The Effects of 24 Hour Exposure to Atrazine and Carbaryl on Juvenile Spotted Salamander (*Ambystoma maculatum*) Locomotive Performance

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The Effects of a 24-hour Exposure to Carbaryl or Atrazine on the Locomotive Performance of Juvenile Spotted Salamanders (*Ambystoma maculatum*)

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ABSTRACT

Amphibian ecotoxicology research has traditionally focused on chronic aquatic exposure of early life stages. However, multiple studies have shown that the survival of juvenile salamanders, frogs, and toads has the largest impact on population growth. Studies on species within the genus *Ambystoma* have shown that juvenile salamanders are the most likely of any life stage to disperse to new ponds. Because chemical contaminants are widely used throughout agricultural lands in the U.S. and have been shown to have effects on the central nervous system, it is important to determine any potential role they might have on locomotive performance. Our study sought to determine whether a 24-hour exposure to environmentally relevant concentrations of carbaryl (a neurotoxic insecticide) and atrazine (an herbicide) affected the locomotive performance of juvenile spotted salamanders (*Ambystoma maculatum*). Exposed salamanders were tested for either speed (max-burst in a U-shaped Plexiglas track) or endurance (time spent running at a constant speed on a treadmill). We found that carbaryl and atrazine treatment did not significantly affect speed or endurance ability. However, we did find that individuals exposed to carbaryl in the speed test experienced significantly increased fatigue throughout the speed trials. In addition, we found that mass to be significantly correlated with speed ability. Overall, our study suggests that a 24-hour exposure to environmentally relevant concentrations of carbaryl and atrazine does not directly significantly affect maximum burst speed or endurance ability in juvenile spotted salamanders.

INTRODUCTION

The recent disappearance and decline of many amphibian species is a phenomenon that has been well documented and a cause for concern over the past several decades—particularly because amphibians have been considered an indicator of the health of an ecosystem (Collins and Storfer, 2003). Habitat destruction, introduction of invasive species, disease, and chemical contamination of habitats have all been implicated as contributing factors in this decline (Corn, 1994; Dodd, 1997; Carey, Cohen and Rollins-Smith, 1999). Despite some evidence suggesting pesticides have contributed to these declines (Davidson et al., 2001), the role of chemical contaminants remains among the least understood. Amphibians are at particular risk to chemical contaminants due to their permeable eggs, skin, and gills (Bishop and Petit, 1992). Agricultural sites, common areas for pesticide use, have been associated with lower amphibian species richness and abundance compared to nearby non-agricultural sites (Berger, 1989; Blaustein and Kiesecker, 2002).

Amphibian ecotoxicology research has traditionally focused on chronic aquatic exposure of early life stages (Sparling et al., 2000). While there is some evidence that embryos and larvae are more sensitive than juveniles and adults (Hall and Swineford, 1980; Schuytema et al., 1991), there has been very little research on the potential effects of contaminant exposure on the terrestrial juvenile lifestage (Brühl et al., 2011). However, multiple studies have shown that the survival of juvenile salamanders, frogs, and toads has the largest impact on population growth (Biek et al., 2002; Vonesh and De la Cruz, 2002; Trenham and Shaffer, 2005). Thus, a reduction in juvenile survival or performance has the potential to impact a population more severely than increased mortality at another life stage (Biek et al., 2002).

Once aquatic larvae metamorphose into terrestrial juveniles, most of these juveniles will migrate to an area near the breeding site, reach sexual maturity, and return to breed in their natal pond. However, some juveniles will disperse into the terrestrial habitat and colonize nonnatal ponds (Semlitsch, 2008). Gamble et al. (2007) showed that an average of 9% of successfully breeding marbled salamanders (*Ambystoma opacum*) dispersed to new ponds as juveniles compared to 1.7-1.9% of experienced breeding adults. The fact that juveniles are most likely to disperse to new ponds further increases their importance to the persistence of a species, especially when habitats have become increasingly fragmented and altered (Rothermel, 2004; Semlitsch, 2008). Considering the small size of amphibians, these dispersal distances are relatively far. The juvenile marbled salamanders averaged a dispersal distance of 440 m (Gamble et al., 2007). Madison (1997) used radio telemetry to show that spotted salamanders (*Ambystoma maculatum*) overwinter an average of over 100 m from the nearest pond. Locomotive performance and dispersal ability are critical factors in influencing an individual's success in the terrestrial environment (Austin and Shaffer, 1991) and factors that affect these endpoints could have important ramifications for the population. Because chemical contaminants are widely applied across the agricultural landscape (EPA 2011), it is important to examine any direct effect an exposure might have on the locomotive capacity terrestrial juvenile amphibians.

Carbaryl (1-naphthyl-N-methyl carbamate) and atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) are two common pesticides used in the US (EPA, 2011). The 2007 EPA Pesticides Industry Sales and Usage Report (2011) estimates that 73-78 million pounds of

atrazine and 4-6 million pounds of carbaryl are used annually in the United States. Both atrazine and carbaryl are known to enter the aquatic environment through direct application or agricultural runoff (Davis et al., 2011). Because many amphibian species breed and live within or around ephemeral ponds in agricultural areas, many are vulnerable to chemical exposure (Boone et al., 2001). However, field concentrations generally do not reach concentrations great enough to cause direct mortality, emphasizing the need to study sublethal effects (Boone et al., 2001). Maximum environemental concentrations of carbaryl in wetlands are estimated to be ~4.8 mg/L (Norris et al., 1983; Peterson et al., 1994). Atrazine concentrations in the environment can reach 50 µg/L and frequently exceed 500 µg/L in small wetlands in agricultural areas—reaching levels as high as 2.3 mg/L (Solomon et al., 1996).

Both carbaryl and atrazine have been shown to affect the central nervous system of amphibians (Marian et al., 1983; Bridges, 1997; Trijasse, 1998; Sparling et al., 2001; Rohr et al., 2003; Relyea and Edwards, 2010). Carbaryl, a neurotoxic insectide, has been shown to directly inhibit acetylcholine esterase activity in frog tissues (Sparling et al., 2001) and decrease larval activity in several anuran species (Relyea and Edwards, 2010). Sublethal levels (2.0-3.5 mg/L) of carbaryl have also been shown to affect anuran feeding and swimming behavior (Marian et al., 1983; Bridges 1997). Atrazine can act as an endocrine disruptor (Hayes et al., 2002), but some evidence suggests it may disrupt the proper functioning of the central nervous system as well. Goldfish exposed to concentrations of atrazine as low as 0.5 µg/L have been shown to have significantly increased burst-swimming reactions (Trijasse, 1998). However, Allran and Karasov (2001) showed no significant difference in swimming speed of *Rana pipiens*' larvae exposed to atrazine, even at concentrations as high as 20 mg/L. Rohr et al. (2003) found that atrazine (400 µg/L) caused skittishness, or increased activity associated with disturbances in streamside salamander larvae (*Ambystoma barbouri*). Yet, little work has been done examining the effects of either pesticide on either the juvenile stage or the caudate central nervous system.

Because locomotive performance is important in juvenile dispersal, predator evasion, and prey capture (Austin and Shaffer, 1992), it is important to examine potential effects resulting from short-term contaminant exposure. The purpose of this study was to evaluate the effects of a 24-hour exposure to ecologically relevant concentrations of carbaryl (200 or 2000 µg/L) and atrazine (50 or 500 µg/L) on the endurance, speed, and fatigue of juvenile spotted salamanders (*Ambystoma maculatum*). We hypothesized that both chemicals would negatively affect locomotive performance, and that the highest concentration of carbaryl (a neurotoxin) would most negatively affect locomotive performance.

METHODS

Animals

We collected spotted salamander (*Ambystoma maculatum*) egg masses on March 10, 2011 from the Indian Creek Wildlife Preserve in Reily, Ohio (Butler County). The egg masses were transported and reared in aquatic mesocosms at Miami University's Ecological Research Center until they hatched. Larvae completed hatching on May 6, 2011 and were placed into experimental mesocosms (15 larvae/tank) on May 9, 2011. Mesocosms have proven beneficial in ecological research because they provide an experimental system that offers more control over

variables while still enabling exposure of the system to the natural environment (Rowe and Dunson 1994). Experimental mesocosms (1.85 m dia.; 1480 L vol.) were filled with 1000 L of aged tap water, 1 kg of deciduous forest leaf litter (primarily Maple [*Acer spp.*] and American Sycamore [*Platanus occidentalis*]), and plankton from natural ponds (500 mL). Ponds were covered with black screen mesh lids to minimize predation and colonization by other species. Metamorphs were collected and returned to the lab to record time at metamorphosis, mass, and SVL from June 18, 2011 to July 10, 2011. Sex could not be determined. Metamorphs were used in the speed or endurance trials within 3 days of metamorphosing and were not fed while held in the lab.

We randomly designated 100 juveniles to either speed or endurance testing to avoid overstressing the animals. Each individual was randomly assigned into one of five treatments: low (200 µg/L) carbaryl, high (2000 µg/L) carbaryl, low (50 µg/L) atrazine, high (500 µg/L) atrazine, or a water control. The concentrations chosen were within expected environmental concentrations (de Noyelles et al., 1982; Howe et al., 1998; Norris et al., 1993; Peterson et al., 1994; Solomon et al., 1996). To expose the salamanders, we used a micropipet injection of 10 mL of solution onto a dry paper towel in small shoebox containers (17 x 12 x 8.7 cm). Individuals were stored in these containers to remain exposed for 24 hours in Miami University's Animal Care Facility at 20ºC with a 12:12 photoperiod. After 24 hours of exposure, the locomotive performance of each individual was tested. Prior to testing, each individual was rinsed with room temperature distilled water (22ºC) to prevent cross contamination of the tracks.

Chemical Stock Solutions

Carbaryl stock solution was made by adding 0.8826 grams of liquid Sevin™ (22.5% carbaryl; GardenTech) to 1 L of distilled water to make an initial concentration of 0.2 g/L. This stock solution was then diluted into either 1/100 or 1/1000 by adding 10 mL or 1 mL, respectively, of stock solution to 1000 mL of water) to reach the desired concentrations of 200 and 2000 µg/L carbaryl. Atrazine stock solution was made by adding 0.1196 g of Aatrex™ (42.2% atrazine) to 1 L of water to make an initial concentration of 0.05 g/L. This stock solution was then diluted by 1/100 and 1/1000 by adding 10L or 1 mL, respectively, to reach the desired concentrations of 50 µg/L and 500 µg/L atrazine. Stock solutions were covered in foil and refrigerated. Initial experimental concentration samples were sent to Mississippi State Chemical Laboratory (Mississippi State, MS) for analysis. Analysis revealed that the stock solution contained 27.1 mg/L atrazine and 60.0 mg/L carbaryl, which would have resulted in experimental concentrations of 27.1 or 271 µg/L atrazine, or 60.9 or 609 µg/L carbaryl.

Locomotor performance

To estimate speed or "burst time," a U-shaped rectangular Plexiglas racetrack (102 x 4 x 5 cm), lined with wet paper towels, was set up in the lab at room temperature (22ºC). Tape was used to mark off a 0.25 m segment of the track. All speed trials were recorded with a Sony Handicam™ shooting at 30 frames per second. Each salamander was removed from its container and placed on one end of the track. Each salamander was then given 15 seconds to acclimate prior to testing after being placed on the track. Light touches to the tail were the applied stimulus used to promote bursts in movement. After each run, every individual was placed at the start of the track and allowed approximately 30 seconds before beginning the next run. Each individual made three runs. Using the program iMovie, we analyzed each run frame by frame and convert the

total number of frames it took to complete the run into a time value (seconds). All speed data evaluated using iMovie was done blindly. In all video recordings, only the ID number of each individual was shown to prevent any potential bias. Treatment information for each individual was stored with the same ID numbers separately. To be used for the max-burst time analysis, an individual had to complete at least one run. For individuals that completed three runs, we also evaluated the max burst time among runs to determine if metamorphs "fatigued" to different degrees among treatments.

Using a stopwatch, we measured endurance as the amount of time an individual could remain walking at a constant speed (0.025 m/s) on a custom-built, burlap-lined treadmill (dimensions: 25 x 10 cm). The burlap lining was kept moistened to prevent dehydration. Treadmill speed was kept constant at 0.025 m/s, predetermined to be a submaximal speed for spotted salamanders (Austin and Shaffer, 1992). When necessary, the salamanders were stimulated with a light tapping of the tail. Exhaustion was determined when an individual hit the back of the treadmill three times in a row despite repeated stimulation (Austin and Shaffer, 1991).

All statistical analyses were ran using the program SAS. We used analysis of variance (ANOVA) to test for difference in salamander mass at metamorphosis among pesticide treatments to ensure individuals did not vary in mass with treatment by chance. We examined how pesticide exposure affected endurance and maximum speed (burst time) using separate ANOVAs. We used a repeated-measure ANOVA to examine how maximum speed changed over repeated trials to examine if metamorphs differed in fatigue among treatments. Mass at metamorphosis was used as a covariate in all analyses because mass can influence locomotor performance. Initially, we used Dunnett's multiple comparison tests to determine if any individual treatment differed significantly from controls. In the event no individual treatment differed significantly, we used planned orthogonal comparisons to examine if (1) control versus carbaryl treatments (200 or 2000 mg/L) or (2) control versus atrazine treatments (50 or 500 mg/L) were significantly different. Additionally, we examined the correlations between metamorph mass and maximum speed, average speed, or endurance to examine how metamorph mass may influence these responses. All data were log-transformed to meet assumptions of ANOVA. Additionally, we performed a post-hoc power analysis (effect size = 1, alpha = 0.05) to examine the likelihood of finding a significant difference if it existed. We had a moderate power with 18-20 replicates (power = 0.635-0.691).

RESULTS

Statistical analysis revealed no significant differences in mass among treatments in the endurance ($P = 0.6228$; $f = .66$; treatment $df = 4$; error $df = 92$) and speed ($P = 0.1969$; $f = 1.54$, treatment $df = 4$; error $df = 90$).

Endurance was not significantly affected by carbaryl or atrazine exposure (Fig. 1). We observed high variation in average run times among all groups. Three individuals reached the previously set maximum run time of 20:00 (1 A50, 1 C200, and 1 Control). Total distance traveled ranged from 1.96-24 meters.

Max-burst time was not significantly affected by carbaryl or atrazine exposure (Fig. 2). As with our endurance trials, we observed high individual variation among groups. Individual mass ranged from 0.627-1.45 g. Burst-times ranged from 0.600-4.3667 seconds. Overall, pesticide treatment had a marginal effect ($P = 0.0617$) on speed over time. An orthogonal contrast showed no significant difference between carbaryl treatments and controls in the first and second run; however, the burst-run times between the carbaryl treatments and control were significantly slower at the third run ($P = 0.0228$) (Fig. 3).

The correlation between mass and endurance was not significant ($P = 0.0949$; $df = 1$; $r = 0.1868$; Fig. 4a) but the correlation between mass and speed was significantly correlated ($P = 0.0079$; $df = 1$; $r = 0.2144$; Fig. 4b).

DISCUSSION

Most amphibian ecotoxicological studies focus on exposure occurring in the larval environment during development. However, most amphibians spend the majority of their lives in the terrestrial environment. Determining how terrestrial exposure could influence individuals at critical life stages is paramount to evaluating the impacts of pesticides on population dynamics. The goal of this experiment was to determine whether an acute exposure to carbaryl or atrazine would significantly compromise juvenile spotted salamander locomotive performance. While we predicted that either pesticide would compromise locomotive performance, we expected carbaryl to have the strongest effect due to its nature as an acetylcholine esterase inhibitor (Sparling et al., 2001). Overall, we found that neither carbaryl or atrazine exposure had strong negative impacts on locomotor performance. However, maximum burst speed slowed over successive trials for animals that were exposed to the insecticide carbaryl, suggesting that these individuals were more fatigued.

We predicted that both pesticides would affect locomotor performance, but neither endurance nor maximum burst speed were affected. This could be because 1) terrestrial life stages are less sensitive than aquatic larval stages to pesticide exposure, 2) the exposure period was not long enough to produce an effect, or possibly because 3) spotted salamanders can detect and avoid contaminated areas. In theory, exposing terrestrial amphibians to wet paper towels simulates the natural environment, where they are more likely to absorb contaminants from the ground than they are likely to be exposed from a direct overhead spray. However, terrestrial exposures on paper towels are sometimes problematic because individuals can avoid exposure by climbing the walls, which they were frequently observed to do (MGM, personal observation). Chivers et al. (1996) showed that *Ambystoma macrodactylum* was capable of sensing chemical alarm cues of conspecifics and, subsequently, avoided areas treated with these cues. Thus, it may be possible that individuals in this study were sensing pesticide exposure and limited their exposure by climbing the walls of the containers. Using containers with the shortest possible walls would still allow for a wet paper towel exposure while potentially minimizing the variation in exposure between individuals.

Furthermore, it will be important for future studies to consider the potential effects of multiple stressors on locomotive performance. This study only examined the effects of a single pesticide per treatment. While single factor studies are important for establishing cause and effect

relationships, they may underestimate the impact since chemicals are often applied in mixtures of insecticides and herbicides in agriculture (Solomon et al., 1996). Many studies have shown that multiple stressors can produce effects not seen in single factor experiments alone (Boone et al., 2007).

Finally, previous studies have shown that, across a wide range of species, smaller individuals are at greater risk for desiccation, travel shorter distances, have lower rates of travel, and have lower stamina, than larger individuals (Goater et al., 1993; Beck and Congdon, 2000; Semlitsch, 2008). While we did not find mass to be significantly correlated with endurance, we did find a significant positive correlation between mass and max-burst speed (Fig. 1b). Because mass is often significantly, positively correlated with locomotive performance, it is possible that any larval exposure to chemical contaminants resulting in decreased mass at metamorphosis may indirectly result in compromised locomotive performance in the terrestrial juvenile stage. Therefore, the effects of pesticides on aquatic life stages may carry over into the terrestrial environment, even if effects or likelihood of terrestrial exposure is low.

For example, studies have shown that high concentrations of carbaryl nearly eliminated some larval spotted salamander communities by killing off their zooplankton prey (Boone and James, 2003; Metts et al., 2005). Zooplankton have been shown to be very sensitive to carbaryl with a 48 hr LC50 ranging from 6.4 to 13 µg/L (Mayer and Ellersieck, 1986), well within ecologically relevant concentrations (Norris et al., 1983; Peterson et al., 1994). Larvae surviving to metamorphosis in ponds exposed to carbaryl had decreased SVL and mass (Boone and James, 2003; Boone et al., 2007), which may reduce endurance and speed. Furthermore, the timing of exposure to carbaryl, and subsequent starving of larvae through prey elimination, could potentially influence juvenile terrestrial performance. Audo et al. (1995) showed that *Hyla chrysoscelis* starved early and midway through development reached the same SVL and mass at metamorphosis as controls, although at a cost of a longer development time. However, the larvae starved late in development metamorphosed at the same time as controls, presumably because they had met the minimum mass to metamorphose, and were 55% the mass of controls, 85% the length, and suffered mortality rates fifteen times greater than controls. Thus, a late larval exposure to carbaryl wiping out the zooplankton prey population may stress larval spotted salamanders enough to stimulate metamorphosis at the minimum required size. This would then result in smaller terrestrial juveniles with reduced locomotive capacity.

CONCLUSIONS

We found a single 24 hour exposure of juvenile spotted salamanders to carbaryl significantly increased fatigue, but exposure to carbaryl or atrazine had no significant effect on endurance or max-burst speed. This study suggests that some pesticides, such as atrazine, may not significantly affect locomotive performance in terrestrial juvenile salamanders while insecticides, such as carbaryl, may need further study. Because the juvenile stage is a critical dispersing stage and often has the biggest impact on population growth, more studies are needed to evaluate the potential direct effects of individual pesticides and multiple stressors on locomotive performance in the juvenile stage. In addition, because numerous studies have shown that pesticides can result in smaller sizes at metamorphosis and because there is a significant correlation between mass and locomotive performance, it would be useful to study the potential impacts larval exposure to contaminants might have on locomotive performance in the juvenile terrestrial state.

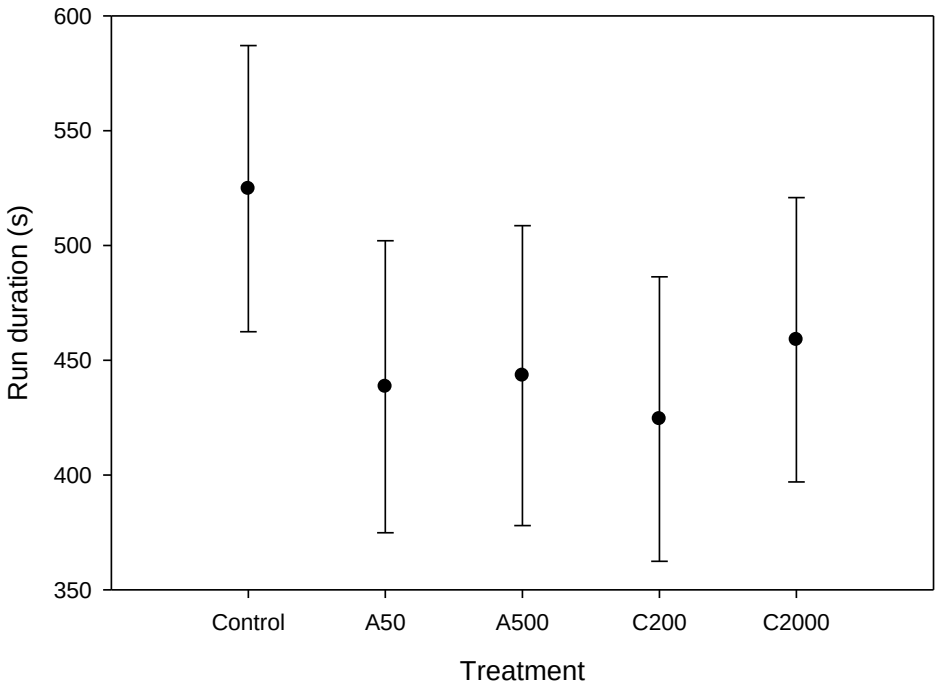
Figure Legends

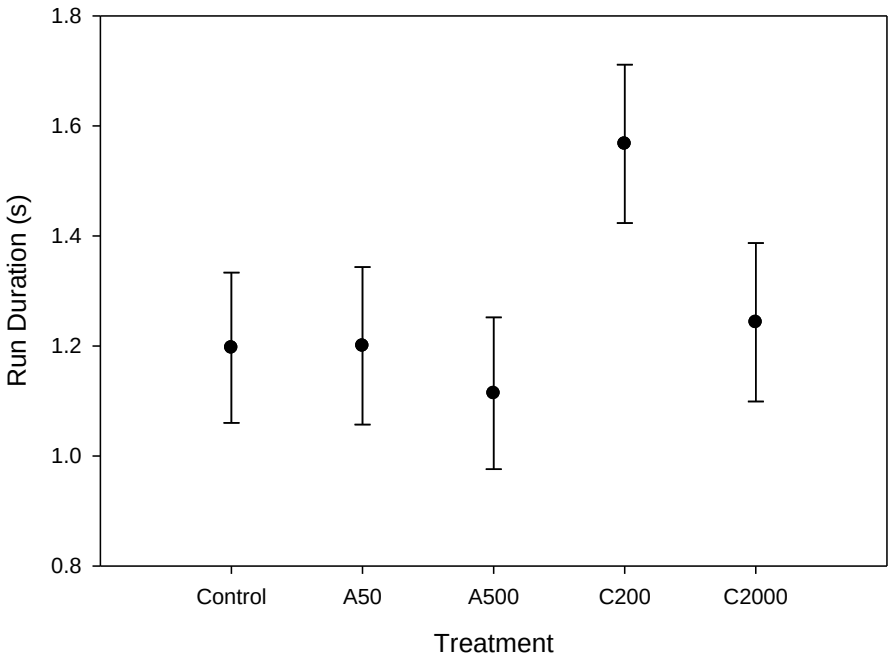
Figure 1. Endurance run times across treatments. Error bars represent ± 1 SE. A50 = atrazine (50 µg/L); A500 = atrazine (500 µg/L); C200 = carbaryl (200 µg/L); C2000 = carbaryl (2000 µg/L).

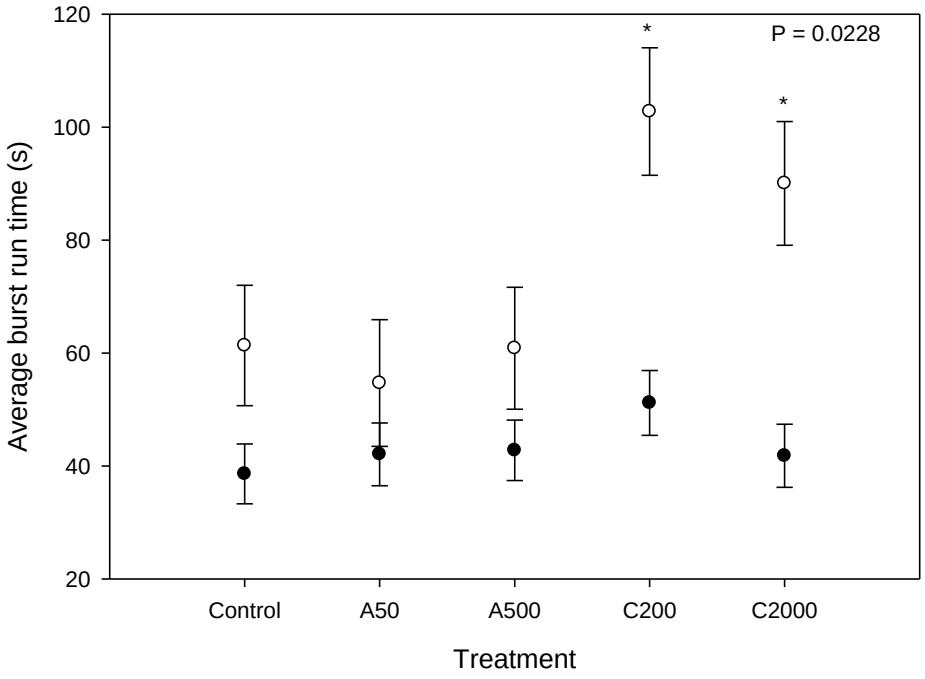
Figure 2. Max burst time across treatment. Error bars represent ± 1 SE. A50 = atrazine (50 µg/L); A500 = atrazine (500 µg/L); C200 = carbaryl (200 µg/L); C2000 = carbaryl (2000 µg/L).

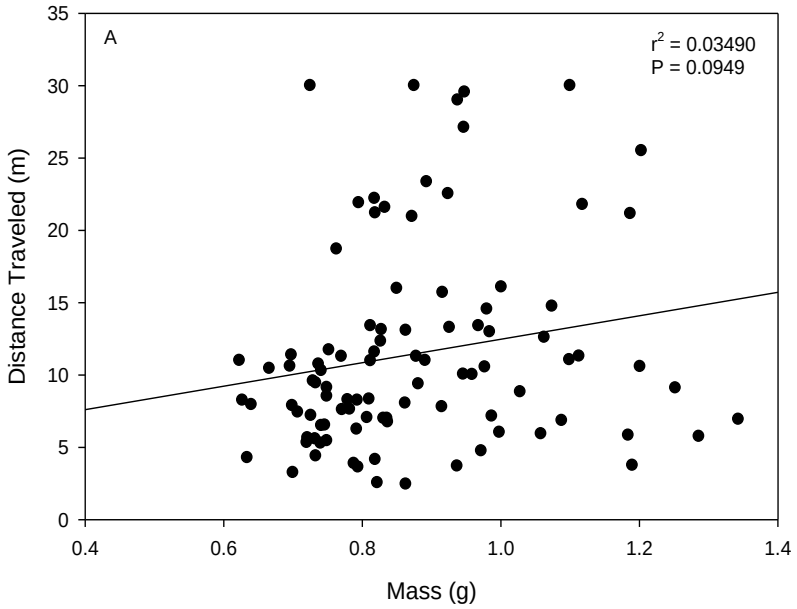
Figure 3. Average burst run times across treatments. Dark circles represent the summed treatment times for the first of three speed trials. Open circles represent the third of three speed trials. Error bars represent ± 1 SE. The *p* value represents the contrast of the combined carbaryl treatments vs. control via an orthogonal contrast. A50 = atrazine (50 µg/L); A500 = atrazine (500 µg/L); C200 = carbaryl (200 µg/L); C2000 = carbaryl (2000 µg/L). Significant results are noted with an asterisk.

Fig. 4. Relationship between mass at metamorphosis on (A) endurance and (B) max burst time.


600
550
500
450
400
350
Run duration (s)
Control
A50
A500
C200
C2000
Treatment


1.8
1.6
1.4
1.2
1.0
0.8
Run Duration (s)
Control
A50
A500
C200
C2000
Treatment


120
100
80
60
40
20
Average burst run time (s)
P = 0.0228
*
*
Control
A50
A500
C200
C2000
Treatment


A
r^2 = 0.03490
P = 0.0949
Distance Traveled (m)
Mass (g)
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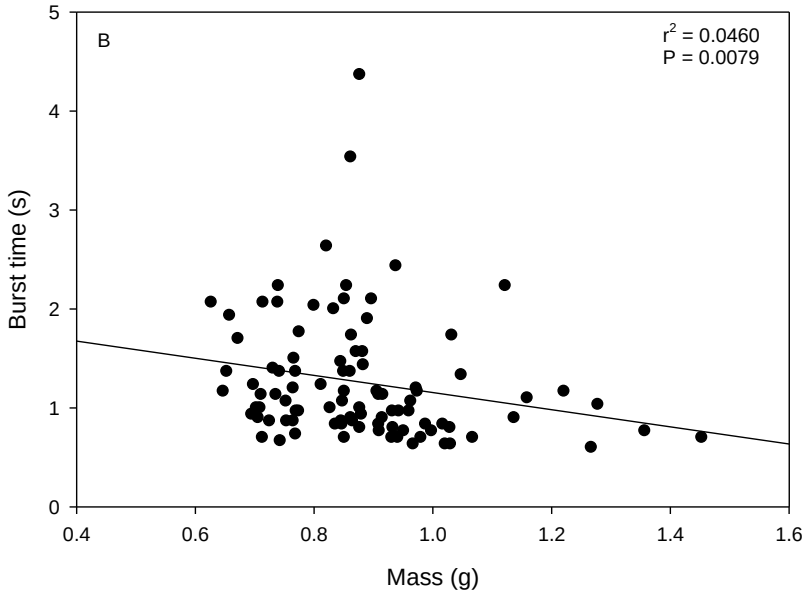
5
10
15
20
25
30
35
0.4
0.6
0.8
1.0
1.2
1.4


B
r^2 = 0.0460
P = 0.0079
Burst time (s)
Mass (g)
0
1
2
3
4
5
0.4
0.6
0.8
1.0
1.2
1.4
1.6

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