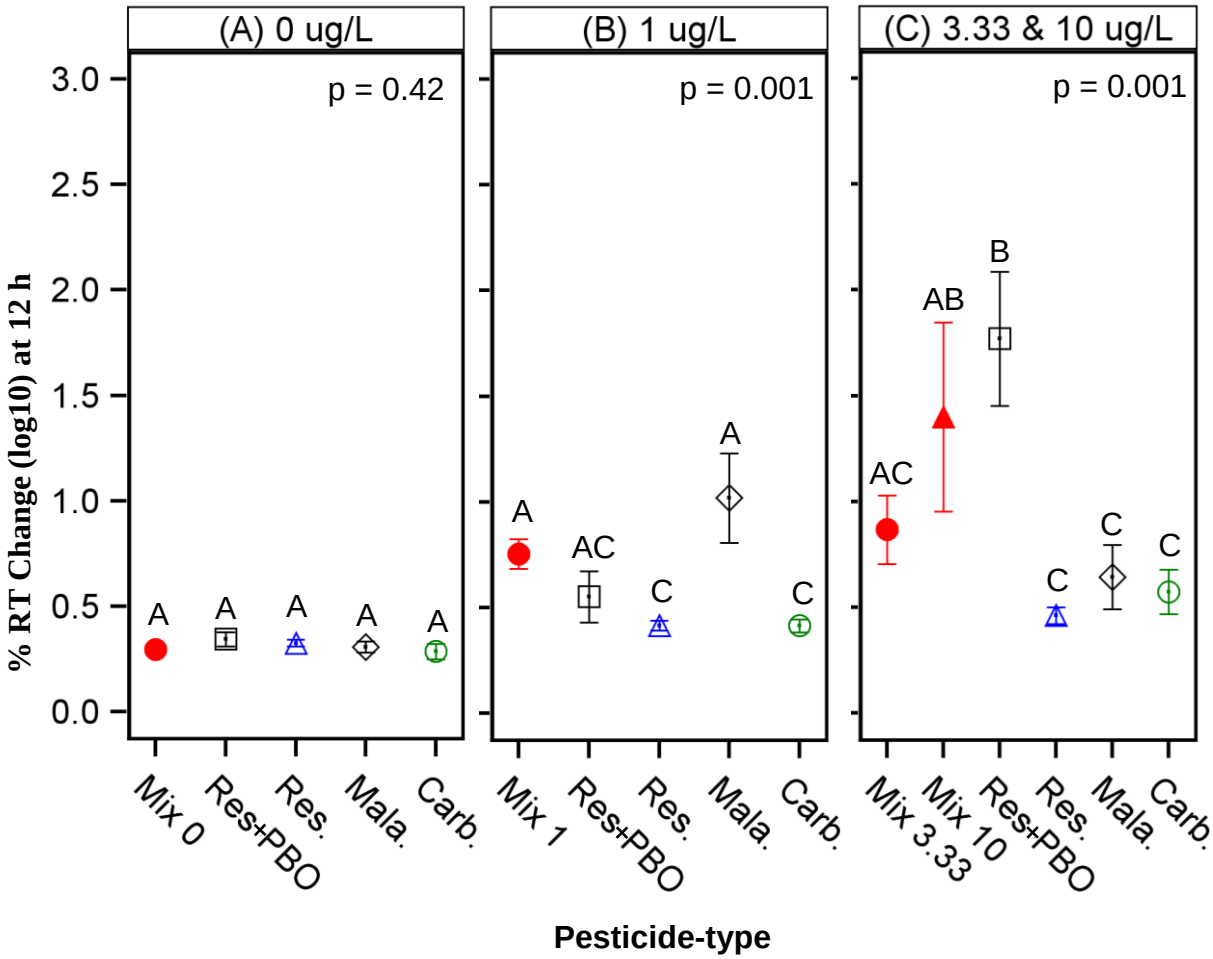

Figure 3.4. Juvenile blue crab RT after 12 hours of exposure to carbaryl, malathion, resmethrin, resmethrin + PBO, or mixture treatments. Letters indicate significant differences in RT between pesticide-types within the same concentration panel, as determined by post hoc comparisons (ANOVA, Holm Method adjustment). 5 µg/L mixture treatment (not shown) had similar effects on RT as the 3.33 µg/L mixtures (see Figure 3.3A).

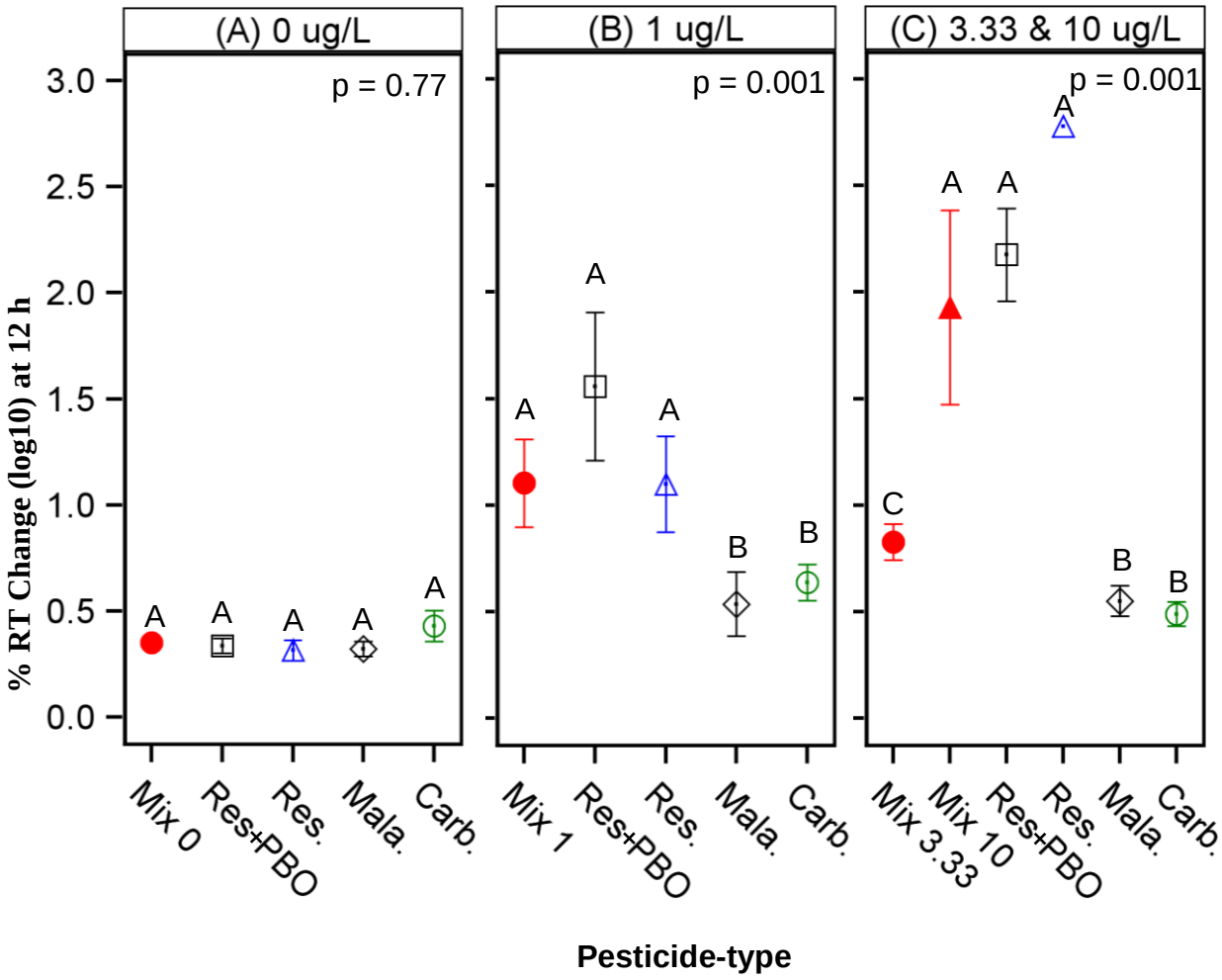

Figure 3.5. Adult blue crab RT after 12 hours of exposure to carbaryl, malathion, resmethrin, resmethrin + PBO, or mixture treatments. Letters indicate significant differences in RT between pesticide-types within the same concentration panel, as determined by post hoc comparisons (ANOVA, Holm Method adjustment). 5 µg/L mixture treatment (not shown) had similar effects on RT as the 3.33 µg/L mixtures (see Figure 3.3B).

DISCUSSION

Carbamate, organophosphate (OP), and pyrethroid insecticides are three of the most commonly applied insecticide-families in the U.S. and have the potential to negatively affect non-target species like blue crabs because of their effects on invertebrate nervous systems (Table 3.1). Under natural conditions, crabs are likely exposed to pesticide mixtures at sublethal concentrations similar to the levels tested in this study (e.g., ≤10 μg/L, Table 3.1). Juveniles and adults exposed to mixtures consisting of malathion, carbaryl, resmethrin and PBO, had significantly reduced survival time, and increased RT. Thus, at legally allowable concentrations, exposure to pesticide mixtures can negatively affect two life-stages of blue crabs, and may contribute to population declines.

Lethal and sublethal effects of mixture exposures were concentration and time dependent; effects peaked within the first 12-24 hours, with most deaths occurring within 36 hours of exposure. RT tended to increase before mortality was observed. Thus, increases in RT in the first 24 hours corresponded with increased mortality. After 24-48 hours, many crabs remaining were either more tolerant or were able to recover from pesticide exposure. In natural populations, pollution can act as a selective force [7]. For example, blue crabs in estuaries polluted by heavy metals developed an ability to depurate heavy metals to decrease body burden and bioaccumulation [118]. But this adaptation likely comes at a cost; the same crabs had decreased predatory abilities, and their diet consisted primarily of algae and detritus instead of carrion [30]. Increased diet breadth may be adaptive for crabs living in polluted estuaries to decrease bioaccumulation, but blue crabs with such diets may also have reduced fitness [137]. If pesticides create a similar selective pressure, and alter blue crab predation and foraging behaviors, blue crabs may be less able to adapt to other natural and anthropogenic stressors.

Differences between life-stages were also concentration dependent. In higher exposures (5-10 µg/L), lethal and sublethal effects were more severe for adults than juveniles. In contrast, in lower exposures (1 µg/L, 3.33 µg/L), lethal and sublethal effects were more severe for juveniles. Early life-stages and smaller-bodied individuals are frequently more sensitive to toxicant exposure than older, larger adult forms [72], though not always [107]. For example, in previous experiments with blue crabs exposed to 1-10 µg/L malathion (Chapter 1), increases in RT were higher for juveniles than adults (also see [64]). In contrast, adult crabs were extremely more vulnerable to 1:3 µg/L resmethrin + PBO than juveniles, which increased mortality and RT more so in adults than juveniles (Chapter 2). In that study, 12 hours of exposure to 1:3 µg/L Res-PBO increased the juvenile average RT to 4.2 seconds versus an average RT of 82 seconds for adult crabs. Life-stage differences in mixture exposures may be explained by interactions between individual pesticides, but may also be related to differences in metabolic capacity of juvenile and adult crabs.

The specific interactions between individual pesticides are not discernable in the current study, but in general such interactions may be considered neutral, additive, synergistic, or antagonistic. Mixtures increased juvenile mortality and RT compared to individual exposures, suggesting additive or synergistic interactions. In contrast, mixture effects on adults may indicate antagonistic interactions at low concentrations. Specifically, only 10% mortality was observed in 1 µg/L mixtures compared to 100% mortality in 1:3 µg/L resmethrin + PBO exposures. Results may be partially explained by pesticide mode of action. For example, PBO synergizes resmethrin (pyrethroid) by inhibiting MFO enzymes (e.g., P450s), but antagonizes malathion (OP) toxicity by decreasing the bioactivation of malathion into malaoxon [33, 36, 91, 92]. PBO + carbaryl interactions are not yet well understood [138]. Simiarly, pyrethroids have complex interactions

with other pesticides, as recent studies show pyrethroids have secondary effects on AChE synthesis [139-141] and some are endocrine disruptors [104]. Further investigation of physiological responses or different exposure combinations may help explain the mechanisms underlying results.

Regardless of the physiological mechanism, severe sublethal effects (i.e., increased RT) were observed in all mixture exposures and indicate impaired neurological functioning [66, 93]. Increased RT was associated with reduced foraging ability and increased vulnerability to predation in blue crabs exposed to resmethrin + PBO for 12 hours (Chapter 2). Similar and even more severe increases in RT were observed in mixtures, especially adult crabs exposed to 1 and 3.33 µg/L. This suggest that even though 90% of adults survived low mixtures exposures, surviving adult crabs would likely be vulnerable to predators and possibly other stressors because of reduced coordination at these low concentrations. Thus, results may be conservative, as all organisms experience multiple stressors in their natural environments [45, 63, 142, 143].

Much of our current understanding of pesticide toxicity relies on individual exposure experiments focused on lethal effects, though a growing number of studies have begun to shift focus to include both lethal and sublethal effects of pesticide mixtures. In estuarine systems, the effects of fipronil, imidacloprid, and atrazine on grass shrimp have been investigated [144]. Shrimp were exposed to pesticides individually, in pair-wise mixtures, and a mixture including all three pesticides. Effects on survival were greater than additive in the mixture containing all three pesticides, in comparison to individual and pair-wise mixtures. These results differ from the present study, as effects on adult blue crabs may indicate antagonist interactions among pesticides at the lower concentrations tested. In freshwater systems, the effects of pesticides mixtures on amphibians and food webs containing amphibians have been investigated [8, 63,

145]. In one such study, mesocosm experiments were conducted in which organisms were exposed to 5 insecticides (malathion, carbaryl, chlorpyrifos, diazinon, and endosulfan) and 5 herbicides glyphosate, atrazine, acetochlor, metolachlor, and 2,4-D), individually, as a mixture of 5 pesticides, a mixture of 5 herbicides, and a mixture of all 10 [63]. The effects of mixture exposures were somewhat predictable from individual exposures. For example, endosulfan was the driving insecticide in those studies, and effects of mixtures containing endosulfan were equal to or more severe than individual endosulfan exposures. Likewise, in the present study the effects of mixtures on juvenile crabs were largely predictable from individual exposures, with resmethrin + PBO seeming to drive the toxicity of mixture exposures. That same study also found that low concentrations of mixtures altered trophic relationships via direct and indirect effects, causing changes in species survival and abundances [63]. Given the results of the present study, it is likely the same effects on trophic relationships may occur in estuarine systems were pesticides mixtures are present.

In summary, pesticide mixtures at environmentally occurring and legally allowable concentrations can negatively affect blue crab neurological functioning, suggesting that crabs may be less able to forage efficiently and may be more vulnerable to predators in the environment**.** Because highly coordinated locomotor skills are critical to blue crab survival, reduced coordination and overall well-being of individual crabs in response to mixtures may have propagating negative effects on blue crab populations, and coastal ecosystem functioning (e.g., fisheries and other services) in which blue crabs or other invertebrate predators have an important role.

ACKNOWLEDGEMENTS

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CHAPTER IV. Increased AChE activity: Effects of carbaryl on AChE activity in adult blue crab tissues

ABSTRACT

Carbamate and organophosphate (OP) insecticides are two of the most commonly applied insecticide-types/families worldwide, both of which target and inhibit the neurotransmitter acetylcholinesterase (AChE) in invertebrates and vertebrates. Given this, AChE is a common biomarker used to evaluate carbamate and OP exposure. Yet, despite its ubiquity, our understanding of how sublethal pesticide exposures affect both AChE activity and behavior is limited. Here, I investigated the effects of sublethal carbaryl exposures on AChE in several target tissues of an ecologically and commercially important estuarine crustacean, the blue crab (*Callinectes sapidus*). Briefly, (1) adult male blue crabs were exposed to carbaryl treatments (0, 1, 10, or 100 µg/L) for 36 hours; (2) gill, muscle, and hepatopancreas tissues were collected from each crab; and (3) AChE activity of tissues were determined spectrophotometrically. AChE activity was dependent on tissue-type and carbaryl concentration, but was highest in both gill and muscle tissues of crabs exposed to carbaryl; whereas, hepatopancreas AChE activity was not significantly affected by carbaryl exposures. Increased AChE activity suggests that blue crabs may compensate physiologically to sublethal exposures of AChE inhibitors by increasing AChE production. Carbaryl can also reduce blue crab neuromuscular functioning (e.g., increased righting time), suggesting reduced foraging and predator escape abilities. Thus, increased AChE activity is associated with increased RT. This suggests that despite a physiological response to compensate for AChE inhibition, critical blue crab behaviors are still be negatively impacted by low, single exposures to carbaryl, which may contribute to blue crab population declines. These

results highlight the importance of studying changes in both AChE activity and behavioral responses.

INTRODUCTION

Pesticides may enter estuarine systems from several sources including run-off and wind-borne drift from municipal vector control programs, agricultural applications, and private at-home use [58]. Pesticides may affect several levels of biological organization including molecular, organismal, and population levels [7]. By studying toxicant effects at the molecular level, biomarkers may serve as an early warning system and provide a mechanistic understanding for observed changes in organismal behavior or population numbers [50]. Identification of reliable biomarkers and data interpretation remains a complex issue and should be carefully linked with effects at higher levels of biological organization within a relevant ecological context [49, 146]. One approach is to study toxicant induced changes in behaviors related to fitness (e.g., changes in locomotor functioning may affect scope for growth and reproduction) and measure biomarkers that indicate stress and/or specific target enzymes [52, 53]. For example, reduced AChE activity in fish has been linked with decreased swimming stamina [50], which in-turn indicates a reduced ability to forage or escape from predators [54] and may lead to reduced population numbers. In this way, effects at the physiological, organismal, and population level may be linked to provide a more holistic understanding of how ecosystems and ecosystem components may be affected by common AChE inhibitors. In the U.S., carbamates (e.g., carbaryl) and organophosphates (OP) insecticides are two of the most commonly applied insecticide-families that have the potential to negatively affect both target and non-target species because of their mode of action. Both inhibit cholinesterase enzymes (e.g., acetylcholinesterase), which are present in invertebrate and vertebrate species and are important for nervous system

function [36, 58]. Estuarine crustaceans, like blue crabs (*Callinectes sapidus*), may be especially vulnerable to AChE inhibitors because they are arthropods, like the insects targeted by insecticides.

AChE is a common biomarker used to evaluate exposure to AChE inhibitors [49-51], but relationships between AChE activity and changes in organismal survival or in ecologically relevant behaviors are not known for most species (but see [50, 55, 56], Table 4.1). A growing number of studies have investigated the effects of AChE inhibitors on marine invertebrates, though research has primarily focused on fishes and linkages between AChE activity and mortality (Table 4.1). Mortality may occur over a wide range of AChE inhibition (e.g., 30-90% in fishes), however, making predictions difficult and non-exact. Even fewer studies have investigated pesticide effects on AChE activity in multiple tissue types or linked effects with changes in behavior (but see Table 4.1). Moreover, toxicants that do not directly target AChE, including pyrethroids, PAHs, and heavy metals, can nonetheless affect AChE activity [139, 141], further complicating the utility of AChE as a biomarker. Together, these studies indicate that the relationship between AChE activity, behavior, and pesticide exposure is complex and requires further study.

Table 4.1. Literature Review: AChE studies in invertebrates and select vertebrates [a]

AChE response	Pesticide	Species	Tissues	Other responses	Cite
Inhibition	Dimethoate (OP)	Shore crab, *C. maenas*	Hemolymph	Cardiac activity: % inhibition correlated with % reduced heart rate	[147]
	Fenitrothion (OP)	Red swamp crayfish, *P. clarkii*	Muscle	N/A	[148]
	Folidol 600 (OP)	Nile tilapia, *O. niloticus*	Plasma	Oxygen consumption: Increased consumption in lowest exposures, decreased consumption in highest exposures	[149]
	Chlorpyrifos (OP), parathion (OP), DEF (synergist)	Channel Catfish, *I. punctatus*	Brain, muscle, gill, liver, plasma	N/A	[150]
	Malathion, malaoxon (OP)	Blue Catfish, *I. furcatus*	Brain, liver	BChE, monoamine activity	[151]
	Malathion (OP), diazinon (OP)	Rainbow trout, *O. mykiss*	Brain	Swimming behavior: Increased distance, speed and turns, correlated with CbE inhibition. *malathion decreased turns.	[152]
	Malathion (OP), parathion (OP)	Channel Catfish, *I. punctatus*, blue crab, *C. sapidus*	Brain, ventral ganglia	Biochemistry characterization (AChE Km), crabs 3-10 x sensitive than fish	[153]
	Malathion (OP), chlorpyrifos (OP), carbofuran (carbamate)	Water flea, *D. magna*	Whole animal	CbE	[55]
	Malathion (OP), chlorpyrifos (OP)	Grass shrimp, *P. pugio*	Whole embryo	N/A	[154]
	Methamidophos (OP)	*White shrimp, L. vannamei*	Muscle, eye	Behavior, feeding rate: Increased movements, no effect on feeding rate	[155]
	Methyl parathion (OP)	Estuarine crab, *C. granulata*	Ventral ganglia	Mortality: weak correlation with increased mortality	[156]
	Imidacloprid (neonicotinoid)	harlequin fly, *C. riparius*	Whole animal	Ventilation, locomotion: Low exposures increased locomotion and ventilation, high decreased locomotion and ventilation	[157]

Table 4.1. Literature Review: AChE studies in invertebrates and select vertebrates [a]

AChE response	Pesticide	Species	Tissues	Other responses	Cite
Activation	Triclorfon (OP)	Red swamp crayfish, *P. clarkii*	Muscle, hepatopancrease * muscle AChE only measured	Glycogen, mortality: studied sublethal concentrations, mixed effects on glycogen	[158]
	Phosalone (OP), carbaryl (carbamate) [b]	Common prawn, *P. serratus*	Abdominal muscle	Mortality: increased mortality	[159]
	Deltamethrin (pyrethroid)	Tadpole, *X. laevis*	Whole animal	GST, lactate, dehydrogenase, acid phosphatase, aspartate aminotransferase activity	[139]
	Deltamethrin (pyrethroid) +/- pirimicarb (carbamate)	Honeybee, *A. mellifera* [c]	Whole animal	n/a	[141]

[a] Also see reviews by: [49] [50]

[b] AChE increased after 13 d exposure, but decreased after 29 d

[c] increased AChE activity occurred in surviving honeybees only

Here, I investigated the sublethal effects of carbaryl (carbamate) exposures on an important ecological and commercial estuarine species, the blue crab (*Callinectes sapidus*) [28, 62], which may be physiologically and behaviorally affected by AChE inhibitors. In natural systems, crabs and other estuarine organisms are likely to be exposed to sublethal concentrations of pesticides, rather than lethal concentrations. A decade-long study of U.S. streams conducted by the USGS revealed that insecticides were present in 90% of streams throughout the country [41]. Survey data in coastal systems are limited, but it is likely that pesticides found in tributaries are also present in coastal systems and may therefore affect non-target species. For example, in Corpus Christi Bay (TX), malathion (organophosphate) was recorded at 11 µg/L [40] and carbamates were recorded at 3.64 µg/L [123]. Given that AChE is a common biomarker of pesticide exposure, it could be a useful tool to investigate how AChE activity in different tissue types may be altered by sublethal exposures. The effects of pesticide exposure *in vivo* and *in vitro* have been studied in blue crabs [49, 147, 153, 160, 161], but none have linked AChE activity with behavioral effects following carbamate exposure. Linking effects across levels of organization (e.g., physiological and organismal levels) may help provide the context in which biomarkers may be best used and understood.

The goal of this study was two-fold: (1) investigate effects on AChE activity in three tissue-types of adult blue crabs following a single, sublethal carbaryl exposure, and (2) to link changes in AChE with behavioral changes (i.e., righting time) observed in a previous study (Chapter 1). This work builds on several behavioral and predator-prey mesocosm studies with blue crabs exposed to carbamates, OPs, and pyrethroid pesticides (Chapter 1, 2, and 3). Sublethal carbaryl exposures (1-100 µg/L) increased adult blue crab righting time (RT) within 1-12 hours of exposure, and effects peaked at 36 hours. RT, defined as the amount of time it takes a crab to

resume its normal position after being placed on its back (180°), is a common proxy measurement used to indicate crab health and neurological functioning [66, 93] and may therefore, be affected by AChE inhibitors like carbaryl. In that study (Chapter 1), increased RT corresponded with reduced coordination, and suggests that survival and coordinated activities like foraging and predator avoidance may be reduced (also see [64]). In another study (Chapter 2), increased RT caused by sublethal pyrethroid exposures was linked with increased predation risk and decreased foraging abilities in blue crabs. By studying biomarkers, like AChE, in the context of known behavioral effects, we may better understand the relationship between toxicant mode of action and effects on ecologically relevant changes in organismal survival and behaviors.

MATERIALS & METHODS

Animal collection, care, and carbaryl exposures

Adult male blue crabs (avg. 112.5 mm, ± 6.2 mm CW; avg. weight 203 g, ± 31.5 g) were caught in estuaries near Corpus Christi, TX, USA and housed in indoor tanks at Texas A&M University-Corpus Christi, TX. Crabs were acclimated to experimental conditions at salinity 20 and temp 22-24^{o} C for a minimum of 48 hours. Crabs were fed shrimp daily, and starved 24 hours prior to experiments. All seawater was made using dechlorinated tap water and Instant Ocean™.

Carbaryl exposures and tissue collection

A stock solution (10 mg/L carbaryl + 1mL/L ethanol) was made 1-2 hours before each replicate experiment. Ethanol was used to increase toxicant solubility in stock solutions, resulting in 100 µL/L (v/v) of ethanol in the highest treatment. All control treatments included 100 µL/L

ethanol to discern any effects possibly caused by ethanol. Carbaryl and ethanol (absolute) were ordered from Sigma-Aldrich.

Crabs were exposed to one of four treatments, 0 µg/L, 1 µg/L, 10 µg/L, or 100 µg/L of carbaryl, for 36 hours using a static, non-renewal design. Animals were exposed in individual glass bowls (salinity 20), containing 10-L of solution and an aerator stone. Exposure concentrations and duration (i.e., 36 hours) were selected based on previous carbaryl experiments with blue crabs (Chapter 1), which determined that carbaryl increased adult blue crab RT within 1-12 hours of exposure and effects often peaked at 36 hours. Thus, crabs were exposed to the same carbaryl concentrations in the present study for 36 hours. Two replicate exposure experiments were completed (August 2014) because of space limitations and animal availability. Both experiments included all treatment levels, with 3 replicate crabs per treatment level. Exposure times were staggered in groups to account for time required to dissect and store tissues.

Immediately following 36-hour carbaryl exposures, crabs were cryoanesthetized using liquid nitrogen. Hepatopancreas, gill, and muscle tissues were dissected from each crab and placed in ice-cold PB buffer (0.25 M PB, 20% glycerol, pH 7.4). Tissues were flash frozen using liquid nitrogen at a final tissue-to-buffer ratio of 1:3 (1 mg tissue-to-3 mL buffer) and stored at -80˚C until AChE enzyme assays were conducted. This ratio was chosen to limit AChE reactivation that may occur as result of dilution [162].

AChE enzyme assays

On the day of each enzyme assay, tissues were first thawed on ice. Sample processing (sonication, centrifugation, and dilutions) for AChE assays were all completed on the same day for each tissue-type. Once thawed, tissue samples were sonicated (amplitude = 45; on = 5

seconds; off = 15 seconds) and then centrifuged at 1,000g for 20 min (4˚C). Next, for each replicate, three 1.5 mL aliquots of the first supernatant were transferred to new microcentrifuge tubes and centrifuged at 10,000g for 20 min (4˚C). The second (final) supernatant was used as the AChE source in activity assays, which were completed in triplicate. Samples were brought to room temperature (25-26˚C) for assays, but care was taken to minimize time between the initial thawing of a sample and when AChE assays were completed to minimize AChE reactivation. A similar protocol is detailed in [153, 163].

AChE activity was measured spectrophotometrically using a commercial test kit (Amplite ™ Colorimetric Acetylcholinesterase Assay Kit, purchased from AAT Bioquest). Assays were completed following the kit manual, which were based on the Ellman method [164] adapted for a microtiter plate (Figure 4.1). Absorbance at 410 nm was measured after assay reactions were incubated for 10 minutes at 25-26 ˚C, and was used to determine product production and enzyme activity. Sample absorbance was compared with a standard curve to determine AChE activity (mU/mL). Protein concentration of AChE assay fractions were determined using the Bradford method adapted for a microtiter plate [165]. BSA (bovine serum albumin) was used as a protein standard, and absorbance was measured at 595 nm. AChE results were normalized for protein (mU/mL/mg protein).

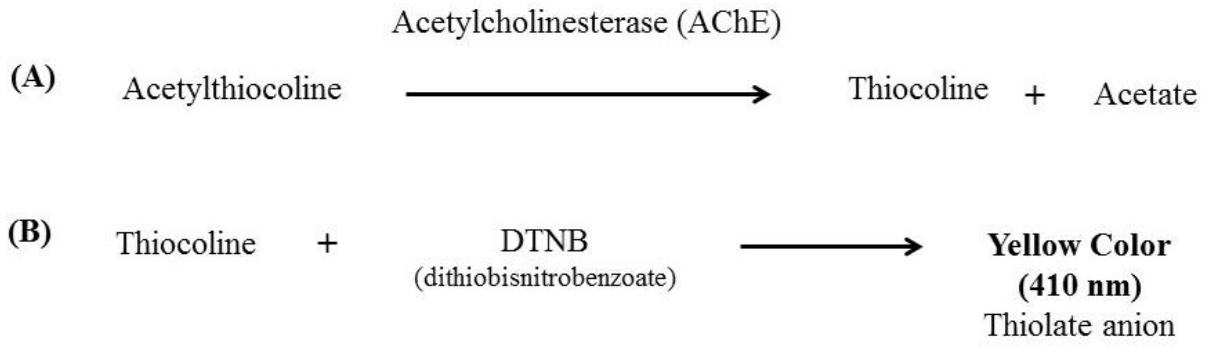

Figure 4.1. Colorimetric reactions used to determine enzyme activity. (A) Substrate ACTC (acetylthiocholine) is hydrolyzed by AChE to thiocholine and acetate. (B) Thiocoline reacts with DTNB to form a yellow thiolate anion, which has a strong absorbance at 410-412 nm. Absorbance measured at 410 nm was directly proportional to thiocholine concentration, which depends on enzyme activity [164], e.g., reduced absorbance indicates reduced enzyme activity.

Statistical analysis

Experimental replicates were individual crab-tissue samples (n = 6). Data were analyzed separately for each tissue-type (gill, muscle, hepatopancreas). AChE activity (mU/mL/mg protein) was analyzed using a 1-way ANOVA, with carbaryl concentration as a fixed factor. Post hoc analysis was completed using Dunnett's test to compare AChE activity of controls vs. each carbaryl concentration [69, 70].

RESULTS

All control treatments included 100 µg/L ethanol to discern any effects possibly caused by ethanol (carrier solvent), and none were observed.

Carbaryl concentration had a significant effect on AChE activity in gill tissue ($F_{3,34} = 5.10$, $p < 0.01$, Figure 4.2), causing an increase in AChE activity. Compared to controls (mean = 13.3), AChE significantly increased in 10 µg/L ($p = 0.03$, mean = 19.1) and 100 µg/L ($p < 0.01$, mean = 21.6) carbaryl exposures, but not in 1 µg/L exposures ($p = 0.32$, mean = 16.8).

Carbaryl concentration effects were significant at $\alpha = 0.10$ on AChE activity in muscle tissue ($F_{3,34} = 2.21$, $p = 0.10$, Figure 4.3), causing an increase in AChE activity. Compared to controls (mean = 162.4), AChE significantly increased in 1 µg/L ($p = 0.06$, mean = 189.8) carbaryl exposures, but not in 10 µg/L ($p = 0.15$, mean = 182.2) or 100 µg/L ($p = 0.60$, mean = 173.2) exposures.

Carbaryl concentration did not significantly affect AChE activity in hepatopancreas tissue ($F_{3,34} = 0.13$, $p = 0.94$). Though, there was slight decrease in activity (Figure 4.4).

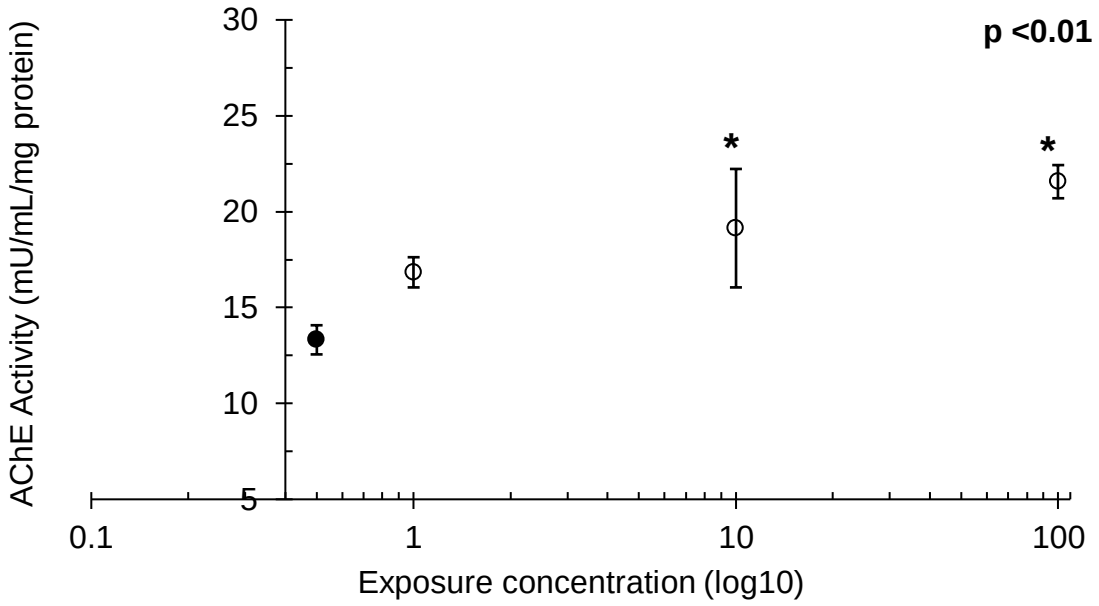

Figure 4.2. Mean (±SE) AChE activity (mU/mL/mg protein) of gill tissue in adult blue crabs exposed to controls (filled circle) or carbaryl (open circles). AChE activity is normalized to protein concentration. Stars (*) indicate significance from controls (Dunnett's test). Carbaryl concentration had a significant effect on AChE activity in gill tissue ($F_{3,34} = 5.10$, $p < 0.01$). Compared to controls AChE significantly increased in 10 ug/L ($p = 0.03$) and 100 ug/L ($p < 0.01$) carbaryl exposures, but not in 1 µg/L exposures ($p = 0.32$).

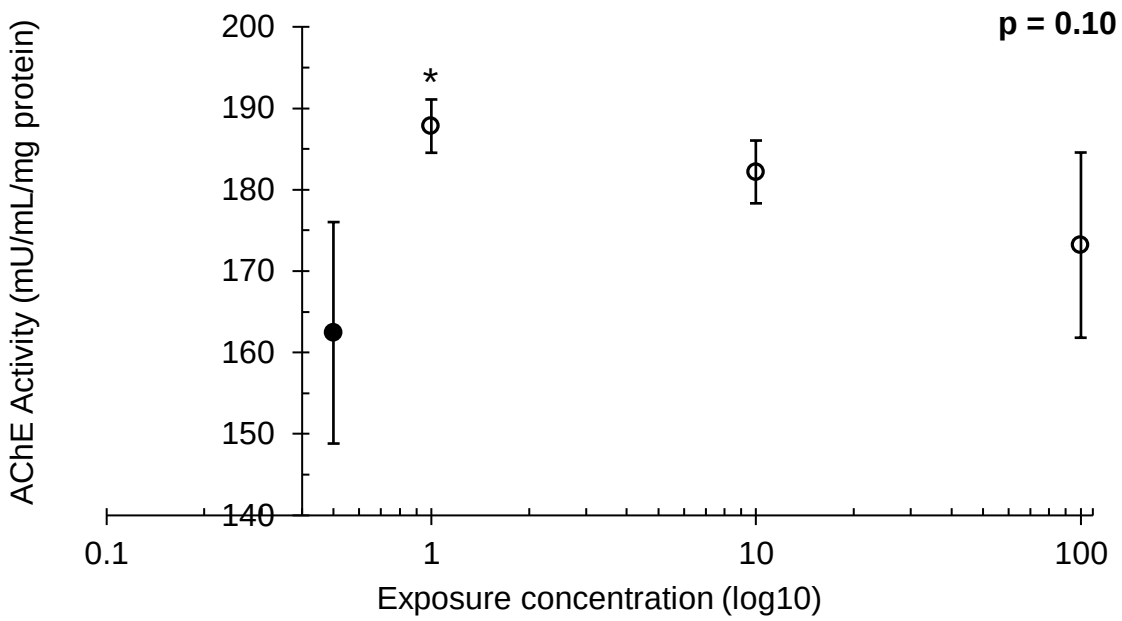

Figure 4.3. Mean (±SE) AChE activity (mU/mL/mg protein) in muscle tissue from adult blue crabs exposed to control (filled circle) or carbaryl (open circles). AChE activity is normalized to protein concentration. Stars (*) indicate significance from controls (Dunnett's test). Carbaryl concentration had a marginally significant effect on AChE activity in muscle tissue ($F_{3,34} = 2.21$, $p = 0.10$, $\alpha = 0.10$). Compared to controls AChE significantly increased in 1 ug/L ($p = 0.06$, mean = 189.8), but not in 10 µg/L ($p = 0.15$) or 100 µg/L ($p = 0.60$) exposures.

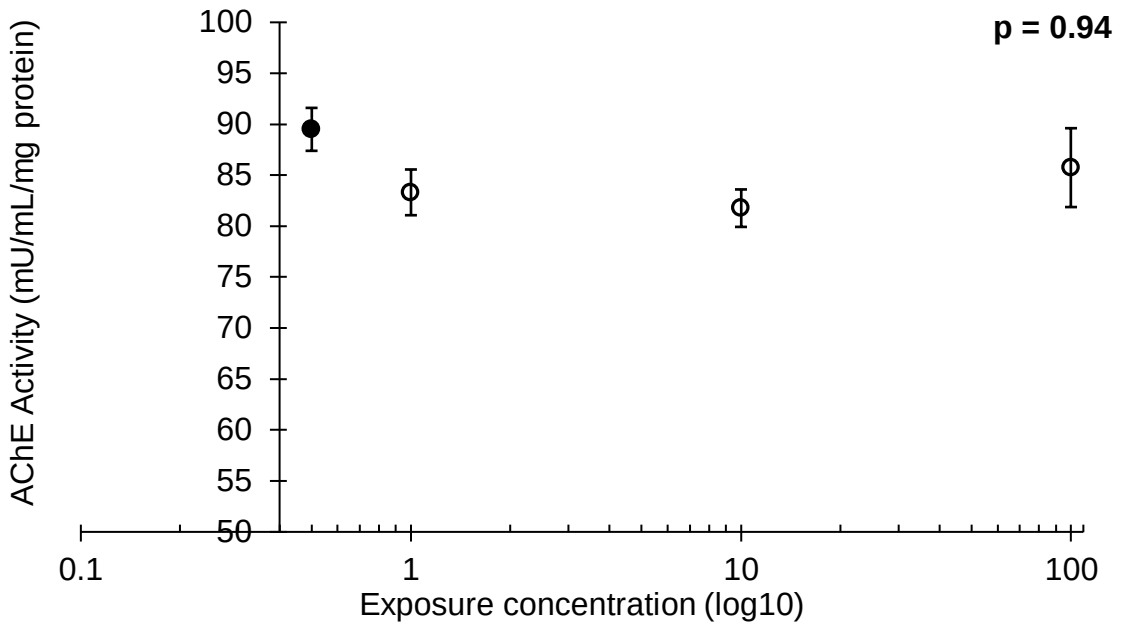

Figure 4.4. Mean (±SE) AChE activity (mU/mL/mg protein) in hepatopancreas tissue from adult blue crabs exposed to control (filled circle) or carbaryl (open circles). AChE activity is normalized to protein concentration. Carbaryl concentration did not significantly affect AChE activity in hepatopancreas tissue ($F_{3,34} = 0.13$, $p = 0.94$).

DISCUSSION

This study assessed the effects of sublethal carbaryl (carbamate) exposure on AChE activity in gill, muscle, and hepatopancreas tissues of an important ecological and commercial estuarine crustacean, the blue crab (*C. sapidus*). Carbamates, like carbaryl, are one of the most commonly applied insecticide-families in the United States used to target urban and agricultural pests [58]. Carbamates inhibit cholinesterase enzymes, including AChE, via a reversible bond [36]. When inhibited, the neurotransmitter acetylcholine can persist, resulting in uncontrolled movements, spasms, stiff muscles, and sometimes death [33]. Given this, AChE is a common biomarker used to study pesticide exposure. Studies are often conducted at lethal or nearly lethal exposure levels [50], however, blue crabs are likely exposed to pesticides at sublethal rather than lethal concentrations in nature [41]. The effects of AChE inhibitors on marine invertebrates and other organisms has been studied (Table 4.1), but few have investigated effects in several tissue types or linked effects with changes in behavior. In crabs, including blue crabs, several studies have investigated the effects of OP exposure on AChE activity [49, 147, 153, 160, 161], but none have linked AChE effects with effects at the organismal level. Further, the effects of carbamates on blue crabs have not been evaluated.

In the present study, carbaryl exposures increased AChE activity in gill (10 µg/L, 100 µg/L) and muscle (1 µg/L) tissues. Given that carbaryl is an AChE inhibitor and that blue crab RT increased following carbaryl exposure (see Chapter 1), AChE activity was expected to decrease in crabs exposed to carbaryl. Likewise, reduced AChE activity has been linked with decreased swimming stamina in fishes [50], as well as reduced foraging and antipredator behaviors [54]. In those studies, fish were exposed to near lethal limits, whereas in the present study carbaryl exposures were nonlethal and therefore, may not have been high enough to reduce

AChE activity below that of the controls [131]. Additional study of AChE activity at higher and lower exposure levels could provide useful insight into the mechanisms behind responses, and may confirm if results constitute a hormetic response [166].

Increased AChE activity in response to pesticide and toxicant exposure has been reported in other studies [49, 50, 139, 158, 167]. For example, grass shrimp larvae exposed to 0.4 μg/L chlorpyrifos (organophosphate) had increased AChE activity [107]. Similarly, exposure to a carbamate, e.g., pirimicarb or propoxur, and/or pyrethroids (e.g., deltamethrin) increased AChE activity in tadpoles (*Xenopus laevis)* [139], honeybees (*Apis mellifera)* [141], and *Daphnia magna* [168] at sublethal concentrations (Table 4.1).

Mechanisms explaining increased AChE activity are complex and diverse [141], and three common mechanisms include: *de novo* synthesis of AChE, decreased substrate (acetylcholine) production, or decreased AChE enzyme affinity or activation [53, 150, 157]. Of these, increased AChE activity in gill and muscle tissues may most likely be the result of *de novo* synthesis of AChE for the following reasons. First, crabs may be conditioned (i.e., non-naïve) to synthesize AChE to compensate for AChE inhibition, even at low concentrations. *De novo* synthesis has been reported following exposure to OPs, but OPs form a more stable bond (irreversible) with AChE whereas carbamates are reversible [150]. Second, in the absence of *de novo* AChE synthesis, a decrease in AChE activity would likely follow carbaryl exposure (relative to controls) because less AChE would be active. Indeed, in a study with honeybees exposed to deltamethrin (pyrethroid), the authors propose the mechanism behind an increase in AChE activity may include *de novo* synthesis and a regulatory overcompensation or production of AChE [141]. Lastly, if crabs simply decreased choline production (i.e., AChE substrate), or decreased AChE affinity and/or availability for binding/signaling, no difference in control vs.

treatment activity levels, or a decrease in activity would be likely. An increase in AChE activity wouldn't be likely in either scenario. For these reasons, it's likely that blue crabs may have synthesized AChE in gill and muscle tissues in response to sublethal exposures of carbaryl. Additional experiments are required to confirm this working hypothesis, however.

Several pesticide families (e.g., carbamates, OPs, and pyrethroids) and heavy metals have been also been shown to induce oxidative stress via production of reactive oxygen species (ROS) [169, 170]. In turn, ROS's may induce changes to antioxidant enzymes or cause oxidative damage to membranes, DNA, and/or proteins [171]. Such effects have been studied more commonly in vertebrates, e.g., fishes and humans, but could play a role in carbamate toxicity in invertebrates as well [151, 169]. For example, in juvenile rainbow trout (*Oncorhynchus mykiss*), carbaryl increased Cytochrome P450-1A (CYP1A), glutathione S-transferase (GST), and catalase (CAT) activity in hepatic tissues after 24 hours of exposure, and reduced carboxylesterase (CbE) activity [172]. These enzymes represent four important antioxidant enzymes involved in xenobiotic metabolism and changes in activity suggest oxidative stress in fish exposed to carbaryl and other pesticides [173-175]. Such effects have also been studied in mussels [176], gastropods, oligochaetes [177] and other invertebrates [50], suggesting oxidative stress may be induced by carbaryl exposures in blue crabs.

Results may also indicate resistance to carbamate and OP exposure, as increased activity levels indicate that choline is still broken down in crabs exposed to carbaryl at concentrations at or below 100 µg/L. Pesticide resistance has primarily been studied in insect pests [178], but the same principles may apply to crabs. For example, in the cotton-pest *Helicoverpa armigera* (bollworm), pyrethroid resistance can be attributed to the overproduction of esterases, which target and sequester insecticides [179]. Likewise, the cattle tick *Rhipicephalus* (*Boophilus*)

microplus has developed pyrethroid resistance. Resistance to OPs is often a consequence of multiple mechanisms, and in locust has been linked with increased detoxification enzymes (e.g., GST), as well as reduced sensitivity and increased activity of AChE [180]. By further studying pesticide resistance in blue crabs, we may in turn gain new insight into how pesticide resistance in pests may develop, and potential adaptations that both pest and beneficial species may develop in response to toxicant stress.

In an ecological context, toxicant-induced enzyme synthesis (e.g., AChE, antioxidant enzymes, P450s) may be an energy-expensive strategy for blue crabs [61, 181, 182], as crabs may divert energy away from growth and reproduction (i.e., fitness) towards new enzyme production [183]. In turn, crabs may be smaller-bodied, which is correlated with increased predation and reduced reproductive capacity [184, 185]. Increased energy requirements may also alter blue crab foraging or antipredator behaviors that mediate predator-prey interactions [11, 30, 119], thus potentially altering benthic and salt marsh community structure where blue crabs are important predators [23-25, 27, 186]. Increased stress may also reduce an organism's ability to adapt to other stressors [29, 142, 187, 188]. For example, lobsters were more vulnerable to disease and faced increased mortality following large scale pesticide applications in the northeastern United States [46, 47]. Like lobsters, blue crabs exposed to pesticides may be unable to adapt to multiple stressors, including disease and fishing pressure. In this context, biomarkers may be a useful tool to assess responses to multiple stressors, but remains a daunting challenge.

In summary, sublethal carbaryl exposures affected adult blue crabs physiologically, as indicated by increased AChE activity in the present study. In previous works (Chapter 1), carbaryl also reduced blue crab neuromuscular functioning (e.g., increased righting time),

suggesting reduced foraging and predator escape abilities in blue crabs. Taken together, increased AChE activity in gills and muscles were associated with increased RT. This might indicate that despite a physiological response to compensate for AChE inhibition, critical blue crab behaviors are still be negatively impacted by low, single exposures to carbaryl. These results highlight the importance of studying changes in behaviors in combination with AChE activity.

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SUMMARY

Carbamates, organophosphates (OP), and pyrethroid insecticides represent 3 of the top 4 most commonly applied insecticide-families in the United States, and each can have lethal and sublethal effects on important estuarine predator, prey, and fishery species, like the blue crab (*Callinectes sapidus*). At sublethal, legally allowable concentrations of carbaryl, malathion, resmethrin, and PBO, individually and in mixture, juvenile and adult life-stages were negatively affected, causing increased physiological costs, reduced survivorship, reduced locomotor functioning, and altered predator-prey interactions via changes on foraging rates and increased vulnerability to predators. Results underscore the importance of studying the effects of pesticide mixtures at sublethal concentrations on several life-stages, as effects may vary non-linearly with concentration (Chapter 1), interactions between individual pesticides may be complex (Chapter 3), and juvenile life-stages are not always the most vulnerable (Chapter 1, Chapter 2, Chapter 3). Notably, blue crabs were most sensitive to exposures including pyrethroid (resmethrin) + PBO, which are representative of common co-components of vector and pest management products. Given that the use of pyrethroids is expected to increase in the coming decades, the application of such products should be carefully evaluated.

Pesticides affect several levels of biological organization (molecular, organismal, and populations), but linkages across levels are not well understood for most species. In blue crabs, behavioral changes (e.g., RT) provided a reliable and sensitive endpoint, indicating physiological (i.e., increased AChE) and altered predator-prey interactions (i.e., reduced adult foraging, increased juvenile vulnerability to predators) in the pesticides exposures studied. Results also highlight the importance of studying individual responses with increasing levels of biological organization, e.g., changes in species interactions, as increases in RT unexpectedly corresponded

with increased consumption rates in juvenile crabs (e.g., hyperactivity, Chapter 2). In the context of fisheries management and environmental regulations, RT may be a useful endpoint when measured in combination with other responses to indicate chances of survival or altered trophic relationships [2, 3].

Further study of underlying mechanisms (e.g., modes of action) is also required, especially those between AChE activity and behavioral responses. By studying biomarkers, like AChE, in the context of known behavioral effects, we may better understand the relationship between toxicant mode of action and effects on ecologically relevant changes in organismal survival and behaviors, that in-turn, may be used to predict altered survival and species interactions that influence coastal and estuarine food web structure and ecosystem services that human coastal economies and communities rely on, e.g., oyster reefs, sea grass beds, and salt marsh [23-25, 27, 186].

In summary, I investigated: (1) lethal and sublethal effects of a single exposure to malathion, carbaryl, and resmethrin + PBO, individually and in mixture, on juvenile and adult blue crab survival and muscular functioning by measuring changes in mortality, righting time (RT), and eyestalk reflexes. Effects observed at the individual level, were further evaluated and linked with changes in (2) predator-prey interactions, and (3) physiological responses. (4) Lastly, differences in susceptibility between juvenile (post-planktonic) and adult life-stages were evaluated in behavioral and mesocosm experiments. Results for each level of effect are summarized in greater detail, across chapters, below:

Organismal effects, individual and mixture exposures

Lethal and sublethal effects varied with pesticide-type, concentration, time, and life-stage. The relative effects of individual exposures (1 µg/L, 10 µg/L, and 100 µg/L) on survival,

RT, and eyestalk reflexes where as follows: carbaryl < malathion < < resmethrin + PBO. Carbaryl and malathion increased RT and abnormal eyestalk reflexes, but were largely non-toxic at the concentrations tested (Chapter 1, but see: 100 µg/L malathion exposures increased adult blue crab mortality). Resmethrin + PBO exposures (1:3 µg/L, 10:30 µg/L, and 100:300 µg/L) were extremely lethal and increased RT of both life-stages, though adults were more vulnerable.

Compared to individual exposures, mixtures (1 µg/L, 3.33 µg/L, 5 µg/L,10 µg/L) severely increased RT of both juveniles and adults, but lethal effects varied with concentration and life-stage (Chapter 3). Thus, the relative toxicity of all exposures for each life-stage were: (a) ***juveniles***: carbaryl < malathion << resmethrin + PBO < mixtures and (b) ***adults***: carbaryl < malathion << mixtures ≤ resmethrin + PBO. An important nuance of mixture results is that, despite a 90% survival rate of adults exposed to the lowest mixture exposure (I µg/L), increased RT suggests adult crabs would likely be extremely vulnerable to predators, and possibly other stressors (Chapter 2 and 3). Results may therefore be conservative, as all organisms experience multiple stressors in their natural environments [45, 63, 142, 143].

Life-stage and concentration trends may be explained by differences in metabolic capacities, and/or interactions between individual pesticides. For example, adult mortality > juvenile mortality in 100 µg/L malathion; this may have occurred if adult crabs metabolized malathion into malaoxon more quickly than juveniles, and thus, became overwhelmed at the highest concentration (Chapter 1). Additionally, molting observed in juveniles tended to increase pesticide effects on RT in malathion experiments. Suggesting juveniles may be more vulnerable to pesticide exposure than adults because of increased molt frequency relative to adult crabs [64, 75]. Regardless, both life-stages were negatively affected by pesticide exposures.

Lastly, effects varied with time and tended to peak within 36-72 hours of exposure; though effects on RT persisted for 5-7 days in all exposures. Notably, large increases in RT were observed within 1-12 hours of Res-PBO (Chapter 2) and mixture exposures (Chapter 3), indicating rapid declines in neurological functioning and coordination following short exposures at sublethal concentrations. In natural systems, this may have profound effects by removing juvenile and adult clue crabs (lethal effects), or by altering behavioral traits (sublethal effects). For example, reductions in blue crab abundances associated with commercial and recreational fishing have been linked with increased grazer populations and salt marsh die-offs [23, 27]. Like fishing, pesticides may cause similar effects on populations even at low concentrations (<10 µg/L).

Predator-prey interactions

Effects of Res-PBO exposures observed at the organismal level (i.e., survival and RT) were linked with changes in juvenile and adult blue crab predator-prey interactions (Chapter 2). Briefly, toxicant-induced changes in predator-prey interactions may be the result of lethal and sublethal effects on predators, prey, or both. Adult and juvenile crabs are both predators, and cannibalism by adults is important, so separate mesocosms using: (1) adults-as-predators + juvenile-prey and (2) juveniles-as-predators + shrimp-prey were completed.

Both life-stages were negatively affected by all Res-PBO exposures, but adult blue crabs were more susceptible (i.e., higher mortality and RT) to 1:3 and 10:30 Res-PBO treatments than juveniles. These individual effects (specifically RT) corresponded with changes in predator-prey interactions: Exposure to 1:3 decreased adult blue crab foraging ability (increased RT). Exposure to 1:3 increased juvenile vulnerability to predation (increased RT), but only if adults (predators) were not exposed. Unexpectedly, 1:3 stimulated juvenile (predator) consumption of shrimp. This

may indicate hyperactivity in juveniles, which could explain increased vulnerability to adult predators.

Overall, predator health was the primary driver in both mesocosm experiments, but importantly, the effects on adults-as-predators and juveniles-as-predators were drastically different. In natural systems where predators (adults) are more vulnerable, prey (juveniles) may indirectly benefit from predator release. In turn, this may alter other species interactions if adults target less active prey or if juveniles increase consumption of forage species.

Physiological effects

The linkages between effects observed at the organismal level (i.e., peak effects on RT) and physiological level (i.e., AChE activity) were also investigated. By studying biomarkers in the context of known behavioral effects we may better understand the relationship between toxicant mode of action and effects on ecologically relevant changes in organismal survival and behaviors. Briefly, (1) adult male blue crabs were exposed to carbaryl treatments (0 µg/L, 1 µg/L, 10 µg/L, and 100 µg/L) for 36 hours; (2) gill, muscle, and hepatopancreas tissues were collected from each crab; and (3) AChE activity of all tissues was compared spectrophotometrically. An exposure time of 36 hours was selected because this time corresponded with peak effects on RT (Chapter 1).

Increased RT was associated with increased AChE activity in gill and muscle tissues of adult blue crabs exposed to sublethal concentrations of carbaryl for 36 hours (Chapter 4). This might suggest that despite a physiological response to compensate for AChE inhibition (e.g., AChE synthesis), critical blue crab behaviors are still negatively impacted by low, single exposures to carbaryl. In an ecological context, toxicant-induced enzyme synthesis (e.g., AChE, stress enzymes, P450s) may be an energy-expensive strategy for blue crabs [61, 181, 182]. Crabs

may divert energy away from growth and reproduction (i.e., fitness) towards new enzyme production [183]. In turn, crabs may be smaller-bodied, which is correlated with increased predation and lower reproductive capacity [184, 185].

In summary, results demonstrate that at sublethal, legally allowable concentrations of carbaryl, malathion, resmethrin, and PBO, individually and in mixture, juvenile and adult life-stages were negatively affected, causing increased physiological costs, reduced survivorship, reduced locomotor functioning, and altered predator-prey interactions via changes on foraging rates and increased vulnerability to predators.

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APPENDIX. SUPPLEMENTARY DATA

Table S1.1. Malathion Exposures: concentration and life-stage specific post-hoc contrasts for juvenile and adult blue crab behavioral responses (RM-GEE A, post-hoc contrast)

	Righting Time (RT)		Eyestalk Retraction			Eyestalk Touch-Response		
Treatment Contrast (µg/L)	Estimate (SE) [a]	Z-value	Estimate (SE) [a]	Odds ratio	Z-value	Estimate (SE) [a]	Odds ratio	Z-value
Juveniles only								
0 vs. 1	1.68 (0.29) ***	5.8	5.79 (0.97) ***	327	6.0	3.53 (0.95) ***	34.3	3.7
0 vs. 10	1.88 (0.30) ***	6.3	5.64 (0.94) ***	280	6.0	3.51 (1.03) ***	33.4	3.4
0 vs. 100	1.09 (0.27) ***	4.1	5.79 (0.94) ***	326	6.1	3.34 (0.93) ***	28.3	3.6
10 vs. 1	-0.20 (0.22)	-0.91	0.15 (0.29)	1.17	0.52	0.03 (0.50)	1.03	0.05
100 vs. 1	0.59 (0.18) ***	3.3	0.00 (0.28)	1.00	0.01	0.19 (0.26)	1.21	0.73
100 vs. 10	0.78 (0.19) ***	4.1	-0.15 (0.24)	0.86	-0.63	0.17 (0.47)	1.18	0.35
Adults only								
0 vs. 1	0.99 (0.41) *	2.4	4.76 (0.98) ***	117	4.9	2.74 (1.00) **	15.6	2.8
0 vs. 10	1.46 (0.38) ***	3.9	4.81 (0.97) ***	123	5.0	2.93 (1.02) **	18.7	2.9
0 vs. 100	2.03 (0.41) ***	5.0	4.67 (0.99) ***	107	4.7	2.1 (1.02) *	8.13	2.1
10 vs. 1	-0.48 (0.33)	-1.5	-0.05 (0.30)	0.96	-0.15	-0.18 (0.49)	0.83	-0.37
100 vs. 1	-1.05 (0.36) **	-2.9	0.09 (0.36)	1.10	0.25	0.65 (0.48)	1.91	1.3
100 vs. 10	-0.57 (0.32) [b]	-1.8	0.14 (0.35)	1.15	0.39	0.83 (0.54)	2.29	1.6
Juvenile vs Adult								
0 vs.0	0.57 (0.40)	1.4	-0.21 (1.32)	0.81	-0.16	-0.21 (1.3)	0.81	-0.16
1 vs.1	1.26 (0.29) ***	4.3	0.81 (0.33) *	2.25	2.5	0.59 (0.37)	1.8	1.6
10 vs.10	0.98 (0.26) ***	3.8	0.61 (0.27) *	1.84	2.3	0.38 (0.59)	1.46	0.64
100 vs.100	-0.38 (0.27)	-1.4	0.90 (0.32) **	2.46	2.8	1.0 (0.40) **	2.84	2.6

[a] Significance indicated with stars (*) where raw p-values were less than or equal to threshold p-values to maintain a 5% FDR

[b] Marginally significant, $0.05 < p \leq 0.10$

* $p \leq 0.05$

** $p \leq 0.01$

*** $p \leq 0.001$

Table S1.2. Carbaryl Exposures: concentration and life-stage specific post-hoc contrasts for juvenile and adult blue crab behavioral responses (RM-GEE A, post-hoc contrast)

	Righting Time (RT)		Eyestalk Retraction			Eyestalk Touch-Response		
Treatment Contrast (µg/L)	Estimate (SE) [a]	Z-value	Estimate (SE)	Odds ratio	Z-value	Estimate (SE)	Odds ratio	Z-value
Juveniles only								
0 vs. 1	1.05 (0.39) **	2.7	5.03 (1.0) ***	152	4.9	2.81 (1.1) **	16.5	2.6
0 vs. 10	1.07 (0.30) ***	3.5	4.83 (1.0) ***	125	4.7	2.68 (1.1) **	14.7	2.5
0 vs. 100	1.01 (0.29) ***	4.5	5.2 (1.0) ***	181	5.1	2.91 (1.0) **	18.3	2.8
10 vs. 1	-0.02 (0.38)	-0.06	0.2 (0.33)	1.22	0.60	0.12 (0.42)	1.13	0.29
100 vs. 1	-0.25 (0.38)	-0.65	-0.17 (0.31)	0.84	-0.56	-0.10 (0.4)	0.90	-0.25
100 vs. 10	-0.22 (0.28)	-0.79	-0.37 (0.26)	0.69	-1.4	-0.22 (0.34)	0.80	-0.65
Adults only								
0 vs. 1	1.14 (0.36) ***	3.2	3.4 (0.64) ***	29.9	5.3	3.42 (1.1) **	30.5	3.0
0 vs. 10	0.84 (0.37) *	2.3	4.37 (0.60) ***	79.2	7.3	4.61 (1.0) ***	101	4.6
0 vs. 100	1.07 (0.35) **	3.0	4.19 (0.62) ***	66.2	6.8	4.18 (1.1) ***	65.3	4.1
10 vs. 1	0.30 (0.26)	1.2	-0.97 (0.37) **	0.38	-2.7	-1.2 (0.65) [b]	0.30	-1.9
100 vs. 1	0.06 (0.24)	0.27	-0.79 (0.40)	0.45	-2.0	-0.76 (0.65)	0.47	-1.2
100 vs. 10	-0.24 (0.25)	-0.93	0.18 (0.32)	1.2	0.56	0.43 (0.36)	1.54	1.2
Juvenile vs Adult								
0 vs.0	-0.30 (0.38)	-0.79	-1.27 (1.2)	0.28	-1.1	0.06 (1.4)	1.06	0.04
1 vs.1	-0.39 (0.35)	-1.1	0.36 (0.41)	1.43	0.88	-0.55 (0.68)	0.58	-0.81
10 vs.10	-0.07 (0.29)	-0.23	-0.81 (0.29) **	0.44	-2.8	-1.87 (0.36) ***	0.15	-5.2
100 vs.100	-0.08 (0.27)	-0.30	-0.26 (0.30)	0.77	-0.88	-1.21 (0.35) ***	0.30	-3.5

[a] Significance indicated with stars (*) where raw p-values were less than or equal to threshold p-values to maintain a 5% FDR

[b] Marginally significant, $0.05 < p \leq 0.10$

* $p \leq 0.05$

** $p \leq 0.01$

*** $p \leq 0.001$

Table S1.3. Pesticide-type comparisons: malathion vs. carbaryl concentration specific post-hoc contrasts for juvenile and adult blue crab behavioral responses (RM-GEE B, post-hoc contrast)

	Righting Time (RT)		Eyestalk Retraction			Eyestalk Touch-Response		
Treatment Contrast (µg/L)	Estimate (SE) [a]	Z-value	Estimate (SE)	Odds ratio	Z-value	Estimate (SE)	Odds ratio	Z-value
Juveniles	0.17 (0.37)	0.46	0.32 (1.35)	1.4	0.24	0.32 (1.32)	1.4	0.23
0 vs. 0	0.85 (0.35) **	2.4	1.03 (0.36) **	2.8	2.9	1.07 (0.40) **	2.9	2.7
1 vs. 1	0.97 (0.25) ***	3.9	1.08 (0.28) ***	2.9	3.9	1.15 (0.53) *	3.1	2.2
10 vs. 10	-0.08 (0.24)	-0.33	0.84 (0.23) ***	2.3	3.7	0.74 (0.24) **	2.1	3.1
100 vs. 100	0.17 (0.37)	0.46	0.32 (1.35)	1.4	0.24	0.32 (1.32)	1.4	0.23
Adults								
0 vs. 0	0.65 (0.45)	1.5	- [b]	-	-	-0.66 (1.4)	0.52	-0.49
1 vs. 1	0.78 (0.31) **	2.5	- [b]	-	-	0.04 (0.67)	1.0	0.06
10 vs. 10	0.03 (0.29)	0.10	- [b]	-	-	1.03 (0.46) *	2.8	2.26
100 vs. 100	-0.34 (0.29)	-1.2	- [b]	-	-	1.43 (0.46) **	4.18	3.13

[a] - Significance indicated with stars (*) where raw p-values were less than or equal to threshold p-values to maintain a 5% FDR

[b] - RM-GEE parameter effects insignificant (Type III generalized score tests)

* $p \leq 0.05$

** $p \leq 0.01$

*** $p \leq 0.001$

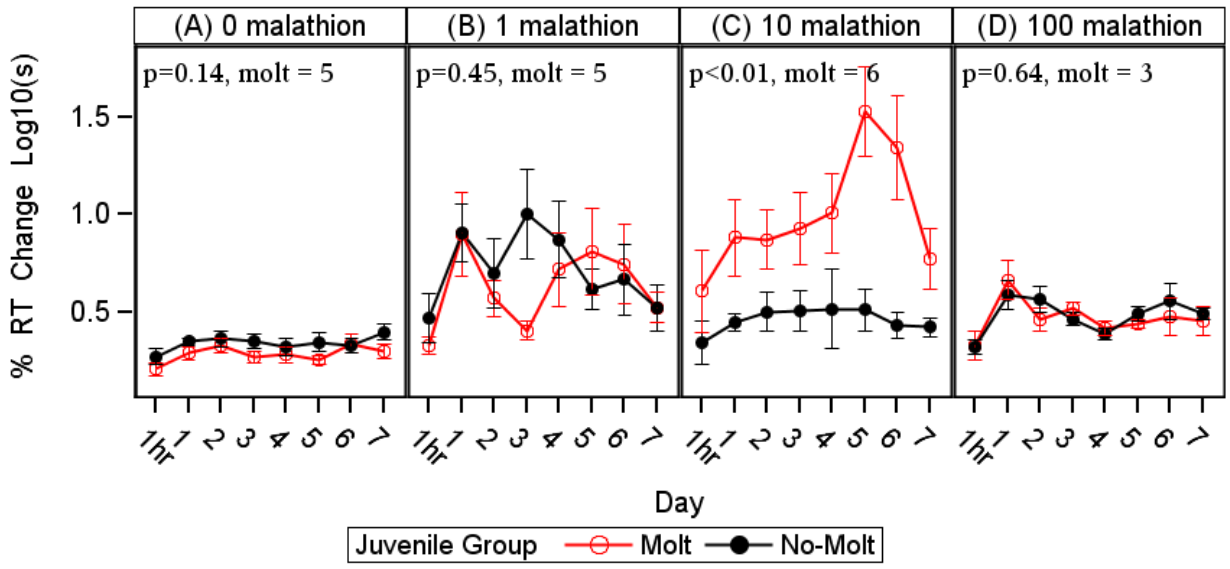

Figure S1.1. Juvenile crab RT response of molt (open circles, red) vs. non-molt (filled circles, black) crabs exposed to malathion for seven days. Values are log-transformed mean (± SE) % change in RT from Time-0 (pre-exposure). Positive RT values indicate an increase in RT. P-values indicate outcome of post-hoc contrast between molt vs. non-molt crabs (exploratory RM-GEE, post-hoc contrast). Within each panel (i.e., treatment level), molt frequency (number of crabs molted) is indicated. Trends require further study, however, given the limited sample size (n = 10 per treatment).

Table S2.1. 48-hr Res-PBO exposures (experiment 1): concentration and life-stage specific post-hoc contrasts for juvenile and adult blue crab survival and RT [a]

	Survival	Righting Time (RT)	
Treatment Contrast (µg/L)	Chi-Sq	Estimate (SE) [b]	Z-value
Juvenile crabs			
0 vs. 1:3	0.54	0.18 (0.07) **	2.6
0 vs. 10:30	21.8 ***	0.57 (0.07) ***	7.9
0 vs. 100:300	23.9 ***	0.38 (0.08) ***	4.9
0 vs. PBO-300	17.8 ***	0.61 (0.09) ***	6.3
Adult crabs			
0 vs. 1:3	24.2 ***	0.63 (0.16) ***	3.6
0 vs. 10:30	26.9 ***	0.47 (0.12) ***	3.8
0 vs. 100:300	7.32 **	0.45 (0.12) ***	3.6
0 vs. PBO-300	29.4 ***	0.67 (0.11) ***	6.2
Juvenile vs. Adult			
0 vs. 0	0.00	0.02 (0.04)	0.47
1:3 vs. 1:3	16.0 ***	0.47 (0.17) **	2.7
10:30 vs. 10:30	0.14	-0.08 (0.14)	-0.6
100:300 vs. 100:300	0.15	0.09 (0.14)	0.6
PBO-300 vs. PBO-300	1.50	0.08 (0.14)	0.6

[a] Results from Log-rank Lifetest (survivorship curve analysis) and RM-GEE. Significance indicated with stars (*) where raw p-values were less than or equal to threshold p-values to maintain a 5% FDR

** $p \leq 0.01$

*** $p \leq 0.001$

Table S2.2. Adult + juvenile cannibalism mesocosms: time specific post-hoc contrasts of prey survival in control vs. pesticide mesocosms at each hour (experiment 2) [a]

	Both Expo 1:3 vs. Control		Adult Expo 1:3 vs. Control		Juv. Expo 1:3 vs. Control		Juv. Expo 10:30 vs. Control	
Time	Estimate (SE)	Z-value	Estimate (SE) [b]	Z-value	Estimate (SE) [b]	Z-value	Estimate (SE) [b]	Z-value
1 h	0.01 (0.03)	0.18	0.01 (0.03)	0.18	0.03 (0.05)	0.75	0.38 (0.12) ***	3.24
3 h	0.21 (0.07) ***	3.09	0.20 (0.07) **	2.91	0.08 (0.11)	0.72	0.42 (0.20) *	2.14
8 h	0.22 (0.14) [b]	1.57	0.28 (0.13) *	2.14	0.26 (0.2)	1.35	0.37 (0.24)	1.56
12 h	0.18 (0.18)	1.02	0.31 (0.16) *	1.92	0.45 (0.24) *	1.86	0.56 (0.38) [b]	1.47
24 h	-0.32 (0.31)	-1.04	0.02 (0.31)	0.05	0.95 0.34) **	2.82	0.42 (0.38) [b]	1.09

[a] Contrasts compare prey survival in control vs. pesticide mesocosms at each hour. Significance indicated with stars (*) where raw p-values were less than or equal to threshold p-values to maintain a 10% FDR

[b] $0.05 \leq p \leq 0.10$

* $p \leq 0.05$

** $p \leq 0.01$

*** $p \leq 0.001$

Table S2.3. Juvenile + shrimp mesocosms (experiment 3): post-hoc contrasts of prey survival in control vs. pesticide mesocosms [a]

Mesocosm treatment	Estimate (SE) [b]	Z-value
Both Expo 1:3 vs. Control	0.42 (0.10) ***	4.27
Juv. Expo 1:3 vs. Control	0.37 (0.08) ***	4.72
Shrimp Expo 1:3 vs. Control	0.22 (0.12)	1.79

[a] Contrasts are controlled for the effect of hour.

[b] Significance indicated with stars (*) where raw p-values were less 4than or equal to threshold p-values to maintain a 5% FDR

*** $p \leq 0.001$

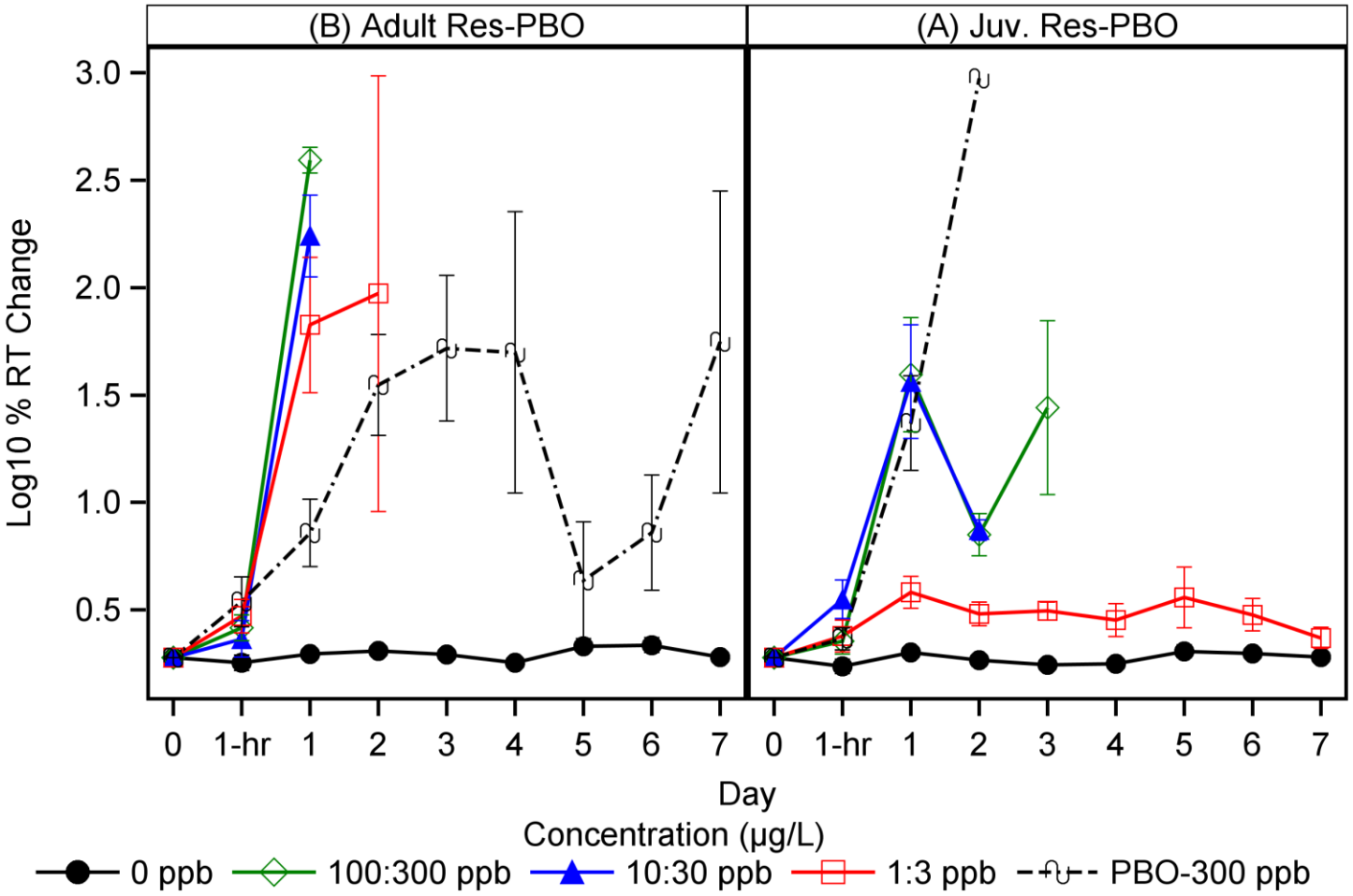

Figure S2.1. RT response: mean (± SE) percent change of juvenile (A) and adult (B) blue crab RT during a single exposure to Res-PBO for 7 days. Positive values indicate an increase in RT. (A and B) Compared to controls, all treatments significantly increased juvenile and adult crab RT ($p < 0.001$, RM-GEE post-hoc contrasts). (A vs. B) Life-stage comparisons: In 1:3 µg/L treatments, adult RT was significantly higher than juvenile RT ($p = 0.03$, RM-GEE post-hoc contrasts). Other life-stage RT differences were non-significant (Supplemental Data Table S2.1).

Table S3.1. Mixtures exposures: concentration and life-stage specific post-hoc contrasts for juvenile and adult blue crab behavioral responses (pairwise post-hoc contrast)

	Survival Time	Righting Time (RT)	
Treatment Contrast (µg/L)	Chi-Square [a]	Estimate (SE) [a]	Z-value
Juveniles only			
0 vs. 1	**5.8 ***	**-0.69 (0.09) *****	**-7.3**
0 vs. 10	**19.2 *****	**-0.70 (0.09) *****	**-7.8**
0 vs. 3.33	**20.8 *****	**-0.50 (0.14) *****	**-3.7**
0 vs. 5	**49.7 *****	**-0.89 (0.21) *****	**-4.3**
1 vs. 3.33	4.1 [b]	-0.01 (0.12)	-0.10
1 vs. 5	3.7 [b]	0.19 (0.15)	1.3
1 vs. 10	**20.2 *****	-0.19 (0.21)	-0.9
3.33 v 5	0.11	0.20 (0.15)	1.4
3.33 v 10	**4.8 ***	-0.19 (0.21)	-0.90
5 v 10	**8.4 ****	-0.39 (0.24)	-1.6
Adults only			
0 vs. 1	**4.7 ***	**-0.59 (0.11) *****	**-5.3**
0 vs. 10	**5.4 ***	**-0.75 (0.12) *****	**-6.0**
0 vs. 3.33	**31.2 *****	**-1.45 (0.13) *****	**-11.6**
0 vs. 5	**32.1 *****	**-1.38 (0.18) *****	**-7.8**
1 vs. 3.33	0.12	-0.16 (0.15)	-1.1
1 vs. 5	**17.9 *****	**-0.86 (0.15) *****	**-5.7**
1 vs. 10	**18.8 *****	**-0.79 (0.20) *****	**-4.0**
3.33 v 5	**11.9 *****	**-0.71 (0.15) *****	**-4.6**
3.33 v 10	**12.5 *****	**-0.63 (0.20) *****	**-3.2**
5 v 10	0.004	0.08 (0.19)	0.40
Juvenile vs Adult			
0 vs.0	0.21	0.03 (0.08)	0.41
1 vs.1	0.08	-0.07 (0.12)	-0.60
3.33 vs.3.33	**5.2 ***	0.08 (0.13)	0.60
5 vs. 5	0.63	**0.98 (0.17) *****	**5.8**
10 vs.10	**7.1 ****	**0.52 (0.25) ***	**2.1**

[a] Significance indicated with stars (*) where raw p-values were less than or equal to threshold p-values to maintain a 5% FDR

[b] Marginally significant, $0.05 < p \leq 0.10$

* $p \leq 0.05$

** $p \leq 0.01$

*** $p \leq 0.001$

Table S3.2. Hours alive (Min, Max, Average) and mortality of juvenile and adult crabs exposed to mixtures that died

	1 µg/L			3.33 µg/L			5 µg/L			10 µg/L		
Life Stage	Mortality (%)	Avg. hour alive	Min, max death hour	Mortality (%)	Avg. hour alive	Min, max death hour	Mortality (%)	Avg. hour alive	Min, max death hour	Mortality (%)	Avg. hour alive	Min, max death hour
Juvenile	38 (9/24)	75	24, 120	60 (18/30)	30	12, 168	63 (12/19)	30	12,132	92 (22/24)	23	12,120
Adult	10 (1/10)	36	36, -	29 (4/14)	57	24, 120	100 (10/10)	22	12, 48	100 (10/10)	18	12, 36

Table S3.3. Mixture vs. individual exposures: Concentration and life-stage specific post-hoc contrasts for juvenile and adult blue crab survival

Pesticide 1 vs.	Pesticide 2	Concentration contrast	Juvenile $\chi 2$ [a]	Adult $\chi 2$ [a]
Mixture	Carbaryl	0 vs. 0	1.5	0.22
Mixture	Carbaryl	1 vs. 1	3.8	0.53
Mixture	Carbaryl	3.33 vs. 1	**21.7 *****	1.7
Mixture	Carbaryl	3.33 vs. 10	**22.0 *****	1.7
Mixture	Carbaryl	5 vs. 10	**17.8 *****	**23.4 *****
Mixture	Carbaryl	10 vs. 10	**64.2 *****	**24.7 *****
Mixture	Malathion	0 vs. 0	2.9	2.3
Mixture	Malathion	1 vs. 1	2.7	0.09
Mixture	Malathion	3.33 vs. 1	**20.6 *****	0.95
Mixture	Malathion	3.33 vs. 10	**20.6 *****	0.32
Mixture	Malathion	5 vs. 10	**16.3 *****	**22.1 *****
Mixture	Malathion	10 vs. 10	**67.3 *****	**23.7 *****
Mixture	Resmethrin	0 vs. 0	2.9	1.9
Mixture	Resmethrin	1 vs. 1	2.7	0.22
Mixture	Resmethrin	3.33 vs. 1	**20.6 *****	0.06
Mixture	Resmethrin	3.33 vs. 10	**20.6 *****	**15.00 *****
Mixture	Resmethrin	5 vs. 10	**16.3 *****	0.07
Mixture	Resmethrin	10 vs. 10	**67.3 *****	0.03
Mixture	Res-PBO	0 vs. 0	2.9	1.9
Mixture	Res-PBO	1 vs. 1	**18.5 *****	**21.9 *****
Mixture	Res-PBO	3.33 vs. 1	0.06	**12.2 *****
Mixture	Res-PBO	3.33 vs. 10	**12.5 *****	**12.8 *****
Mixture	Res-PBO	5 vs. 10	0.38	0
Mixture	Res-PBO	10 vs. 10	0.85	0
Res-PBO	Carbaryl	0 vs. 0	0.24	0.85
Res-PBO	Carbaryl	*1 vs. 1*	2.8	**23.2 *****
Res-PBO	Carbaryl	10 vs. 10	**27.4 *****	**24.00 *****
Res-PBO	Malathion	0 vs. 0	0	0.02
Res-PBO	Malathion	*1 vs. 1*	1.7	**26.4 *****
Res-PBO	Malathion	10 vs. 10	**27.1 *****	**22.8 *****
Res-PBO	Resmethrin	0 vs. 0	0	0
Res-PBO	Resmethrin	*1 vs. 1*	1.7	**17.7 *****
Res-PBO	Resmethrin	10 vs. 10	**27.1 *****	0.05
Resmethrin	Carbaryl	0 vs. 0	0.24	0.85

Table S3.3. Mixture vs. individual exposures: Concentration and life-stage specific post-hoc contrasts for juvenile and adult blue crab survival

Pesticide 1 vs.	Pesticide 2	Concentration contrast	Juvenile $\chi 2$ [a]	Adult $\chi 2$ [a]
Resmethrin	Carbaryl	*1 vs. 1*	0.24	1.3
Resmethrin	Carbaryl	10 vs. 10	0.36	**27.3 *** **
Resmethrin	Malathion	0 vs. 0	0	0.02
Resmethrin	Malathion	*1 vs. 1*	0	0.63
Resmethrin	Malathion	10 vs. 10	0	**27.00 *** **
Carbaryl	Malathion	0 vs. 0	0.24	1.1
Carbaryl	Malathion	*1 vs. 1*	0.24	0.23
Carbaryl	Malathion	10 vs. 10	0.36	0.76

[a] Significance indicated with stars (*) where raw p-values were less than or equal to threshold p-values to maintain a 5% FDR

[b] Marginally significant, $0.05 < p \leq 0.10$

* $p \leq 0.05$

** $p \leq 0.01$

*** $p \leq 0.001$

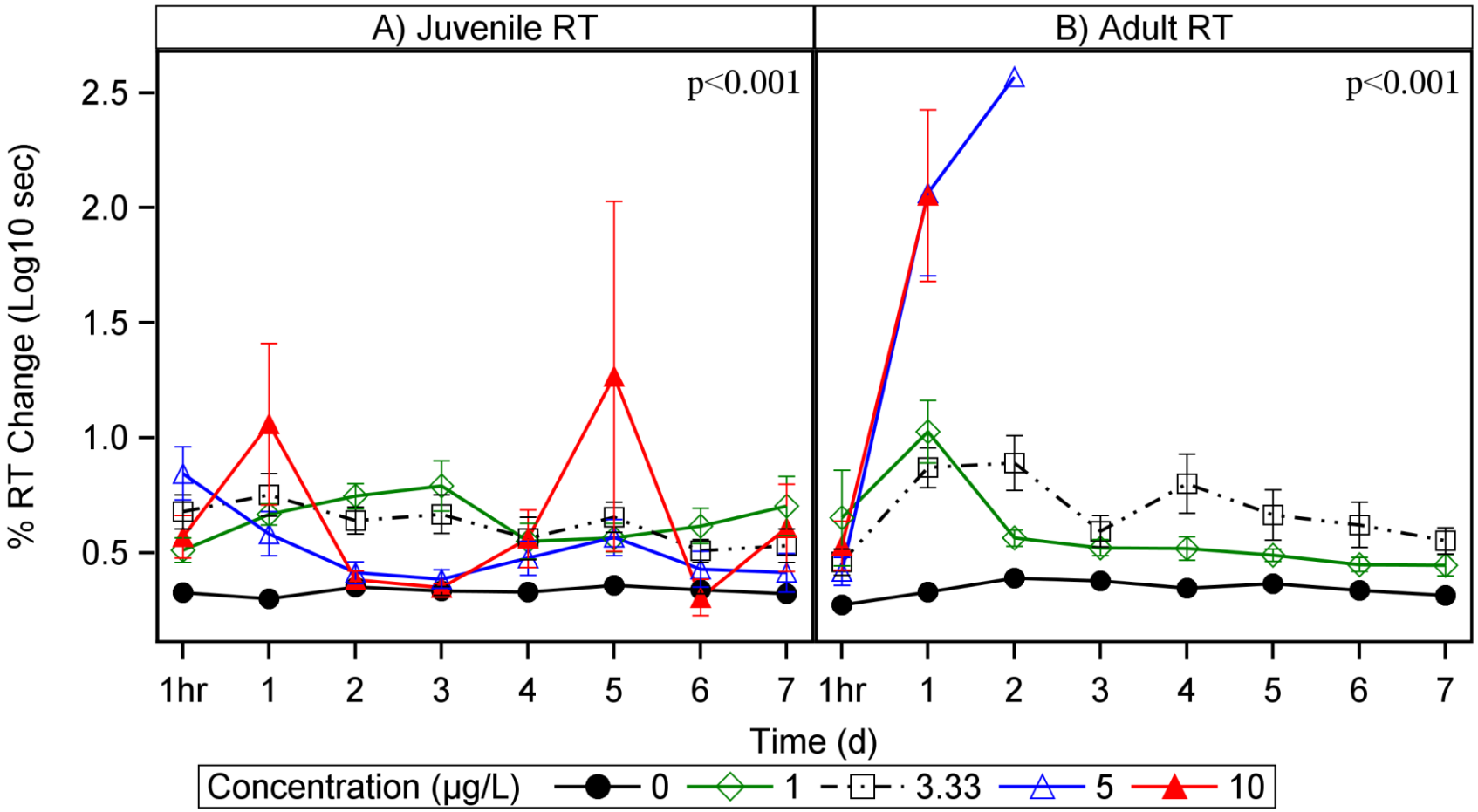

Figure S3.1. RT response (all data): mean (± SE) percent change of juvenile (A) and adult (B) blue crab RT during a single exposure to mixtures for 7 days. Positive values indicate an increase in RT. (A and B) Compared to controls, all treatments significantly increased juvenile and adult crab RT ($p \leq 0.01$, RM-GEE post-hoc contrasts). (A) Note that after 2 d, juvenile 10 µg/L n = 3. (B) 100% mortality in 5 µg/L and 10 µg/L. (A vs. B) Life-stage comparisons: In 5 µg/L treatments, adult RT was significantly higher than juvenile RT ($p < 0.01$, RM-GEE post-hoc contrasts). Other life-stage RT differences were non-significant.