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Acute toxicity and sub-lethal effects of common pesticides in post-larval and juvenile blue crabs, *Callinectes sapidus*

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**A B S T R A C T**

The coastal plains of the southeastern United States support extensive agricultural operations that apply pesticides and herbicides. The shallow tidal creeks and ditches that directly drain agricultural fields are home to the young of the ecologically and economically important blue crab, *Callinectes sapidus* (Rathbun, 1896). Massive mortality observed by peeler crab fishers led us to investigate the acute toxicity of several commercial pesticide formulations and their active ingredients to blue crab megalopae and J1–J4 stage juveniles. Twenty-four hour acute toxicity assays were conducted with the organophosphate acephate (Orthene®), the carbamate aldicarb, the chloro-nicotinyl imidacloprid (Trimax™), the pyrethroid lambda-cyhalothrin (Karate® with Zeon Technology), and the glyphosate-based herbicide Roundup® Pro. LC50 values ranged from 0.22 μg/L for megalopae exposed to lambda-cyhalothrin to 316,000 μg/L for juveniles exposed to Roundup. The acute toxicities of active ingredient insecticides to blue crabs followed the order: lambda-cyhalothrin ≈ imidacloprid > aldicarb > acephate. Megalopae were almost always more sensitive than juveniles and there was little difference between the LC50 of each commercial formulation and its active ingredient. Treatment of intermolt megalopae with LC20 levels of Roundup resulted in significantly reduced time to metamorphosis (TTM) compared to estuarine water controls, while no differences resulted from treatment with the four active ingredient insecticides. Treatment with acephate, aldicarb, imidacloprid, and Roundup significantly increased the frequency of juveniles that died within 6 h of molting. The sensitivity of molting blue crabs to these pesticides makes frequently molting juveniles particularly vulnerable to pesticides in estuaries.

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1. Introduction

The southeastern United States consists of extensive coastal plains drained by creeks, streams, and rivers that flow into estuaries and sounds. The land supports widespread agriculture, particularly cotton, corn, soybeans, and tobacco that forms the base of much of the local economy and is associated with abundant pesticide and herbicide application. The water bodies that drain this region make up the center of the U.S. blue crab fishery, which is often one of the most economically important fisheries in the southeastern U.S. Herein we report the acute toxicities and sub-lethal effects of common pesticides in various blue crab life stages. We focused our study on the effects of pesticides commonly used around the North Carolina blue crab fishery.

The blue crab fishery is the most profitable and highest landing fishery in North Carolina with over 29.7 million pounds of hard blue crabs harvested in 2010, worth over $23.8 million (NCDMF, 2011). This fishery is centered on the Albermarle and Pamlico Sounds, which are bordered by counties that host thousands of acres of crop- and a valuable agriculture industry. Over 225,000 acres of soybeans, corn, and cotton were harvested from Hyde, Beaufort, and Pamlico counties (NC) in 2010, contributing over $161 million to the economy (NCDAC&S, 2011). Brackish tidal creeks that drain these agricultural fields are important habitats for juvenile and adult stage blue crabs (Posey et al., 2005; Ramach et al., 2009) and their prey and are frequently less than 10 cm deep and 2 m wide. With two of North Carolina’s most important economic engines in close proximity, it is essential to understand the interaction and subsequent effects of pesticides on blue crabs.

After mating in spring and summer, gravid female blue crabs migrate downstream and out of the estuary to release larvae. Dispersed
The genetic modification of several crops including cotton, corn, and soybeans with glyphosate resistant genes has greatly changed which herbicides are used on crops with a trend towards glyphosate-based products. So called Roundup Ready® (Monsanto Company) cotton has been available since 1997 and nearly all North Carolina cotton is now of this variety. A total of 951,000 lbs of glyphosate-based products (including glyphosate-isopropylamine salt, the form used in Roundup) were applied to at least 86% of NC cotton fields in 2007 at a rate averaging 0.733 lbs/A per application (USDA, 2008). Glyphosate is a highly water soluble phosphonoglycine herbicide that inhibits an enzyme required to produce the amino acids tryptophan, phenylalanine, and tyrosine (Amrein et al., 1980). It is often found in commercial products (Roundup) with a polyethoxylated tallow amine (POEA) surfactant that has proven more toxic than the active ingredient itself (Giesy et al., 2000).

Twenty-four hour acute toxicity experiments were performed on megalopae and juvenile blue crabs, Callinectes sapidus, with both commercial formulation pesticides and their active ingredients (Table 1). The effects of active ingredient compounds and commercial Roundup on the metamorphosis of megalopae to the first juvenile stage were also investigated.

2. Methods

2.1. Blue crab collection

Blue crab (C. sapidus Rathbun) megalopae were collected from the water column as they migrated inshore during summer 2008 nighttime rising tides. Two 0.75 m diameter plankton nets were deployed for 1 h straddling the maximum flood tide current from the NOAA sampling platform located beneath the Pivers Island Bridge, Beaufort, North Carolina, USA. Plankton samples were coarsely sieved to remove ctenophores and visually identified blue crab megalopae were sorted out. Megalopae were maintained in filtered aged estuarine seawater (ASW: salinity 35, 25 °C) on an ambient light:dark cycle and reared in 6 in. diameter glass finger bowls. Each finger bowl contained 100–200 individuals in ~800 mL of ASW. Filtered and aged estuarine seawater was used to mimic natural conditions as closely as possible while minimizing any potential effects of chemical cues. Megalopae were fed with ~5 mL (~1500 individuals) of newly hatched brine shrimp (Artemia) nauplii following daily water changes. Newly molted juvenile (J1) blue crabs and dead megalopae were removed from the finger bowls daily. Megalopae were used in toxicity testing within three days of collection or allowed to molt to juveniles. This method of collecting megalopae from near-oceanic water ensured that the crabs had not been exposed to any significant amount of pesticide for several weeks.

Juvenile blue crabs were reared in similar conditions to megalopae but were kept at lower densities (~50 per 800 mL) and fed crushed shrimp pellets (dry fish food) daily. Strips of nylon

Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Formulation</th>
<th>Class</th>
<th>Megalopae</th>
<th>Juveniles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>24 h LC50 (95% confidence interval) (μg/L)</td>
<td></td>
</tr>
<tr>
<td>Karate®</td>
<td>Comm.</td>
<td>Pyrethroid</td>
<td>0.2560 (0.351–0.789)</td>
<td>3.565 (1.721–7.385)</td>
</tr>
<tr>
<td>λ-Cyhalothrin</td>
<td>A.I.</td>
<td>Pyrethroid</td>
<td>0.2233 (0.1833–0.2720)</td>
<td>2.701 (2.215–3.294)</td>
</tr>
<tr>
<td>Tralomethrin</td>
<td>Comm.</td>
<td>Chloro-nicatinyl</td>
<td>312.7 (222.4–439.9)</td>
<td>816.7 (692.9–962.6)</td>
</tr>
<tr>
<td>Imidacloprid</td>
<td>A.I.</td>
<td>Chloro-nicatinyl</td>
<td>10.04 (6.381–15.79)</td>
<td>1112 (841.9–1468)</td>
</tr>
<tr>
<td>Aldicarb®</td>
<td>A.I.</td>
<td>N-methyl carbamate</td>
<td>311.6 (281.6–344.8)</td>
<td>291.1 (2277–372.3)</td>
</tr>
<tr>
<td>Orthene®</td>
<td>Comm.</td>
<td>Organophosphate</td>
<td>61210 (48500–77260)</td>
<td>191,300 (141,100–259,000)</td>
</tr>
<tr>
<td>Acrephate</td>
<td>A.I.</td>
<td>Organophosphate</td>
<td>50,380 (44,300–57,300)</td>
<td>137,300 (132,800–141,900)</td>
</tr>
<tr>
<td>Roundup® Pro®</td>
<td>Comm.</td>
<td>Phosphonoglycine</td>
<td>6279 (5937–6640)</td>
<td>316000 (167,000–595,200)</td>
</tr>
</tbody>
</table>

Comm., commercial; A.I., active ingredient.

a Aldicarb was only tested as A.I.

b Roundup Pro was only tested as a commercial product.
window screening were added to each bowl to provide structure and minimize interactions among juveniles, including cannibalism. First stage (J1) through fourth stage (J4) juveniles were reared and used in acute toxicity assays.

2.2. Chemicals

Active ingredient insecticides used for megalopae and juvenile acute toxicity and time to metamorphosis assays were acquired through Fisher Scientific and were manufactured by Chem Service (West Chester, PA). The four insecticides were dry powder acephate (99.3% purity), aldicarb (99.0% purity), imidacloprid (99.5% purity), and λ-cyhalothrin (99% mix of isomers). Experimental samples of granular Temik® 15G (15% aldicarb) and liquid Trimax® Pro (40.80% imidacloprid) were acquired from Bayer CropScience (Kansas City, MO). Karate® with Zeon Technology™ (Syngenta Crop Protection, Greenville, NC) was graciously donated by Open Grounds Farm (Carteret County, NC). Ortho Orthene® Fire Ant Killer (Ortho Group, Marysville, OH) with 50% acephate and Roundup® Pro Concentrate (Monsanto Company, St. Louis, MO; 50.2% glyphosate isopropylamine salt) were purchased from Lowe’s Home Improvement. Karate and Temik 15G are restricted use pesticides and were obtained and used under North Carolina Pesticide Board Pesticide Applicator License number 026–26323.

2.3. Experimental solutions

All chemicals were kept in the dark to prevent photolysis. Working stocks were made immediately before use with nanopure water in glass vials and kept in a light-tight metal container for up to one week before being discarded. Lambda-cyhalothrin was the only compound that required a solvent so stocks were made with methanol so that dosing solutions never exceeded 0.05% methanol. Toxicant dilutions were made immediately before use and diluted with ASW. Concentrations of commercial products in this study are presented as nominal concentrations of active ingredient.

2.4. Acute toxicity

We performed 24 h acute toxicity assays on megalopae and juvenile crabs. Experiments were dosed once at the beginning of the experiment under still conditions (static, non-renewal). Between two and six assays were performed for each experimental toxicant, with each assay testing progressively narrower ranges of concentrations around the effective concentration for 50% mortality. Each assay included several concentrations of pesticide as well as aged seawater (ASW) control and a solvent control when needed. Test animals in a given round were haphazardly taken from all of the available rearing finger bowls and allocated to their respective containers with a small amount of ASW while pesticide dilutions were made. The ASW was then removed and replaced with toxicant solution. Megalopae were tested in glass test tubes containing 10 mL of test solution (n = 5 individuals per tube, 3 replicates per concentration). Juveniles were tested in cell-culture-treated (hydrophilic) polystyrene 24-well microplates with one individual per well in 1.5 mL of test solution (n = 24 individuals per concentration). These two container materials were chosen so that they had similar hydrophilicity. Test containers were covered to minimize evaporation and the number of dead crabs counted at 24 h. No feeding, aeration, or mixing took place during the 24 h assay. Death was judged by lack of movement of the appendages and antennae in response to shaking the container. Dead juveniles usually had an opaque coloration and were often upside down with legs curled.

Acute toxicity was modeled as four-parameter log dose–response nonlinear regressions using GraphPad Prism 5 (GraphPad Software, San Diego, CA). To avoid the logarithm of zero, ASW and solvent controls were entered into models as concentration at least 3 log units below the lowest experimental concentration. This was 0.0001 μg/L for λ-cyhalothrin and Karate; 0.001 μg/L for imidacloprid, Trimax, and aldicarb; and 1 μg/L for Orthene, acephate, and Roundup. Since 100% mortality was reached with all test solutions, model maxima were constrained to a value of 100%. If control mortality was zero for a toxicant then its model was constrained to a minimum of 0%. No lower constraints were placed on models of toxicants with control mortalities greater than zero. LC50 values with 95% confidence intervals were calculated from each model. Statistical differences between LC50 concentrations of megalopae and juveniles in the same toxicant and between the same ontogenetic stage in the commercial pesticide and its active ingredient were tested using one-way analysis of variance (ANOVA).

2.5. Time to metamorphosis (TTM)

After completing and modeling the 24 h acute toxicity assays, the concentration modeled to kill 20% of the crabs (LC20) was used to test the effects of pesticides on the time to metamorphosis from the megalopae stage to the J1 stage. Megalopae were collected as above and used within 24 h of collection. Only visually identified intermolt megalopae of a single cohort (Forward et al., 1996) were used in this assay. Intermolt megalopae were housed individually in glass test tubes filled with 2 mL of test solution (n = 30 per concentration). Housing megalopae individually in glass eliminated any potential effects of plasticizers, cannibalism, or changes in density with removal of juveniles. Test solutions were made using estuarine seawater (ESW) so that any natural chemical cues would be present. These were static, non-renewal exposures on an ambient light:dark cycle. Water was not changed and megalopae were not fed during the experiment. Megalopae were checked approximately every 6 h until all megalopae had either died as megalopae or molted to juveniles. The time period in which any individual died or molted (or both) was recorded.

Two separate rounds of time to metamorphosis (TTM) assays were completed. The first round tested the ESW control (control 1), 32,000 μg/L acephate, 240 μg/L aldicarb, and 4.8 μg/L imidacloprid. The second round tested the ESW control (control 2), 0.15 μg/L λ-cyhalothrin, and 3500 μg/L Roundup Pro. Statistical analyses were performed using GraphPad Prism 5 between experimental results and those of their respective control; i.e. only within round 1 and within round 2. This was done because different cohorts of megalopae, collected on different days with different experiences, were used in each round.

Time to metamorphosis data were analyzed using three different methods: ANOVA on the mean TTM (Forward et al., 1994, 1996, 1997); Log-rank Mantel–Cox survival analysis (similar to Tankersley and Wieber, 2000); and ANOVA of regression-derived ET50. For the first method, all of the times (h) of metamorphosis for each treatment were averaged together (Brumbaugh and McConaugha, 1995; Fitzgerald et al., 1998; Forward et al., 1994, 1996, 1997; Gebauer et al., 1999; O’Connor and Gregg, 1998; O’Connor and Judge, 1997; Rodriguez and Epifanio, 2000; Wolcott and De Vries, 1994). Only those megalopae that successfully molted to the juvenile stage, whether alive or dead when checked were included in the calculation of the mean time to metamorphosis. Two one-way ANOVAs (one for each round) with Dunnett’s post-hoc tests were employed to compare the mean TTM.

A survival analysis on metamorphosis was employed as the second method of TTM analysis similar to Tankersley and Wieber (2000). “Death” was replaced in the traditional survival analysis with “metamorphosis.” This method has the benefit of taking into account megalopae that die during the experiment when calculating the percentage of megalopae or juveniles at each point. A log-rank
Mantel–Cox test (Peto and Peto, 1972) was employed for each round of testing.

The third method of TTM analysis mirrored our acute toxicity analysis. TTM was modeled using a four-parameter curve similar to the dose–response curve. The percentage of megalopae metamorphosed at each time point, as calculated during the survival analysis (above), was used as the input data. The times modeled to produce 50% molting for each treatment (ET50; effective time 50) were compared using two separate one way ANOVAs with Bonferroni-corrected pairwise post-hoc comparisons.

Fisher’s exact tests were used to compare the frequencies of megalopae that molted to J1 for each treatment. Likewise, the frequencies of molted juveniles found dead when first checked after molting were tested. These tests were performed using SigmaPlot 11 (Systat Software, San Jose, CA).

3. Results

3.1. Acute toxicity

3.1.1. Karate and λ-cyhalothrin

Karate with Zeon technology and its active ingredient λ-cyhalothrin were the most potent of the insecticides tested. LC50 values for megalopae exposed to Karate was 0.5260 μg/L and 3.565 μg/L for juveniles (Fig. 1,

Fig. 1. Mortality of megalopae (●) and juveniles (△) exposed to pesticides. Commercial formulations are presented in the left column and active ingredients in the right. Best fit model (solid line) and 95% confidence interval (dotted line) are drawn. N.B. panels G and H are different active ingredients.
Table 1). Treatment with λ-cyhalothrin produced similar results with a megalopa LC50 of 0.2233 μg/L and a juvenile LC50 of 2.701 μg/L. The LC50 for juveniles exposed to λ-cyhalothrin was significantly greater than that for megalopae (p = 0.0001, F1,29 = 68.7). There was no significant difference between Karate and λ-cyhalothrin LC50 values for either megalopae (p = 0.075, F1,25 = 3.478) or juveniles (p = 0.4413, F1,28 = 0.6101).

3.1.2. Trimax and imidacloprid
Trimax and its active ingredient imidacloprid were the next most potent insecticides after Karate and λ-cyhalothrin. The LC50s for megalopae and juveniles exposed to Trimax were 312.7 μg/L and 816.7 μg/L, respectively (Fig. 1, Table 1). Imidacloprid was significantly more toxic than Trimax (p < 0.0001, F1,51 = 42.68) to megalopae with a LC50 value 10.04 μg/L. Exposure of juveniles to imidacloprid produced a LC50 of 1112 μg/L, which was not significantly different than that for Trimax (p = 0.0969, F1,17 = 3.087). Both Trimax (p = 0.002, F1,44 = 17.07) and imidacloprid (p < 0.0001, F1,24 = 42.87) were significantly more toxic to megalopae than to juveniles.

3.1.3. Aldicarb
We had difficulty getting pelletized Temik 15G into solution so we only present results for its active ingredient aldicarb. Aldicarb exhibited toxicity to blue crabs similar to the toxicity of Trimax and imidacloprid. LC50s for megalopae and juveniles treated with aldicarb were 311.6 μg/L and 291.1 μg/L, respectively (Fig. 1, Table 1). Aldicarb was the only tested insecticide that showed no significant difference in toxicity between megalopae and juveniles (p = 0.8554, F1,55 = 0.0335). The dose-response curve for megalopae treated with aldicarb was particularly steep and had a Hill slope of 6.66 compared to the more gradual Hill slope of 2.05 for juveniles treated with aldicarb (Fig. 1).

3.1.4. Orthene and acephate
Orthene and its active ingredient acephate were by far the least potent of the insecticides tested. The LC50 for Orthene-exposed megalopae was 61,210 μg/L, between two and four orders of magnitude less potent than the other insecticides (Fig. 1, Table 1). The LC50 of Orthene for blue crab juveniles (191,300 μg/L) was significantly higher than for megalopae (p = 0.0069, F1,43 = 8.077). The toxicity of acephate to megalopae (LC50 = 50,380 μg/L) did not differ significantly from that of Orthene (p = 0.1151, F1,80 = 2.531). Acephate was significantly more toxic to megalopae than to juveniles (p = 0.004, F1,64 = 8.892) with a juvenile LC50 of 137,300 μg/L. Acephate was significantly more toxic to juveniles than Orthene (p = 0.0149, F1,18 = 7.247).

3.1.5. Roundup
The toxicity of the herbicide Roundup Pro was generally very low but significantly higher to megalopae than to juveniles (p = 0.0001, F1,41 = 48.63). The LC50 for megalopae exposed to Roundup was 6279 μg/L and that for juveniles was 316,000 μg/L (Fig. 1, Table 1). Similar to the results of aldicarb exposure, roundup exposure produced the particularly large difference in Hill slopes of 11.41 for megalopae and 1.2 for juveniles (Fig. 1).

3.2. Time to metamorphosis (TTM)

Roundup was the only treatment that significantly reduced mean TTM (q = 2.646, p = 0.05) and ET50 (p = 0.002; Fig. 2) but its survival curve was not significantly different than controls (Fig. 3). Round 1 tested acephate, aldicarb, and imidacloprid. There was no significant difference in mean TTM (F2,85 = 0.0653, p = 0.978; Fig. 2), survival curves (χ2 = 1.428, 3 df, p = 0.6990; Fig. 4), or ET50s (F2,35 = 2.265, p = 0.091; Fig. 2). Mean TTM ranged from 64.14 h for aldicarb to 67.13 h for acephate. ET50s ranged from 55.81 h for aldicarb to 66.90 h for acephate.

Round 2 tested λ-cyhalothrin and Roundup. There were significant differences in mean TTM (F2,68 = 4.362, p = 0.0165; Fig. 4) and ET50 (F2,15 = 6.515, p = 0.0070; Fig. 2) but no differences among survival curves (χ2 = 2.212, 2 df, p = 0.3309; Fig. 3). Mean TTM for round 2 ranged from 63.38 h for Roundup to 85.03 h for λ-cyhalothrin. ET50 for Roundup and λ-cyhalothrin were 52.06 h and 81.25 h, respectively. For both rounds all ET50 values were lower (quicker) than their corresponding mean TTM.

Of the 30 intermolt megalopae per treatment, 86.2% of control 1 and 96.7% of control 2 molted to juveniles. Only treatment with

![Fig. 2. Mean time to metamorphosis (±S.E.M.) and modeled ET50. An axis break separates round 1 from round 2 and statistical analyses were only performed within groups. Significance: *p < 0.05.](image1)

![Fig. 3. Metamorphosis curves (with S.E.M.) for round 1 (A) and round 2 (B). Only those megalopae still alive or successfully molted to the J1 stage (alive or dead) at each check point are included in the percentage calculation.](image2)
imidacloprid (56.7%; p = 0.0204) and λ-cyhalothrin (58.6%; p = 0.0004) resulted in significantly fewer megalopae molting than their respective controls (Fig. 4). None of the newly molted juveniles in either control treatments was dead when first checked after molting. Treatment with the three insecticides acephate, aldicarb, and imidacloprid significantly increased the frequency of juveniles that died after molting compared to control 1 (Fig. 4). Aldicarb proved most deadly to the molting crabs with 91.3% (p < 0.0001) of juveniles dead when first checked after molting. Treatment with acephate and imidacloprid resulted in 37.5% (p = 0.0005) and 41.2% (p = 0.0007) of the juveniles dying after metamorphosis, respectively. The commercial herbicide product Roundup Pro (p = 0.04) significantly increased mortality after molting compared to control 2.

4. Discussion

4.1. Acute toxicity

The acute toxicities of the tested active ingredient insecticides to blue crab megalopae and juveniles result in the pattern: λ-cyhalothrin > imidacloprid > aldicarb > acephate (Fig. 5). The commercial herbicide formulation Roundup was similar to acephate in its toxicity to juvenile blue crabs, but was much more toxic to megalopae.

Lambda-cyhalothrin (λ-cyhalothrin) is an extremely potent pyrethroid insecticide whose mode of action is to bind to and open sodium channels on neuron axons, effectively paralyzed subjects (He et al., 2008). Lambda-cyhalothrin, alone and in its commercial formulation Karate, was by far the most acutely toxic compound tested (Fig. 5). Treatment of blue crabs with λ-cyhalothrin produced a megalopa LC50 of 0.2233 μg/L and a juvenile LC50 of 2.701 μg/L. LC50 values for blue crab megalopae and juveniles exposed to the commercial formulation Karate were 0.526 μg/L and 3.565 μg/L, respectively. Our values are similar to 24 h LC50 reported for other crustaceans treated with λ-cyhalothrin-based pesticides such as 0.183 μg/L for the fairy shrimp Stenopelmatopsis salinus (Lahr et al., 2001) and 0.87 μg/L for the freshwater shrimp Cardina laevis (Sucharay et al., 2008). Mokry and Hoagland (1990) reported similar 48 h LC50 values for Daphnia magna and Ceriodaphnia dubia first instars (Table 2).

Imidacloprid is a chloro-nicotinyl insecticide and acetylcholine receptor agonist (Matsuda et al., 2001). We report a two order of magnitude difference between megalopae and juvenile blue crab imidacloprid LC50 values and over a one order of magnitude difference between megalopae values for imidacloprid and the commercial formulation Trimax. Imidacloprid toxicity in other crustaceans ranges broadly with 24 h LC50 as low as 103 μg/L for the amphipod Gammarus pulex (404 nmol/L; Ashauer et al., 2011) to 112 μg/L for the rice paddy ostracod Ilyocypris dentifera to as high as > 320,000 μg/L for D. magna (Sánchez-Bayo and Goka, 2006). 48 h LC50 values show a similarly large range in other crustacean models (Table 2). The blue crab megalopae and juveniles are more sensitive to imidacloprid than the model crustacean D. magna by many orders of magnitude but are similar in sensitivity to the widely used amphipod G. pulex (Ashauer et al., 2011).

Aldicarb is an N-methyl carbamate and cholinesterase inhibitor that is highly acutely toxic to a broad range of organisms, especially crustaceans. 24 h LC50 values of aldicarb to blue crab megalopae and juveniles (Table 1) are similar to that for G. pulex (Ashauer et al., 2011) but are two orders of magnitude less than for 72 h old Artemia salina nauplii (Barahona and Sánchez-Fortún, 1999) (Table 2). In 2010, the U.S. Environmental Protection Agency and Bayer CropScience agreed to reduce and ultimately discontinue the production of aldicarb as well as eliminate all aldicarb product registrations by the end of 2014 (Federal Register, 2010).

Acephate is a broad spectrum organophosphate and a known neurotoxin that inhibits the breakdown of acetylcholine (Eto, 1974). Acephate and Orthene were by far the least toxic insecticides tested with LC50 over an order of magnitude higher than for any other pesticide tested here (Table 1), similar to results from other studies comparing toxicity of acetylcholinesterase inhibitors (Printes and Callaghan, 2004). Venkateswara et al. (2007) reported 24 h LC50 values for 72 h old Artemia salina nauplii-treated with acephate (Table 2) even higher than the LC50s for blue crab megalopae and juveniles reported here. Interestingly the acephate metabolite methamidophos (O,S-Dimethyl phosphoramidothioate) is highly toxic to crustaceans with 24 h LC50 values for D. magna of 108.7 μg/L (Lin et al., 2006) and 0.16 μg/L for mysis stage Penaeus stylostris shrimp (Tamaran® 600; Juarez and Sanchez, 1989).

Glyphosate is a phosphonoglycine herbicide that inhibits an enzyme required to produce certain amino acids (Amrhein et al., 1980) and was only tested here as the commercial product Roundup that includes the POEA surfactant that has proven toxic by itself (Brausch and Smith, 2007; Brausch et al., 2007; Folmar et al., 1979; Giesy et al., 2000). Blue crab megalopae were much more sensitive to Roundup than juveniles (Table 1). Different glyphosate-based products produced drastically different 48 h LC50 values for D. magna with Roundup (Folmar et al., 1979) being much more toxic than Rodeo® (Henry et al., 1994) (Table 2). The amphipods Gammarus pseudolimnaeus and Hyalella azteca were not very sensitive to glyphosate-based herbicides with a reported 48 h LC50 of

![Fig. 4. The percentages of megalopae successfully molting to the J1 stage and the percentages of those successfully molting that were found dead as J1 stage crabs during TTM experiments. An axis break separates round 1 from round 2 and statistical analyses were only performed within rounds. Significance: *p<0.05, **p<0.0005.](image)

![Fig. 5. LC50 (±95% confidence interval) for megalopae (●) and juveniles (○) treated with the various pesticides. Background shading groups commercial products with their active ingredient.](image)
Roundup 48 h LC50 of 177,000 (Henry et al., 1994), respectively. Tsui and Chu (2003) reported a decrease in toxicity with increasing ontogenetic stages in blue crabs (Bookhout and Pesticides like mirex and kepone have shown a decrease in toxicity of several other pesticides as well (Sánchez-Fortún et al., 1995, 1996). An acephate metabolite.

Juvenile crabs occur throughout estuaries and routinely inhabit and forage in very shallow water (Ramach et al., 2009) including shallow drainage areas. Thus they and their prey may be routinely exposed directly to overspray and run-off. Assuming a ditch, stream, or other body of water near agriculture has vertical sides, it can be modeled simply as a cuboid (3D rectangle). The depth a water body would need to be for a direct overspray of the tested compounds at the 2007 average rate for cotton (USDA, 2008) to equal the juvenile 24 h LC50 is easily calculated. Since application rates can be converted to the units g/m2, the depth in meters can be calculated by dividing the LC50 (converted to units of g/m2) by the application rate. Lambda-cyhalothrin and Karate were the most potent insecticide assayed and Karate was applied at a rate of 0.028 lbs a.i./A in 2007. A body of water would need to be greater than 87.7 cm (95% CI: 42–267 cm) deep for Karate application and greater than 1.15 m (0.95–1.41 m) deep for λ-cyhalothrin application to ensure the concentration remains below juvenile blue crab 24 h LC50. This does not take into account any adsorption of λ-cyhalothrin to suspended particles, which would likely be significant. Leistra et al. (2004) reported 24–40% of λ-cyhalothrin remained in the water column in experimental vegetated ditches after one day. If this range of adsorption rates is taken into account our theoretical ditches would need to be greater than 21.1 to 35.5 cm deep for Karate application and greater than 27.8 to 46.4 cm deep for λ-cyhalothrin application. Since many ditches and creeks are less than 20 cm deep (Rittschol, personal observation), many direct oversprays with Karate or λ-cyhalothrin would likely kill over 50% of the blue crab juveniles in the ditch in the first 24 h. Direct overspray by imidacloprid or Trimax at the manufacturer's recommended 0.046 lbs a.i./A would require a water body deeper than 4.1 cm (3.1–5.4 cm) or 5.6 cm (4.7–6.6 cm), respectively, to maintain lower than LC50 concentrations. These depths are common in eastern NC, so direct overspray with Trimax or imidacloprid has a good chance to be acutely toxic to any blue crabs there. Aldicarb is a seed treatment and it will be off the market by the end of 2014 (Federal Register, 2010) so a direct overspray is less likely. Overspray with acephate, Orthene, or Roundup at average NC rates would require a ditch less than 1 mm deep to surpass the juvenile LC50 and so are unlikely to acutely impact blue crabs by direct overspray.

Since tidal creeks and marshes are forage areas for blue crabs as well as nursery areas for many important estuarine species, lethal

Table 2

<table>
<thead>
<tr>
<th>A.I.</th>
<th>Formulation</th>
<th>Species</th>
<th>Organism type</th>
<th>Duration</th>
<th>LC50 (μg/L)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>λ-Cyhalothrin</td>
<td>Karate</td>
<td>Streptocepalus sudanicus</td>
<td>FW fairy shrimp</td>
<td>24 h</td>
<td>0.183</td>
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<td>Matador</td>
<td>Cardina laevis</td>
<td>FW shrimp</td>
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<td>0.87</td>
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<tr>
<td>Karate</td>
<td>Daphnia magna</td>
<td>FW cladoceran</td>
<td>48 h</td>
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<td>Ceriodaphnia dubia</td>
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<td>48 h</td>
<td>0.3</td>
<td>4</td>
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</tr>
<tr>
<td>Imidacloprid</td>
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<td>FW amphipod</td>
<td>24 h</td>
<td>103</td>
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<td>FW ostracod</td>
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<td>&gt;320,000</td>
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<td>301</td>
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<tr>
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<td>Daphnia magna</td>
<td>FW cladoceran</td>
<td>48 h</td>
<td>17,360</td>
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<td>658</td>
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<td>SW brine shrimp</td>
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<td>60,122</td>
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<td>SW brine shrimp</td>
<td>24 h</td>
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<td>Methamidophos</td>
<td>Daphnia magna</td>
<td>FW cladoceran</td>
<td>24 h</td>
<td>108.7</td>
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<tr>
<td>Methamidophos</td>
<td>Penaeus stylirostris</td>
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<td>Roundup</td>
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<tr>
<td>Roundup</td>
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<td>62,000</td>
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<tr>
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<td>48 h</td>
<td>177,000</td>
<td>13</td>
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</tbody>
</table>

References: 1: Lahr et al. (2001); 2: Sucahyo et al. (2008); 3: Mokry and Hoagland (1990); 4: Ashauer et al. (2011); 5: Sánchez-Bayo and Goka (2006); 6: Song et al. (1997); 7: Barahona and Sánchez-Fortún (1999); 8: Venkateswara et al. (2007); 9: Lin et al. (2006); 10: Juezre and Sanchez (1989); 11: Folmar et al. (1979); 12: Henry et al. (1994); 13: Tsui et al. (2003).

A.I., active ingredient; FW, freshwater; SW, salt water.

62,000 μg/L (Folmar et al., 1979) and a 96 h LC50 of 720,000 μg/L (Henry et al., 1994), respectively. Tsui and Chu (2003) reported a Roundup 48 h LC50 of 177,000 μg/L for the marine copepod Acartia tonsa. Blue crab megalopae are similarly sensitive to Roundup as Daphnia magna, but juvenile blue crab sensitivity is even less than amphipods and copepods.

All tested pesticides and formulations, except aldicarb, were significantly more toxic to megalopae than to juveniles (Fig. 5). There was no statistical difference between the aldicarb 24 h LC50 values for megalopae and juvenile blue crabs. Foran et al. (1985) reported significantly greater toxicity of aldicarb to juvenile Daphnia laevis than adults. Alternatively, Barahona and Sánchez-Fortún (1999) showed decreased 24 h LC50 in 24 to 72 h old brine shrimp A. salina exposed to aldicarb. This could be more a function of the species than the toxicant since A. salina showed increased sensitivity with age to several other pesticides as well (Sánchez-Fortún et al., 1995, 1996). Pesticides like mirex and kepone have shown a decrease in toxicity with increasing ontogenetic stages in blue crabs (Bookhout and Costlow, 1975; Bookhout et al., 1980). The greatest difference between LC50 for megalopae and juvenile blue crabs was seen with imidacloprid, which was 100-fold more toxic to megalopae than juveniles. Similarly in the shrimp species, the LC50 for the shrimp Karate was 0.16. Leistra et al. (2004) reported 24–40% of λ-cyhalothrin remained in the water column in experimental vegetated ditches after one day. If this range of adsorption rates is taken into account our theoretical ditches would need to be greater than 21.1 to 35.5 cm deep for Karate application and greater than 27.8 to 46.4 cm deep for λ-cyhalothrin application. Since many ditches and creeks are less than 20 cm deep (Rittschol, personal observation), many direct oversprays with Karate or λ-cyhalothrin would likely kill over 50% of the blue crab juveniles in the ditch in the first 24 h. Direct overspray by imidacloprid or Trimax at the manufacturer's recommended 0.046 lbs a.i./A would require a water body deeper than 4.1 cm (3.1–5.4 cm) or 5.6 cm (4.7–6.6 cm), respectively, to maintain lower than LC50 concentrations. These depths are common in eastern NC, so direct overspray with Trimax or imidacloprid has a good chance to be acutely toxic to any blue crabs there. Aldicarb is a seed treatment and it will be off the market by the end of 2014 (Federal Register, 2010) so a direct overspray is less likely. Overspray with acephate, Orthene, or Roundup at average NC rates would require a ditch less than 1 mm deep to surpass the juvenile LC50 and so are unlikely to acutely impact blue crabs by direct overspray.

Since tidal creeks and marshes are forage areas for blue crabs as well as nursery areas for many important estuarine species, lethal
and sub-lethal effects here could have serious implications for the broader estuarine ecosystem. Changes to lower trophic levels will ultimately impact higher trophic levels throughout the estuary even if they are not directly influenced by the pollutants themselves. Predator–prey interactions have been shown to change in response to pollution (e.g. Brooks et al., 2009; Clements et al., 1998). Blue crabs and mummichogs (Fundulus heteroclitus) along with lower trophic level grass shrimp (Palaemonetes pugio) showed reduced densities in estuaries polluted with the organochlorine endosulfan (Scott et al., 1992). If prey populations are reduced in creeks developing blue crabs may be forced to forage in areas that are less safe from predators.

4.2. Time to metamorphosis

Blue crab megalopae can delay metamorphosis until chemical cues indicating suitable juvenile habitat are sensed (reviewed by Forward et al., 2001). Cues such as ammonium and predator odor delay metamorphosis in estuarine crabs, as do extremes in salinity and oxygen concentration (Forward et al., 1994, 1996; Welch et al., 1997). Delays of metamorphosis can result in smaller and later molting crabs with consequently reduced fitness (Gebauer et al., 1999). Others have noted increased duration of estuarine crab larval development in response to pesticides (Bookhout and Costlow, 1975; Bookhout et al., 1972, 1976, 1980), with the exception of the stone crab Menippe mercenaria treated with the chlorinated hydrocarbon mirex (Bookhout et al., 1972). The acacetol metabolite methamidophos slowed development in the shrimps Macrobrachium rosenbergii and P. stylirostris (Juarez and Sanchez, 1989). We therefore anticipated that all tested pesticides would increase TTM in blue crab megalopae. Since megalopae were collected from the field, different cohorts had different larval experiences and could have been of slightly different ages, which impact TTM, as seen in the difference between the respective controls for round 1 and 2 (Figs. 2–4). For that reason we only performed statistics within cohorts and not across cohorts.

Roundup was the only tested compound that significantly changed the rate of metamorphosis (Figs. 2 and 3). The mean TTM and the modeled ET50 of Roundup were significantly accelerated compared to estuarine water. Most other studies of crab metamorphosis used offshore seawater as the negative control. Those studies have not tested any compounds that have been able to significantly accelerate metamorphosis beyond estuarine water (positive control) (Forward et al., 1994). Roundup and its POEA surfactant alone also significantly effected TTM in the frog Rana pipiens, but in contrast, it significantly increased TTM (Howe et al., 2004). Weis and Mantel (1976) reported that fiddler crabs treated with the organochlorine insecticide DDT (1,1,1-trichloro-2,2-di-(4-chlorophenyl)ethane) increased the rate of limb regeneration and reduced the time to the next molt. They noted no such differences after treatment with the organophosphates malathion (diethyl 2-[dimethoxyphosphorothiolyl]sulfanyl]butanediato) and parathion (O,O-Diethyl O-(4-nitrophenyl) phosphorothioate), or the carbamate carbaryl (1-naphthyl methylcarbamate; Sevin®, Bayer). Weis and Mantel (1976) suggested the excitation of the central nervous system by the neurotoxic DDT played a role in the acceleration of molting in fiddler crabs. Glyphosate, however, is not a neurotoxin and so some other mechanisms are more likely to be responsible. Surfactants are detergents and can alter cell membrane and sensory cell characteristics, increase uptake of extracellular molecules (Riechers et al., 1994), and alter thresholds for neuronal vesicle binding (Zhu and Stevens, 2008). We suggest that the tallow amine surfactant, POEA, might be altering sensory cell membranes and activating the same pathway as humic acids, or allowing natural chemical cues in the estuarine seawater medium to act at lower concentrations. Future studies of the effect of surfactants, and POEA in particular, on settlement, molting, and metamorphosis are warranted.

Survival curve analysis and the ET50 modeling take into the account the megalopae that die by including them into the calculation of percentage molted until they die. This perhaps makes these two methods of analysis more suitable for time to metamorphosis assays with toxic compounds than traditional mean TTM. The inclusion of dead megalopae in the ET50 calculation is responsible for making those values lower than their respective mean TTM. Most megalopa mortality occurred at the beginning of experiments and thus any remaining successful molts accounted for an increased percentage change in the percent molted value. This lead to steeper curves than would be modeled if only the final numbers of molted juveniles were used in the calculation of percentages. Steeper lines produced lower ET50 values.

Treatment with imidacloprid and λ-cyhalothrin resulted in a significantly reduced frequency of successful molting (Fig. 4). Only 56.67% of imidacloprid and 58.62% of λ-cyhalothrin treated megalopae molted compared to all others above 80%. Both of these pesticides showed similar trends in death and molting. Most of the megalopae that died under both treatments occurred within the first two days and before any megalopae molted. The timing of metamorphosis and death under the other treatments were much more interspersed. Where imidacloprid and λ-cyhalothrin differed was survival of the molted juveniles (Fig. 4). All of the molted juveniles treated with λ-cyhalothrin were found alive while 41.18% of imidacloprid treated juveniles were found dead. Most striking was the case of aldicarb, which resulted in a molting rate of 82.14%, but 91.3% of those were found dead as juveniles. It is possible that much of the λ-cyhalothrin had become adsorbed to the glass test tube (Ali and Baugh, 2003) after 2–3 days and therefore was not bioavailable to affect molting megalopae.

Unlike other pesticides on the market, none of those tested here target ecysone, the hormone that controls molting in arthropods (Clare et al., 1992). Imidacloprid, λ-cyhalothrin, and aldicarb all affect the nervous system and induce neurotoxicity but belong to different chemical classes and operate by slightly different modes of action (Gupta et al., 2002; He et al., 2008; Kegley et al., 2011; Matsuda et al., 2001). Only aldicarb is a suspected endocrine disruptor, while the others are unknown (Kegley et al., 2011). The possible ability of aldicarb to affect both nervous and endocrine systems could be the reason it is so toxic to molting blue crabs.

Molting is an especially sensitive time for crustaceans (McCaon and Pascoe, 1988; Mortimer and Connell, 1994; Rebach and French, 1996). This makes the megalopae and juvenile stages of crustaceans particularly sensitive to environmental toxicants since they molt often. Blue crab megalopae usually molt in submerged vegetation soon after entering the estuaries using tidal stream transport (Forward et al., 1994; Welch et al., 1997). It is the juvenile stages that then disperse throughout estuaries, into tidal creeks and the upper estuary (Reyns and Eggleston, 2004). Early stage juveniles can molt as often as once a week effectively resulting in increased sensitivity to pesticide toxicity.

Lambda-cyhalothrin is the only tested compound that is not water soluble and so should have a lower risk of run-off. The adsorption and desorption of pesticides with dissolved or particulate organic matter is a complex issue controlled by several physical and chemical factors. None of those factors were explicitly tested in this project. All of the experiments presented here were performed in sediment-free environments with filtered seawater. Pesticides such as α-cyhalothrin that have low water solubility and strongly adsorb to sediment (Ali and Baugh, 2003) can still impact blue crabs and their prey. It has been shown that the concentration of freely dissolved λ-cyhalothrin in water is the fraction that is toxic (Amweg et al., 2005; Hamer et al., 1999; Maund et al., 1998). The process of adsorption can also inhibit the breakdown of the pesticide, therefore prolonging its persistence in the environment (Schwarzenbach et al., 1993). Thus creek sediments can be repositories of hydrophobic pesticides for years.
beyond their original application. Blue crab juveniles and adults bury in the sediment to hide and to prevent the growth of fouling organisms on parts of their carapace they cannot reach. Buried blue crabs and other benthic fauna are exposed to the pore water, and thus could be exposed to low pesticide concentrations for extended periods of time. The prolonged phase of desorption of pesticides into the pore water makes this habitat particularly vulnerable to chronic toxicity and sub-lethal effects. Maul et al. (2008) reported significant reductions in concentration of larval Chironomus tentans at λ- cyhalothrin concentrations 4.3 times lower than their LC50 when exposed in 10 day sediment-water experiments. Rasmussen et al. (2008) reported changed ecosystem function and decreased mobility and feeding in the amphipod *G. pulex* and the stone fly *Leuctra nigra* when treated with 0.1 μg/L λ- cyhalothrin in experimental stream channels. If blue crabs respond similarly, decreased growth could have significant reproductive and ecological effects. Future studies of λ- cyhalothrin toxicity in blue crabs should focus on sub-lethal effects of chronic low level exposures from spiked sediment.

### 5. Conclusion

Blue crabs are ecologically and economically vital estuarine species in the coastal southeastern United States and often live in shallow creeks that can experience elevated pesticide loads. The pesticides assayed here showed toxicity to blue crab megalopae and juveniles in the order of λ- cyhalothrin > imidacloprid > aldicarb > acephate with the herbicide Roundup showing low toxicity to young blue crabs similar to acephate. The potential for direct oversprays of λ- cyhalothrin and imidacloprid to reach acutely toxic concentrations in shallow tidal creeks makes these two insecticides the most dangerous to developing blue crabs. The metamorphosis-associated mortality at LC50 levels of all but λ- cyhalothrin suggests that frequently molting juvenile blue crabs are at an elevated risk of pesticide toxicity beyond what is suggested by the LC50 alone. Future studies on this subject should include evaluating the sub-lethal effects in blue crabs from exposures to environmentally-relevant concentrations of individual pesticides, pesticide mixtures, and their adjacent or surfacants. Furthermore, the role that pesticides play in the availability or toxicity of blue crab prey species should be explored.

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