

THE EFFECTS OF 2, 4 –DICHLOROPHENOXYACETIC ACID ON SWIM
PERFORMANCE IN LARVAL LONG-TOED SALAMANDERS (AMBYSTOMA
MACRODACTYLUM)

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of
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Abstract

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The effects of sublethal amounts of herbicides on amphibians is poorly understood yet may contribute to amphibian population declines. In this study we tested the effects of environmentally relevant amounts of the herbicide 2, 4-Dichlorophenoxyacetic acid (2, 4-D) on sprint swim performance of a larval salamander native to the Palouse, *Ambystoma macrodactylum*. Larvae were collected from five different ponds which had endogenous levels of 2, 4-D ranging from 0 ppm to 0.0034 ppm. In the laboratory, larvae were exposed to one of 6 treatments of 2, 4-D: 18 ppm, 1.8 ppm, 0.18 ppm, 0.018 ppm, 0.0018 ppm and a control. Swim performance, body mass and snout-vent length were measured on each individual four times between 24 and 334 hours after exposure. Although we hypothesized an effect of 2, 4-D treatment on salamander swim performance, our results were not significant; exposure to 2, 4-D in the

lab did not affect swim speed or body mass. However, there was a significant effect of pond on swim speed and body mass: salamanders from ponds with lower endogenous levels of 2, 4-D were larger and had faster swim speeds. This may have been caused by the differing amounts of 2, 4-D found naturally in the ponds, although other environmental or genetic factors can not currently be ruled out. Other studies indicate that amphibian eggs or hatchlings exposure to herbicides or pesticides can negatively impact individuals as they grow and develop. Future research on effects of 2, 4-D on *Ambystoma macrodactylum* should make use of common garden experiments on eggs and hatchlings to identify the underlying causes of the results reported herein.

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INTRODUCTION

Global amphibian declines continue to be reported (Jung and Jagoe, 1995, Hayes, 2004, Relyea, 2004). Causes for these declines that are well understood include habitat destruction, invasive species, and overexploitation (Davidson et al., 2002, Carey, 2000, Boyer and Grue, 1995). Causes for declines that are not clearly understood include global climate change, emerging diseases, and environmental contaminants (Davidson et al., 2002, Carey, 2000). Pollution may play a crucial role in survival for amphibians. Amphibians can absorb chemicals cutaneously, making them more sensitive to many environmental perturbations (global warming, UV radiation, etc.). The ability to avoid predators, forage and the overall fitness of many species may be compromised by contaminants and a decrease in fitness would likely result in population declines. There have been recorded declines in Southeast Asia of a caecilian species (*Ichthyophis glandulosus*) caused by soil pollution (Gower and Wilkinson, 2005). Pesticide runoff can affect small watersheds where larvae are found. Bank et al. (2006) found a decline in dusky salamanders (*Desmognathus fuscus fuscus*) most likely caused by extensive pollution, including mercury accumulation in surface waters for this stream dwelling salamander. Finally, research done by Sanzus & Hecnar (2006) showed that Wood frogs (*Rana sylvatica*), when exposed to NaCl (road salt used for de-icing), exhibited a decrease in weight and activity and displayed physical abnormalities.

Chemical contaminants have been shown to have a wide range of physiological effects on amphibians. Hayes et al. (2002) found that the pesticide atrazine hindered gonadal growth and observed hermaphroditism in animals collected from atrazine polluted

locations around the country. When exposed to sublethal amounts of Carbaryl, Bridges (1997) found that sprint speed and activity in the leopard frog (*Rana pipiens*) were significantly reduced. Furthermore, a study done by Savage et al. (2002) found sublethal effects of polychlorinated biphenyls (PCB) on the Wood frog (*Rana sylvatica*), including behavioral abnormalities and decreased swim performance. Finally, after exposing adult male European common frogs (*Rana temporaria*) to DDT, Leiva-Presa & Jensen (2006) found a distinct interruption in hepatic metabolism.

Effects can also vary from species to species. In experiments done by Berrill et al. (1995), exposure of the spotted salamander (*Ambystoma maculatum*) to 2 ppm of the insecticide fenitrothion caused paralysis; a second exposure caused nearly a 100% mortality rate. However, the same exposure in wood and leopard frogs (*R. sylvatica* and *R. pipiens*, respectively) gave no indication of negative effects. Contaminants can have different harmful effects on amphibians; some may increase activity thereby likely increasing predation rates, whereas others may decrease activity and, in turn, decrease foraging rates (Bridges, 1999).

In southeastern Washington the primary crop is wheat and 2, 4-dichlorophenoxyacetic acid (2, 4-D) is sprayed as an herbicide year round. The ester of 2, 4-D was used as the primary component of Agent Orange, a defoliant used during the Vietnam War (Stellman et. al., 2003). Plant roots absorb the salts and plant leaves absorb the ester, where the active ingredient acts as a growth inhibitor by mimicking the plant hormone auxin (Tomlin, 2003). 2, 4-D can be found in commercial products to control weeds including Weedtrine II, Aqua Kleen, Barrage, Plantgard and Lawn-Keep (WSDA,

2004). Currently, 2, 4-D is one of the most commonly used herbicides for weed control and comes in two forms; an ester form and an amine form (Tomlin, 2003).

The 2, 4-D residue found in aquatic environments is believed to be from runoff; also, when aerial spraying is being employed there can be direct contact with the water and aquatic organisms (Castillo et al., 2006). 2, 4-D can have effects on aquatic organisms that range from loss of equilibrium to hemorrhaging from the body (Gomez et al., 1998). Catfish exposed to sublethal amounts of 2, 4-D show changes in their red blood cells (which are common of 2, 4-D exposure effects) (Gomez et al., 1998). Neurological effects of 2, 4-D have been observed in aquatic organisms such as fish. These can include abnormal swimming (perpendicular and downward), jumping, and abrupt startlings from the water (Sarıkaya and Yılmaz, 2003). Farah et al. (2004) found that mosquito larvae, *Culex pipiens fatigans*, exposed to 2, 4-D showed trouble respiring, in turn causing them to gather at the top of the water and in time they couldn't move upright. In the same experiment, Farah et al. (2004) found that exposure to 2, 4-D caused twitching of the body and muscle spasms. Furthermore, in a study done by Little et al. (1990) using rainbow trout (*Oncorhynchus mykiss*) 2, 4-D caused significant increases in swim performance at low dosages and significant decreases in swim performances at high dosages. The LC50 (the lowest concentration of a contaminant that causes 50% mortality) of 2, 4-D ranges between 1.0 and 100 mg/L in cutthroat trout, depending on the formulation used (WSDA, 2004). A study done by Morgan et al. (1996) indicates that the LC50 level in the African clawed frog (*Xenopus laevis*) is 270 mg/L. In birds, the LC50 ranges from 668 mg/L to 1000 mg/L in mallards and pigeons, respectively (WSDA, 2004).

Burst swimming performance is a component of Darwinian fitness (Watkins, 1996) and is a critical factor in the capture of prey, predator avoidance, social interactions and responses to the environment (Shaffer, 1991). Therefore, amphibian larval swimming performance is a key characteristic for survival. In their most vulnerable state, amphibian larvae are heavily preyed upon and so the ability to escape quickly is important. For example, Watkins (1996) found in Pacific Tree frog (*Pseudacris regilla*) tadpoles that burst swim performance was significantly correlated with survival within a population because predation levels can be as high as 90% and they lack an exterior defense. Selection should favor more rapid swimming in the early life stages because of the high levels of predation encountered (Azizi, 2002).

Swimming performance is strongly influenced by body size and shape, so that larvae that develop short, deep tails and elongated bodies have a higher rate of survival (Van Buskirk & McCollum, 2000b). In burst swimming trials of the striped marsh frog (*Limnodynastes peronii*), Wilson & Franklin (2000) examined the relationship between body length and burst swim, and found that burst swim performance was strongly dependent upon body length. Hence, body size and shape are important determinants of swim performance.

Larval swimming performance is negatively affected by pesticides (Savage, 2002, Bridges, 1999, Bridges, 1997). Bridges (1999) found that when the grey tree frog tadpoles (*Hyla versicolor*) were exposed to sublethal concentrations of Carbaryl, they reduced their activity by 50%. In addition, Bridges (1997) found that leopard frog (*Rana blairi*) tadpoles swim performance was significantly reduced when exposed to sublethal Carbaryl concentrations. Effects of pesticides also lower fitness associated with body

mass, ability to reach maximum growth rate, swim performance and/or risk of predation (Jung and Jague, 1995). Many pesticides have been shown to induce a lack of predator avoidance. Prodding a tadpole is considered to be an effective way to induce an avoidance response; Berrill et al (1994) showed that 3 tadpole species (*R. clamitans*, *R. sylvatica*, and *R. pipiens*) exposed to 1.2 ppm of the herbicide tryclopyr all showed no response to prodding, indicating a lack of avoidance response (Berrill, 1994). When studying the sublethal effects of endosulfan, Berrill et al. (1998) found that tadpoles of wood frogs, American toads, and green frogs (*R. sylvatica*, *B. americanus*, and *R. clamitans*, respectively) showed a significant reduction in total body length. Furthermore, when exposed to 8 ppm of fenitrothion bullfrogs (*R. catesbeiana*) were significantly smaller than controls (Berrill et al, 1994).

The western long toed salamander (*Ambystoma macrodactylum*) is a native amphibian to Washington likely to be affected by 2, 4-D. Pesticide residue is found in aquatic habitats of the long-toed salamander across the Palouse (bioregion of Southeast Washington and Northwest Idaho) because of runoff from aerial spraying of wheat (Castillo et al., 2006, Berenzen et al., 2005, Jergentz et al., 2005, Leight et al., 2005, Palma et al., 2004). The purpose of this study was to test for immediate and long term effects of environmentally relevant levels of 2, 4-D on an important component of fitness, swimming performance, in larval *Ambystoma macrodactylum*. We hypothesized that there would be a significant decrease in swim performance after exposure to the chemical and that this effect would continue for several weeks after a single bout of exposure.

METHODS

Collecting:

Salamanders were collected from five different ponds in the vicinity of Pullman, WA and Moscow, ID in 2006. Pullman, Washington, and Moscow, Idaho, are located approximately 8 miles from one another across the state border. Pond 3 (Campus) is on Washington State University campus, off Forest Way behind Chinook Village housing in Pullman, WA (Approximately at Lat. 46.83875958, Long. -116.98029323). Pond 4 (Rt. 27) is located ~7 miles North at 8232 State Route 27 in Pullman, WA (Lat. 46.80627837, Long. -117.11485837). Pond 5 (Smoot Hill) is located at 4951 Shawnee Road ~ 4 miles North of Albion, WA (Approximately Lat. 46.77354049, Long. -117.2501125). Pond 6 (Hess) is located at 3109 West Twin Road approximately ~5 miles East of Moscow, ID (Lat. 46.77354049, Long. -116.95471515). And Pond 7 (Hardesty) is located at 1250 Four Mile Road, Viola, ID ~ 9 miles Northeast of Moscow, ID (Lat. 46.83875958, Long. -116.98029323). The ponds were chosen based on the abundance of salamanders (personal observation) and permission of the landowners. Salamanders were collected beginning in late May, when all larvae were within 0.5 inch in size to one another. Salamanders were collected over a 5 day period using minnow traps, dip nets and seine nets until approximately 130 individuals had been collected from each of the 5 ponds.

Housing and Caring of Animals:

All experiments were conducted in the School of Biological Sciences at Washington State University under Animal Care and Use Committee approved protocol (LARC #3489). Before swimming and exposure to herbicide, animals were kept in a cooler and allowed to acclimate for 7 days in an environmental chamber set at 12°C.

After 7 days all salamanders were assigned an individual 6 digit ID number that also defined what block and pond they were from and what treatment they would receive. While being introduced to the herbicide, animals shared 9 x 4.5 x 4.5 plastic containers divided by non-toxic aquarium sealant and paper clipped fiberglass netting allowing 2 salamanders per container, giving a total of 300 containers and 600 salamanders. The cold room in which the animals were stored was set on a light timer at 12:12 L: D photoperiod. Animals were fed trout chow ad libitum. Larvae were not fed during the days of experimental treatment, as detailed in Bridges, (1997). Because of possible positioning effects, containers were randomly moved each day of the experiment. Dead larvae when discovered were removed on a daily basis.

Experimental Design:

One hundred and twenty Salamanders were collected from each of five different ponds for a total of 600 individuals. Twenty individuals from each pond were randomly assigned to one of six treatment groups. Each treatment group was randomly divided into four measurement blocks so that there were 150 individuals per block. Individuals in measurement Block I had swimming speed measured on experimental days 1, 3, 11, and 13. Individuals in measurement Block II had swimming speed measured on experimental days 2, 4, 12, and 14. Individuals in measurement Block III had swimming speed measured on experimental days 6, 8, 16, and 18. Individuals in measurement Block IV had swimming speed measured on experimental days 7, 9, 17, and 19.

Block I and Block II were exposed to the herbicide on the same day (day 0), but because of time constraints we could only swim one block per day. Block I was swum on day 1 (24 hrs after exposure), day 3 (72 hrs after exposure), day 11 (264 hrs after

exposure) and day 13 (310 hrs after exposure). Block II was swum on day 2 (48 hrs after exposure), day 4 (96 hrs after exposure), day 12 (286 hrs after exposure), and day 14 (334 hrs after exposure). On day 5, Block III and Block IV were exposed to the herbicide. Block III was swum on day 6 (24 hrs after exposure), day 8 (72 hrs after exposure), day 16 (264 hrs after exposure), and day 18 (310 hrs after exposure) and Block IV was swum on day 7 (48 hrs after exposure), day 9 (96 hrs after exposure), day 17 (286 hrs after exposure), and day 19 (334 hrs after exposure). In summary, Blocks I and III were swum at 24, 72, 264, and 310 hours after exposure, whereas, blocks 2 and 4 were swum at 48, 96, 286, and 334 hours after exposure.

Herbicide Application:

2, 4-D was obtained from Sigma (#064K0011, St. Louis, MO). There were six treatments, including a control. Information obtained through Washington State Agricultural Statistics for 2004 stated that 0.49 kg of 2, 4-D was sprayed per acre on spring wheat in the Palouse region, which would equal 0.18 ppm when applied to an acre of water 0.5 meter deep. No vehicle was used in the application of 2, 4-D on spring wheat. Experimental treatment levels chosen were 0.0018, 0.018, 0.18, 1.8, 18 ppm, which bracketed the amount actually applied to spring wheat. The solubility of 2, 4-D in water is low; therefore a stock solution was prepared by dissolving the desired amount of 2, 4-D in ethanol which was then diluted to the desired concentration with deionized water. Solutions were made from deionized water instead of pond water because water toxicology analysis showed differing levels of 2, 4-D already present in most ponds. Prior to exposure of the herbicidal solution, a pilot study measured no effects of ethanol and deionized water on salamander larvae swim performance.

Pond water analysis:

Water was sampled from each pond and taken to the Holm research center (University of Idaho, Moscow, ID) and analyzed for 2, 4-D and 24 other chemicals. All chemicals other than 2, 4-D and bromoxynil were not detected (ND).

Swimming Tunnel Measurements:

Burst swimming speeds were measured using a phototransistor swimming tunnel made of clear Plexiglass (based on Nelson et al., 2002). The swimming tunnel's measurements were 100 cm long x 10 cm wide x 13 cm high. Another piece of Plexiglass was placed lengthwise down in the middle so that the width was reduced to 5 cm. There was a gate that established a 4 x 5 area where a larva was placed prior to swimming. A continuous electrical current powered three laser pointers which created sets of light beams that the larva had to pass through. Because the laser light had to be dispersed, a 0.25 inch diameter glass rod was used to disperse the laser beam (Reinbold, 2005, M.S. Thesis). Each bank of lasers passed a vertical plane of light through the tunnel. Twelve phototransistors were set up on the opposite side making an upright 'bank' adjacent to the laser light beams. A 9 volt battery was used to provide a continuous charge across each bank of phototransistors. Each bank of lights was positioned 10.2 centimeters apart. When an animal broke a beam for a given bank it changed the voltage of the phototransistor, which was recorded by a Sable data acquisition system (200 samples sec⁻¹) with a time stamp.

Larvae were individually placed in the tunnel in the gated area, closed prior to measurements. When the system was enabled, the gate was lifted and the larva was stimulated to swim the length of the tunnel by prodding its tail base with a pipette. Burst swimming was recorded for a maximum of five times per larvae on succession on a given day. Some larvae could not be swum 5 times due to lack of ability to swim. After each swim trial, individuals were towel dried, weighed to the nearest milligram, and measured for snout-vent length and head-tail length to the nearest millimeter.

Statistical Analysis:

Trials that consisted of unrealistic measurements were discarded, then individual swim performances were totaled and the mean used for the statistical analysis. A regression analysis was used in SAS to analyze the data, using proc mixed with repeated measures and Tukey-Kramer contrast analysis. The SAS model used was:

mean swim speed = day of measure + 2, 4-D treatment + block + pond + mass + (day of measure) (2, 4-D treatment) + (day of measure) (pond) + (day of measure) (block).

RESULTS

Table 1 shows the results of toxicological analyses conducted by the University of Idaho (Moscow, ID) and gives the initial levels of 2, 4-D in the experimental ponds. 2, 4-D was absent from Pond 7, and Pond 5 had levels less than 0.001 ppm. Ponds 3, 4, and 6 had levels equal to or greater than 0.001 ppm.

There was no significant effect of 2, 4-D treatment (P= 0.9366; Table 2) on *Ambystoma macrodactylum* larvae's swim performance (Fig.1). There was also no significant effect of treatment by day (P= 0.1756; Table 2) on salamander larvae swim

performance. Therefore, no significant differences in the position or the shape of the swimming performance curves were detected among 2, 4-D treatments (Fig. 1).

There was a significant effect of pond on swim performance ($P < 0.0001$; Table 2), indicating significant differences in the position of the curves (Fig. 2). Individuals from ponds 5 and 7 were always faster swimmers than other larvae from other ponds. This could be because ponds 5 and 7 had the lowest amount of 2, 4-D, at less than 0.001 and 0.0 ppm, respectively (Table 1). All other ponds had recorded levels of 2, 4-D in amounts greater than 0.001 ppm. There was also wide variation in the shape of performance curves from all ponds, leading to a significant interaction between pond and day ($P = 0.0006$; Fig. 2). As can be seen in Figures 1 and 2, a significant effect of day ($P < 0.0001$; Table 2) was detected; with successive days of swimming, salamanders' swimming speeds increased.

There was a significant block effect ($P = 0.0091$) and a significant interaction of block by day ($P = 0.0002$). This was likely caused by differences in time of exposure before blocks were swum. Regardless, because block was used as a cofactor in the statistical analysis and individuals were randomized across block, block effects were statistically removed and therefore do not impact other results.

As expected, mass had a significant effect on speed ($P < 0.0001$, Table 2) with bigger tadpoles swimming faster. Body mass was also analyzed as a dependent variable (Table 3 and Fig. 3). Body mass was significantly different among ponds, with individuals from pond 7, where 2, 4-D was absent, considerably larger than other pond larvae. Surprisingly, the results reveal an interaction between pond and day, demonstrating a significant difference in the shapes of the curves (Fig. 3). Not

surprisingly, mass decreased across days as the animals progressed toward metamorphosis.

DISCUSSION

Although we hypothesized an effect of 2, 4-D on salamander swim performance, our results were not significant; exposure to 2, 4-D in the lab did not affect swim speed. 2, 4-D has been known to cause nervous system damage in fish and mammals within 24 hours of exposure; Gomez et al.(1998) observed in fish exposed to 400 mg/L, half of the LC50 dose, a loss of equilibrium, erratic swimming behavior and hemorrhaging from the fins and the cloaca, effects that were seen throughout the remaining 11 days of the experiment. In contrast to this, Relyea (2005) found that there was no effect of 0.12 mg/L of 2, 4-D, applied at the manufacturers' suggested maximum rates, on periphyton, zooplankton, predator richness, or biomass when exposed in a mesocosm over a 13 day period and actually increased the survival of a dragonfly species (*Neonecta undulate*) and the spotted salamander (*Ambystoma maculatum*). Relyea's results correspond with our results more closely, probably because of the similar levels of 2, 4-D used, but should be pointed out that Gomez used exposure amounts half of the LC50 dosage for his study organism. Butler and Verrell (2005) hypothesized that because 2, 4-D was present in WEED B GON, an herbicide formulation, it would be toxic to earthworms at 96 hours of exposure, but found no negative affects either.

Although our results showed no effect of 2, 4-D treatment in the lab, there could be differences in swimming performance caused by the varying levels of 2, 4-D found

among ponds. Salamander larvae exposed to higher amounts of 2, 4-D in their natural ponds were poorer swimmers regardless of treatment levels they were exposed to in the lab. This apparently is a threshold effect; if salamanders were exposed to levels higher than 0.001 ppm swimming performance is impaired. If this is the case, then it would seem effects of 2, 4- D have a greater impact on egg masses or hatchlings than in later larvae stages.

Other work has shown the vulnerability of eggs or hatchlings to pesticide exposure. Kadokami et al. (2004) found that two-thirds of contaminants found in female frogs were also detected in their egg masses, and previous research has shown similar negative effects on amphibian egg masses. Permethrin and Endosulfan are insecticides that are neurotoxic. Berrill et al. (1993) found that embryos and tadpoles exposed to sublethal concentrations of permethrin displayed decreased growth rate, and when embryos of wood frogs (*Rana sylvatica*) were exposed to sublethal concentrations of endosulfan (0.28 mg/L), 30% showed a reduction in predator avoidance behavior (Berrill, 1998). Permethrin caused frog limb deformities at exposure levels of 0.0001 mg/L and has been found in amphibian embryos in previous studies (Greulich and Pflugmacher, 2004). In a study by Rohr et al. (2003), permethrin decreased embryo survivability and caused respiratory distress in the streamside salamander (*Ambystoma barbouri*). Both endosulfan and permethrin are insecticides that are considered to be neurotoxic. Berrill et al. (1993) found that sublethal concentrations of permethrin caused abnormalities in newly hatched tadpoles that were exposed as embryos, including twisting and jerking when prodded.

An additional factor that pesticides could affect is growth rate and size. Ponds that were more heavily contaminated with 2, 4-D produced smaller larvae (Fig. 3). As *Ambystoma macrodactylum*'s mass increased their swim speed increased (Fig. 3) and body size has been directly correlated with swim performance in other studies (Van Buskirk & McCollum, 2000a, Wilson & Franklin, 2000). Berrill et al. (1993) found a decrease in feeding behavior in tadpoles that were exposed to 0.05 and 0.1 ppm of permethrin (Berrill et al., 1993). A reduction in feeding can lead to slower growth rate and in turn be associated with lowered reproductive success. Furthermore, if pesticides are interfering with larvae's ability to forage it can lead to an overall smaller size resulting in slower swimming. Hence, the decreased swim speeds among ponds measured could be caused by reduced growth and size.

It is also possible that other unmeasured environmental or genetic factors might have contributed to the measured differences in swim performance among ponds. For example, the ponds may differ in predator density, availability of food or temperature that could result in differences in swim speed among larvae from those ponds. There is also the possibility that genetic variation among ponds caused the differences in swim speeds; Scribner et al (2001) reported that genetic variation among populations is highly associated with fitness traits. Since the ponds used in this study are separated by many miles and are nearby to fragmented agricultural landscapes, dispersal may be low and might result in high genetic variance among ponds. Additional studies are required to determine whether 2, 4-D or some other pond-specific factor is significantly influencing swimming speed and body mass.

In summary, our results suggest that swimming performance in larval long-toed salamanders is sensitive to environmentally relevant exposures of 2, 4-D but only at certain developmental stages; however, this currently can not be distinguished from other possible environmental or genetic differences among populations. Ponds with less than 0.001 ppm of 2, 4-D produced individuals that swam faster and were larger. Swim performance is a direct measure of fitness since it aides in escape performance and prey capture (Wilson & Franklin, 1999), so that being a poorer swimmer may lead to a reduction in predator avoidance. Future studies should focus on partitioning effects of 2, 4-D exposure from possible genetic differences among populations. A common garden breeding study in the lab should result in more conclusive results on the effects of 2, 4-D and show genetic differences among salamander populations. Finally, this study highlights the importance of measuring relevant variables, such as endogenous levels of 2, 4-D, in amphibian populations that are collected for laboratory toxicology trials.

Figure Legends

Figure 1 – *Ambystoma macrodactylum*'s mean swimming speeds plotted across days of swim in 5 treatment levels. Points are means of ≤ 5 swim trials taken from each individual. Vertical lines span one standard error on either side of the mean. Values presented are mean \pm 95% confidence interval (CI). Day of swim labeled at 1, 3, 11, and 13 because all blocks were swum between same day intervals (2, 8, and 2 days between swims).

Figure 2 – *Ambystoma macrodactylum*'s mean swimming speeds plotted across days of swim in the 5 experimental ponds. Points are means of ≤ 5 swim trials taken from each individual. Vertical lines span one standard error on either side of the mean. Values presented are mean \pm 95% confidence interval (CI). Day of swim labeled at 1, 3, 11, and 13 because all blocks were swum between same day intervals (2, 8, and 2 days between swims).

Figure 3 – *Ambystoma macrodactylum*'s mass in grams plotted across days of swim in the 5 experimental ponds. Points are means of ≤ 5 measurements taken from each individual. Vertical lines span one standard error on either side of the mean. Values presented are mean \pm 95% confidence interval (CI). Day of swim labeled at 1, 3, 11, and 13 because all blocks were swum between same day intervals (2, 8, and 2 days between swims).

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Table 1. Natural Levels (ppm) of Herbicides in Experimental Ponds

Experimental Ponds	2, 4-D	Bromoxynil
Pond 3	0.001	<0.0001
Pond 4	0.0012	<0.0001
Pond 5	0.00059	0
Pond 6	0.0034	<0.0001
Pond 7	0	<0.0001

Table 2- Analysis of Variance on the effects of 2, 4-D on burst swim performance of *Ambystoma macrodactylum*

Table 1. Effect	Num DF	F Value	P Value
Day	3	18.55	<0.0001
Treat	5	0.26	0.9366
Block	3	3.87	0.0091
Pond	4	7.01	<0.0001
Treat*Day	15	1.33	0.1756
Pond*Day	12	2.91	0.0006
Block*Day	9	3.62	0.0002
Mass	1	59.67	<0.0001

Table 3. Analysis of Variance on the effects of 2, 4-D of mass on burst swim performance of *Ambystoma macrodactylum*

Effect	Num DF	F Value	P Value
Day	3	26.94	<0.0001
Treat	5	0.76	0.5819
Block	3	2.03	0.1085
Pond	4	79.68	<0.0001
Treat*Day	15	1.41	0.1335
Pond*Day	12	2.66	0.0016
Block*Day	9	1.09	0.3655

Figure1.

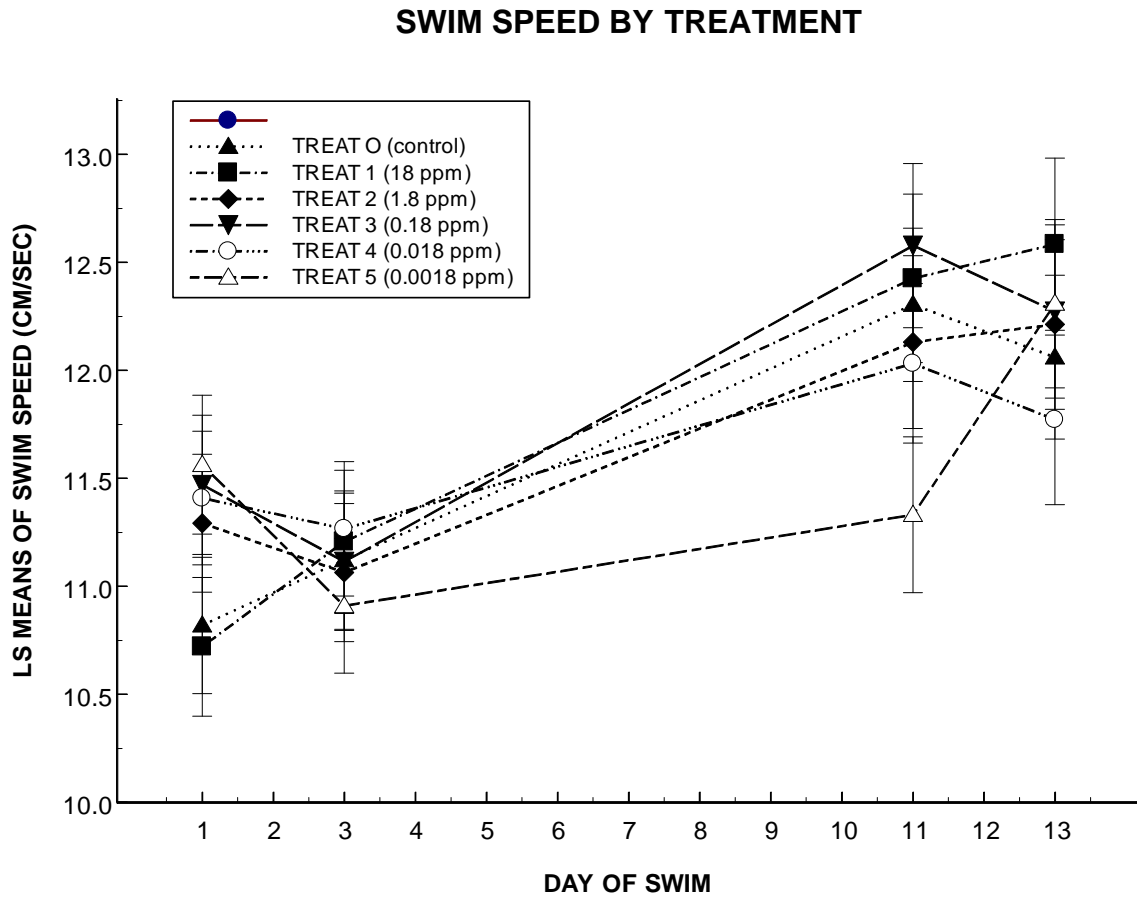


FIGURE 2.

SWIM SPEED BY POND

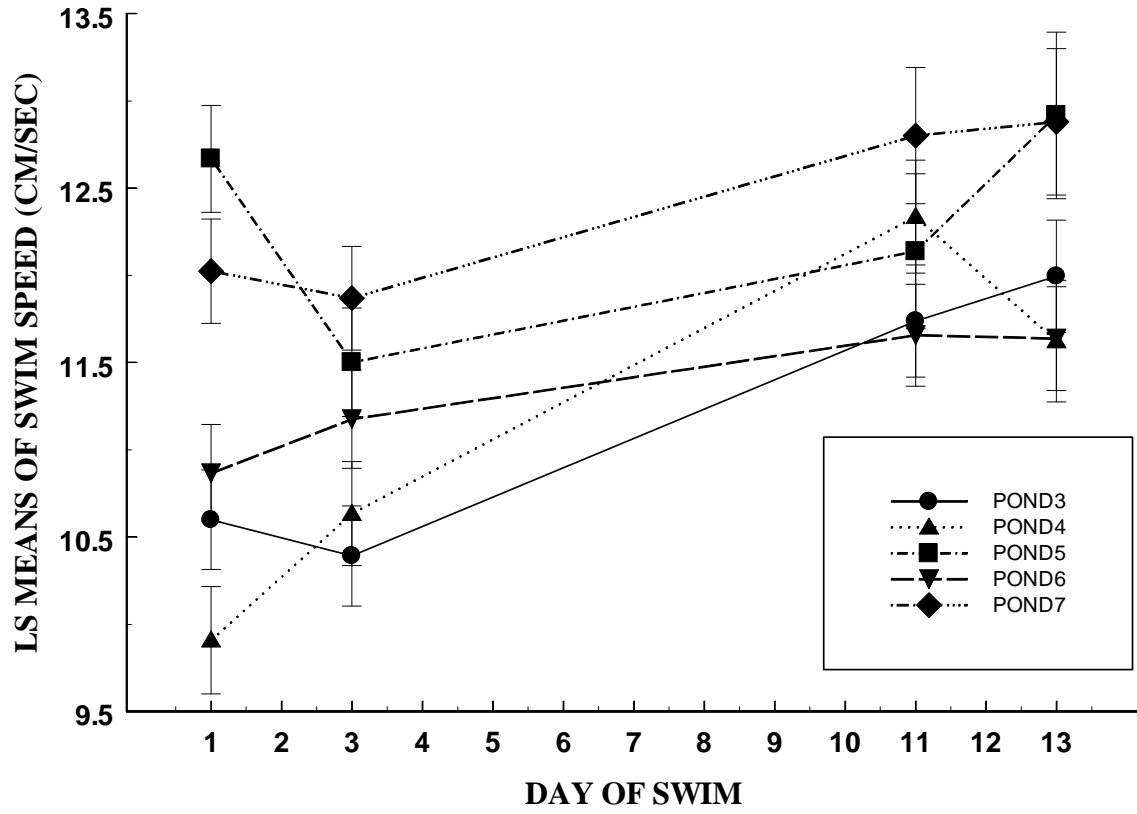


Figure 3

