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Growth and Survival of Salamanders Exposed to Different Formulations of Glyphosate-Based Herbicide

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GROWTH AND SURVIVAL OF SALAMANDERS EXPOSED TO DIFFERENT FORMULATIONS OF GLYPHOSATE-BASED HERBICIDE

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By

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I dedicate this thesis to my supportive family and friends and also my loving fiancé.

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ABSTRACT

Amphibian populations have been experiencing rapid declines worldwide in the past few decades. There are many proposed causations, including the use of agricultural chemicals such as herbicides. Glyphosate based herbicides are one of the most widely used herbicides. This study looks at the effects of different brands of glyphosate-based herbicides, including those intended for aquatic use, on the survival and growth of axolotl salamander larvae. Out of four brands of glyphosate herbicide (Aquamaster, Aquaneat, Helosate plus, and Roundup Pro), the survival rates of Roundup Pro were the lowest. Most mortality occurred between the 3 mg/L and 6 mg/L concentrations, during which all those treated with Roundup Pro died. The growth, measured in terms of total snout to tail length and head width, appeared to be greatest in length for those larvae treated with Aquaneat brand herbicide. These results indicate that Roundup Pro is lethal at concentrations of 6 mg/L, and that the composition, which includes the surfactant POEA, may be responsible. Subsequently, the concentration at which different adjuvant surfactants meant for use with aquatic safe herbicides (Dyne-Amic, Kinetic, and Cygnet) affected larval growth and survival was compared to the results obtained with Roundup Pro. The larvae exposed to the initial 5 mg/L concentration of Roundup Pro had total mortality, but survival was unaffected by exposure to aquatic safe surfactants at low concentrations. At high concentrations, Dyne-Amic and Kinetic significantly increased larval mortality while Cygnet did not. Of the surviving larvae, there was no difference in growth. The findings of this study are significant in that they give insight regarding how the use of herbicides could be contributing to the decline of amphibian populations.

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INTRODUCTION

Globally, scientists have been reporting declines in amphibian populations for the past three decades (Blaustein et al. 2011; Collins 2010; Houlahan et al. 2000; Stuart et al. 2004). In general, there does not seem to be a single cause of the global amphibian decline phenomenon, but often factors such as habitat modification and fungal diseases have been implicated in local declines and extinctions (Blaustein et al. 2011; Collins 2010). Additionally, the role of environmental toxins such as herbicides and pesticides has been widely studied, with some results indicating direct negative effects of these compounds on amphibian survival (Relyea 2005a), indirect negative effects on amphibian communities, or even indirect positive effects on growth and survival (Relyea et al. 2005), and non-lethal developmental and performance effects on amphibian larvae (Levis et al. 2016).

The most widely used herbicides are glyphosate-based herbicides (Myers et al. 2016). Glyphosate is a synthetic compound developed in the 1970s by the biotechnology corporation Monsanto and marketed as an herbicide under the name “Roundup”. Early investigation of the toxicity of commercially available Roundup on amphibian populations demonstrated overall negative effects (Relyea 2005b). However, when the active ingredient glyphosate was tested in isolation, the negative effects were reduced. Other investigations have found that polyethoxylated tallow amine (POEA), which helps bind the glyphosate to the leaf surface of target plants, is significantly more toxic to amphibians than the active ingredient glyphosate (*e.g.*, Howe et al. 2004). The use of glyphosate-based herbicides continues to increase, as Monsanto has genetically engineered crop plants that are resistant to glyphosate, to accommodate large-scale application of Roundup to agricultural fields to control weeds while leaving crop plants unaffected. Additionally, the patent on glyphosate has expired, leading to development of many generic versions of the product. All of the new formulations use glyphosate as the active ingredient, but the adjuvants vary. It is important to understand the effects of these new herbicide formulations on amphibian populations because of the potential for deleterious effects resulting from toxic adjuvants.

Glyphosate-based herbicides, even those intended for terrestrial use, have the potential to come into contact with larval amphibians through aerial drift or runoff from agricultural fields and other locations where herbicides may be used (Giesy et al. 2000). Many amphibian species have a biphasic life cycle and need to breed in aquatic environments, such as ephemeral or semi-permanent pools. As a result, amphibian larvae are subject to the effects of runoff, and herbicides can be found in water bodies at a variety of concentrations (*e.g.*, Battaglin et al. 2009).

It is expected that herbicides marketed for general, or terrestrial use, will contain a surfactant in their formulation, which could potentially increase mortality in amphibian larvae. Through this study, negative effects that glyphosate-based herbicides and their surfactants will be demonstrated. This information can be used to potentially minimize the impacts of runoff resulting from modern agricultural practices and to decrease larval amphibian mortality rates. Thus, the decline of amphibians would be slowed globally through an increase in the number of larvae surviving to adulthood.

Two experiments were performed to evaluate the effects of different formulations of glyphosate-based herbicide and alternative surfactant types on salamander larvae in a laboratory setting. For ‘Experiment 1’ larval axolotls (*Ambystoma mexicanum*; Figure 1) were exposed to two formulations each of ‘terrestrial’ vs. ‘aquatic’ glyphosate-based herbicides without the addition of surfactants. It was hypothesized that these formulations would have variable, although largely negative, effects on developing axolotl larvae due to differences in the adjuvant composition of the herbicides. For ‘Experiment 2’ larval spotted salamanders (*Ambystoma maculatum*; Figure 2) were exposed to an “aquatic safe” glyphosate-based herbicide with three different surfactants intended to be added by the end user of the herbicide. It was hypothesized that the addition of the surfactants would negatively affect salamander growth and development compared to treatments that lacked surfactants.

METHODS

Experiment 1—Study Species

The axolotl, *Ambystoma mexicanum* (Figure 1), has proven to be an effective model organism in ecotoxicology studies as well as others areas of research (Mouchet et al. 2007), and they are easily bred and kept in laboratories. The use of the axolotl over wild caught species for Experiment 1 was due to several factors. First, axolotls used in this study have never been exposed to environmental herbicides because they have been bred in laboratories for many generations, and this means larvae have not inherited resistance mechanisms from preceding generations. If wild caught salamanders had been used, it is possible that the populations have been previously exposed to environmental contaminants at some point, including herbicides and pesticides, which could bias our results.

Experiment 1—Effect of herbicide brand

During Experiment 1, different commercially available formulations of the herbicide glyphosate were used to test effects on survival and growth of larval *A. mexicanum*. The larvae were obtained through three separate crosses of adults breeding pairs present in the lab. The crosses were done by selecting three male-female pairs of axolotls from those in the lab and placing them in tanks set up with ideal mating conditions. These conditions included a lowered water temperature, which was done by adding ice water and monitoring the temperature, which was kept at 12-14 °C and providing gravel and plastic vegetation as substrate for egg deposition. Axolotl pairs were monitored for mating activity, which consists of a ritual "dance" of the male swimming around the female, and once the male released spermatophore packets around the tank he was removed. The female was then left to lay her eggs and was monitored daily for the presence of egg masses. Once the female had laid all of her eggs, the eggs were left to hatch and newly hatched individuals were placed into 150 mL wide mouth glass jars, glass being used to prevent any potential reaction between herbicide mixtures and plastic containers. The larvae were fed brine shrimp (*Artemia spp*.) daily and received clean 40% Holtfreter's water, a solution consisting of NaCl, KCl, $CaCl_2$, and $MgSO_4$ mixed with filtered water, once every 3-4 days. The larvae were reared for three weeks before exposure trials took place.

Four different formulations of commercially available glyphosate-based herbicide were used, two of which were labeled for aquatic usage, meaning that their use is intended for aquatic vegetation control and are expected to be safe for use within water bodies without causing harm to the wildlife within that body of water (Table 1). The herbicides used included Aquamaster, Aquaneat, Helosate Plus, and Roundup Pro, with Aquamaster and Aquaneat being the aquatic use formulations. The experiment was a full factorial replicated experiment (Figure 3), with ten replicates used for each herbicide treatment and the control group. All assignments of individuals into the treatments or control were done using a random number generator. Individuals were assigned to four different blocks, each containing the same number of individuals receiving each different treatment. All individuals were housed in controlled conditions in a laboratory environment.

The herbicides were applied at a concentration of 3 mg/L acid equivalency of glyphosate salt to a 40% Holtfreter's solution in which the larvae were reared. The herbicide-Holtfreter's solution was replaced every 24 hours for 72 hours. In the 72-hour time frame, larvae mortality rates were recorded. The surviving larvae at the end of the 72 hours were then exposed to a higher concentration of herbicide, each time increasing the amount by 3 mg/L, with the ending concentration being at 18 mg/L. The head width and total length from snout to tail tip were measured and recorded for those larvae remaining at the end of the herbicide trials. These measurements of growth were chosen for their impact on larval survival. Total length relates to tail length, which impacts the ability of larvae to swim from predators. Head width relates to the size of the mouth opening in salamander larvae, determining what size food is available for larvae to consume. Both of these have the potential to negatively impact survivability of larvae if reduced significantly.

Experiment 2—Study Species

The spotted salamander, *Ambystoma maculatum* (Figure 2), was used in Experiment 2. The selection of this species was to simultaneously determine whether the treatments that were redundant with Experiment 1 (Roundup Pro and Aquamaster) had the same effect on a wild caught species, and to test the effects of different herbicide surfactants. The spotted salamander typically breeds during early spring in ephemeral pools in wooded areas. The nature of the ephemeral pools makes the organisms that use them for breeding and habitat susceptible to exposure to environmental contaminants by not having permanent water to dilute contaminants and also being filled by precipitation and runoff from the area (Turtle 2000).

Experiment 2—Effect of adjuvant brand

The second experiment was done to further investigate the effects of chemical adjuvants used in conjunction with glyphosate-based herbicides on *A. maculatum* larvae survival and growth. Egg masses were collected from an ephemeral pool near Blue Level in Warren County, Kentucky and then brought into the lab. As the eggs hatched, individuals were transferred to 150 mL wide mouth glass jars with Holtfreter's solution, as done in Experiment 1. Larvae were fed brine shrimp and clean water was replaced every 3-4 days. The larvae were reared for three weeks before surfactant exposure trials.

Experiment 2 was also set up as a full factorial replicated experiment, with replicates of each treatment and randomly placed individuals in four different treatment blocks, all under the same housing conditions in a lab environment. The treatments used included three different commercially available surfactants, Cygnet, Kinetic, and Dyne-Amic, which were combined with Aquamaster, a glyphosate based herbicide intended for aquatic use and formulated without a surfactant, ingredients shown in Table 2. Aquamaster was selected because the results from Experiment 1 suggested that it was not harmful to salamander larvae. The Aquamaster-surfactant combinations were mixed according to manufacturer recommendations and then diluted to 5 mg/L acid equivalent concentration. These herbicide-surfactant combinations were used alongside a positive control, Roundup Pro, which includes the surfactant POEA in its formulation, and a negative control, Aquamaster with no additional surfactant.

The larvae were exposed to the different treatments at 5 mg/L acid equivalency of glyphosate salt and 0.0003% surfactant for a 72-hour period, with treatment water being replaced every 24 hours. Larvae mortality was recorded each day. Surviving larvae from each 72-hour period were then exposed to a surfactant concentration increased by two times the previous amount, continued up to 0.002%. The remaining larvae at the end of the trials were measured for total length and head width, as in Experiment 1.

Statistical Analysis

For both Experiments 1 and 2, differences in larval mortality among treatments were assessed via comparison of mortality curves using survival analysis. Differences in measured larval morphological characteristics, including head width and total length were compared using two-way analysis of variance (ANOVA) with type I sums of squares to partition variances between family groups and treatments. All tests, as well as assessment of the assumptions of normality and homoscedasticity were performed in R (R Core Team 2016). For all analyses the alpha level for type 1 error allowance was set at 0.05.

RESULTS

Experiment 1

During Experiment 1, various commercial formulations of glyphosate- based herbicides were applied to the water of axolotl larvae. The data recorded included daily mortality rates, and measurements of head width and total length.

The mortality was recorded daily and is shown in Figure 4. The different formulations of herbicide used can be divided into two distinct groups, the “aquatic safe” herbicides, which includes the Aquamaster and Aquaneat, and the terrestrial formulations which include a surfactant in the formulation, this includes the Helosate Plus and the Roundup Pro. There was no mortality shown in the larvae that were exposed to the Aquamaster and Aquaneat treatments, even as concentrations reached 18mg/L a.e. (data not shown). The Helosate Plus treatment had almost no mortality, with a 90% survival rate (Fig. 4), despite the presence of proprietary adjuvants including one or more surfactants. The Roundup Pro treatment had no surviving larvae at a concentration of 6 mg/L glyphosate, significantly affecting the mortality across treatments (X^2=112, df=4, $P<0.001$). This result is consistent with previous work regarding the toxicity of name brand Roundup (Relyea and Jones 2009).

For the larvae that survived all concentrations of glyphosate-based herbicide, the total length was measured, starting at the tip of the snout to the tip of the tail, in centimeters. There was a significantly longer total length in those larvae that were exposed to the Aquaneat treatments (Figure 5), but the others were not significantly different from one another ($F_{3,111}$=6.4, $P<0.001$; Table 3). Effects of family are minimal, as shown in Figure 6, and Table 3 ($F_{2,111}$=1.378, P=0.256). Normality was evaluated with a Shapiro-Wilks test and found W=0.990 P=0.536 (Figure 7). The result of a Levene's test homogeneity of variances is $F_{3,113}$=0.469; P=0.705 (Figure 8).

The head width was measured at the widest point, in centimeters, for all those larvae surviving all concentrations of herbicide. There were no significant effects found between the treatments (Figure 9), as shown in Table 4 ($F_{3,111}$=1.495, P=0.220). Families similarly showed little differences (Figure 10), as also indicated in Table 4 ($F_{2,111}$=2.501, P=0.087). Normality was evaluated with a Shapiro-Wilks test and found W=0.986 P=0.284 (Figure 11). The result of a Levene's test for homogeneity of variances is $F_{3,113}$=0.906; P=0.433 (Figure 12).

Experiment 2

During Experiment 2, spotted salamander larvae were exposed to various surfactants in conjunction with glyphosate-based herbicides in order to determine surfactant effect on mortality. Aquamaster herbicide with a non-ionic surfactant was compared to a control of Roundup Pro, which contains the surfactant POEA, and also another control of Aquamaster herbicide with no added surfactant.

The non-ionic surfactant additives used were Kinetic, Cygnet, and Dyne-Amic. The main ingredients comprising these surfactants are found in Table 2. The main ingredients of these are silicone-based (Kinetic and Dyne-Amic) or limonene-based (Cygnet). These surfactants were mixed as directed with an aquatic safe glyphosate herbicide and the concentration was increased every 72 hours starting at 0.0003% and ending at 0.002%. Glyphosate was mixed in with water at a concentration of 5 mg/L acid equivalent as this was at the concentration of Roundup found to be lethal to larvae in Experiment 1.

The mortality was recorded daily and is shown in Figure 13. While Roundup had its typical effect on larvae (*i.e.*, complete mortality), the larvae were not significantly affected by the surfactants in the other treatments until a concentration of 0.001% was reached. After that point, differences in mortality among treatments were significant (X^2=105, df=4, P<0.001).

The surviving larvae had measurements of total length and head width taken, as in Experiment 1. Total length data are shown in Figure 14, and differences between groups were minimal ($F_{2,13}$=1.685, P=0.223; Table 5). The effects of family on total length were also minimal ($F_{2,13}$=0.158, P=0.855; Table 5) as shown in Figure 15. Normality was evaluated with a Shapiro-Wilks test and found W=0.981 P=0.963 (Figure 16). The result of a Levene's test for homogeneity of variances is $F_{2,15}$=2.152; P=0.151 (Figure 17). Head width data are shown in Figure 18, and differences between groups were small ($F_{2,13}$=0.643, P=0.542; Table 6). The effects of family on head width were also small ($F_{2,13}$=0.268, P=0.769; Table 6) as shown in Figure 19. Normality was evaluated with a Shapiro-Wilks test and found W=0.958 P=0.569 (Figure 20). The result of a Levene's test for homogeneity of variances is $F_{2,15}$=0.658; P=0.532 (Figure 21). Due to the elevated mortality compared to Experiment 1, sample sizes probably yielded low power to detect any meaningful effects for total length and head width in Experiment 2.

DISCUSSION

Contaminants are a major constituent of the suspected causations of the decline of amphibian species globally (Collins 2010; Houlahan et al. 2000; Stuart et al. 2004). This study sought to address this through examining the effect of a widely used active ingredient in herbicides, glyphosate, and the associated non-active adjuvants, on larval amphibian survival and growth.

Mortality—The results of Experiment 1 found that Roundup Pro has a negative impact on salamander larvae survival, and was lethal to salamander larvae at a concentration of approximately 5-6 mg/L acid equivalent. Roundup Pro was the only herbicide formulation that was known to contain the surfactant POEA (polyethoxide tallow amine). As a result, Roundup Pro could be considered a positive control in this study, and when compared to the other formulations of herbicides used, it is readily apparent that survival of amphibians is much greater in the presence of non-POEA-containing herbicides. This mortality result is not a novel finding, as previous studies have suggested that POEA and glyphosate herbicides containing POEA as a surfactant are more toxic to amphibians, fish, and invertebrates and have higher mortality rates (Howe et al. 2004; Folmar et al. 1979). This study is consistent with those in that Roundup formulations have a higher acute toxicity than formulations without a surfactant, but still containing the active ingredient glyphosate. However, because of the widespread usage of the Roundup brand, the surfactant POEA is also frequently present in the environment. The widespread usage is exacerbated with the practices of modern agriculture and the use of Roundup ready crops. Ubiquitous use of the herbicide increases the amount that could runoff into nearby water bodies and therefore increases the potential for contact with amphibians at potentially lethal concentrations. The survival of amphibian larvae into adulthood and sexual maturity is essential in preventing populations from declining further.

The results of Experiment 2 confirmed that Roundup Pro has a negative impact on larval survival, even if collected from the wild, and also found that the surfactants added to the Aquamaster "aquatic" herbicide, had no significant impact on larvae survival at the low concentrations expected to be encountered in the wild under typical usage situations. This suggests that the nonionic surfactants used, are not likely having dramatic direct negative effects on larvae. The results of Experiment 2 support the findings of a study by Haller and Stocker (2002), in which POEA surfactants were found to be more toxic to Bluegill Sunfish than surfactants with a silicone-based active ingredient (*e.g.*, Kinetic and Dyne-Amic) and a limonene-based active ingredient (*e.g.* Cygnet).

Size—In both experiments, it was found that among the surviving larvae, there were no significant differences in total length or head width, with the exception of larvae exposed to Aquaneat herbicide having a significantly longer total length. The mechanism leading to the increased size of larvae in the Aquaneat treatment is unclear. However, several studies have also found an increased size associated with glyphosate exposure (Ortiz-Santaliestra et al 2011; Levis and Johnson 2015). Perhaps some unidentified adjuvant in the formulation of the Aquaneat herbicide is enhancing the growth of larvae. Additional studies should be conducted to replicate this result and investigate potential mechanisms and consequences.

The total length and head width measurements were only recorded for the larvae surviving their respective treatment at the end of each experiment. This was to facilitate a comparison of measurements among the treatment groups when they were at the same age. Because the Roundup Pro treatment resulted in total mortality in both experiments, measurements for total length and head width were not recorded, and the effect of the POEA surfactant on larval salamander growth is unknown. Ortiz-Santaliestra et al. (2011) found that Roundup Plus exposure resulted in an increased total length at hatching, indicating that glyphosate-based herbicides with POEA may also have increased length similar to the Aquaneat exposed group in this study (Ortiz-Santaliestra et al. 2011).

CONCLUSIONS

This study sought to assess the effects of various glyphosate-based herbicide formulations and various added surfactants on the survival and growth of salamander larvae. In order to do this two experiments were conducted. Experiment 1 consisted of evaluating various formulations of glyphosate-based herbicide on axolotl larvae and found that Roundup Pro, a terrestrial formulation containing the surfactant POEA, had a significant negative effect on larval survival. It was also found that larvae exposed to Aquaneat resulted in significantly longer total length, but there were no other differences among treatments with respect to total length and head width values. The second experiment evaluated the effects of various surfactant formulations on spotted salamander larvae survival and size. While the negative effects of Roundup remained strong, it was found that when added to Aquamaster at low concentrations, the surfactants Kinetic, Dyne-Amic, and Cygnet did not have a significant impact on survival or size.

The impact of Roundup Pro on survival can possibly be attributed to the surfactant used in its formulation, POEA, which has been suggested to increase herbicide toxicity (Howe et al. 2004; Folmar et al. 1979). The increased length found to be associated with Aquaneat herbicide has an unknown mechanism. However there has been several instances of increased size due to glyphosate exposure (Ortiz-Santaliestra et al. 2011; Levis and Johnson 2015). There are multiple possible causations as to why amphibian populations have been declining globally, and environmental contaminants are one possibility (Collins 2010). This study reaffirms that the surfactant POEA, which is used in Roundup formulations of glyphosate-based herbicides is lethal to salamander larvae at 5-6mg/L acid equivalent. The use of this surfactant is widespread, as is Roundup herbicide and understanding its impacts on non-target organisms, such as salamanders and other amphibians, is important in evaluating the impact of contaminants on amphibian decline.

Recommendations—This study has demonstrated that wildlife populations, specifically of the amphibian variety, would benefit from a reduction in the use of glyphosate herbicide formulations that contain POEA (*e.g.*, Roundup), and has found that alternative surfactants have no obvious direct effects on mortality. From the perspective of a salamander, the obvious recommendation is to encourage the use of 'aquatic safe' herbicides in areas where contaminants can make their way into aquatic habitats unintentionally. What remains to be determined, from the perspective of the human end-user, is the effectiveness of these non-Roundup alternatives in removing unwanted plants. Additional research into the efficacy of glyphosate when paired with alternative surfactant formulations, including organosilicone and limonene based adjuvants, on weed control and crop production compared to formulations containing POEA is needed. Assuming the utility of the herbicide is not adversely affected by the removal of POEA as an adjuvant, these results clearly advocate for reduced reliance on this toxic surfactant. To be clear, the results of this work do not certify that any of the surfactants and herbicides used in this study are completely benign to amphibians, other wildlife, and/or humans. There are certainly active ingredients included in Tables 1 and 2 that have the potential for effects that would not be detectable from the experiments performed here.

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Table 1. Ingredients used in glyphosate-based herbicides.

Treatment	Manufacturer	Surfactant added	Glyphosate	Intended Use
Roundup Pro	Monsanto	Yes: POEA; 13%	50.2%	Terrestrial
Helosate Plus	Helm Agro	Yes: Proprietary	41.0%	Terrestrial
Aquamaster	Monsanto	No	53.8%	Aquatic
Aquaneat	NuFarm	No	53.8%	Aquatic

Table 2. Active ingredients and amounts in surfactants used in Experiment 2.

Surfactant	Manufacturer	Active Ingredients	Amount
Dyne-Amic	Helena Chemical	• Methyl esters of C16-C18 fatty acids • Polyalkyleneoxide • Modified polydimethylsiloxane • Alkylphenol ethoxylate	Combined 99.0%
Kinetic	Helena Chemical	• Polyalkyleneoxide • Modified polydimethylsiloxane • Nonionic surfactants	Combined 99.0%
Cygnet	Cygent Enterprises	• Limonene • Methylated vegetable oil • Alkyl hydroxypoly oxyethylene	75% 15% 10%

Table 3. ANOVA table for total length in Experiment 1. As Type 1 sums of squares was used, the effects of 'Treatment' are estimated after accounting for the effects of 'Family'.

	Df	SumSq	MeanSq	F	P
Family	2	0.083	0.041	1.378	0.256
Treatment	3	0.588	0.196	6.486	<0.001
Error	111	3.355	0.030		

Table 4. ANOVA test for family effects on head width**.** As Type 1 sums of squares was used, the effects of 'Treatment' are estimated after accounting for the effects of 'Family'.

	Df	SumSq	MeanSq	F	P
Family	2	0.007	0.004	2.501	0.087
Treatment	3	0.007	0.002	1.495	0.220
Error	111	0.161	0.001		

Table 5. ANOVA test for family effects on total length in Experiment 2. As Type 1 sums of squares was used, the effects of 'Treatment' are estimated after accounting for the effects of 'Family'.

	Df	SumSq	MeanSq	F	P
Family	2	0.083	0.042	1.378	0.256
Treatment	3	0.588	0.196	6.486	<0.001
Error	111	3.355	0.030		

Table 6. ANOVA test for family effects on head width in Experiment 2. As Type 1 sums of squares was used, the effects of 'Treatment' are estimated after accounting for the effects of 'Family'.

	Df	SumSq	MeanSq	F	P
Family	2	0.007	0.004	2.501	0.087
Treatment	3	0.007	0.002	1.495	0.220
Error	111	0.161	0.001		

Figure 1. Larval *Ambystoma mexicanum*, the axolotl.

Figure 2. *Ambystoma maculatum*, the spotted salamander (post metamorphosis).

Figure 3. Experimental setup for experiments. There were four replicated treatment blocks, divided into six sections, one for each treatment. Within each section was an equal amount of larvae from each family, randomly distributed into jars.

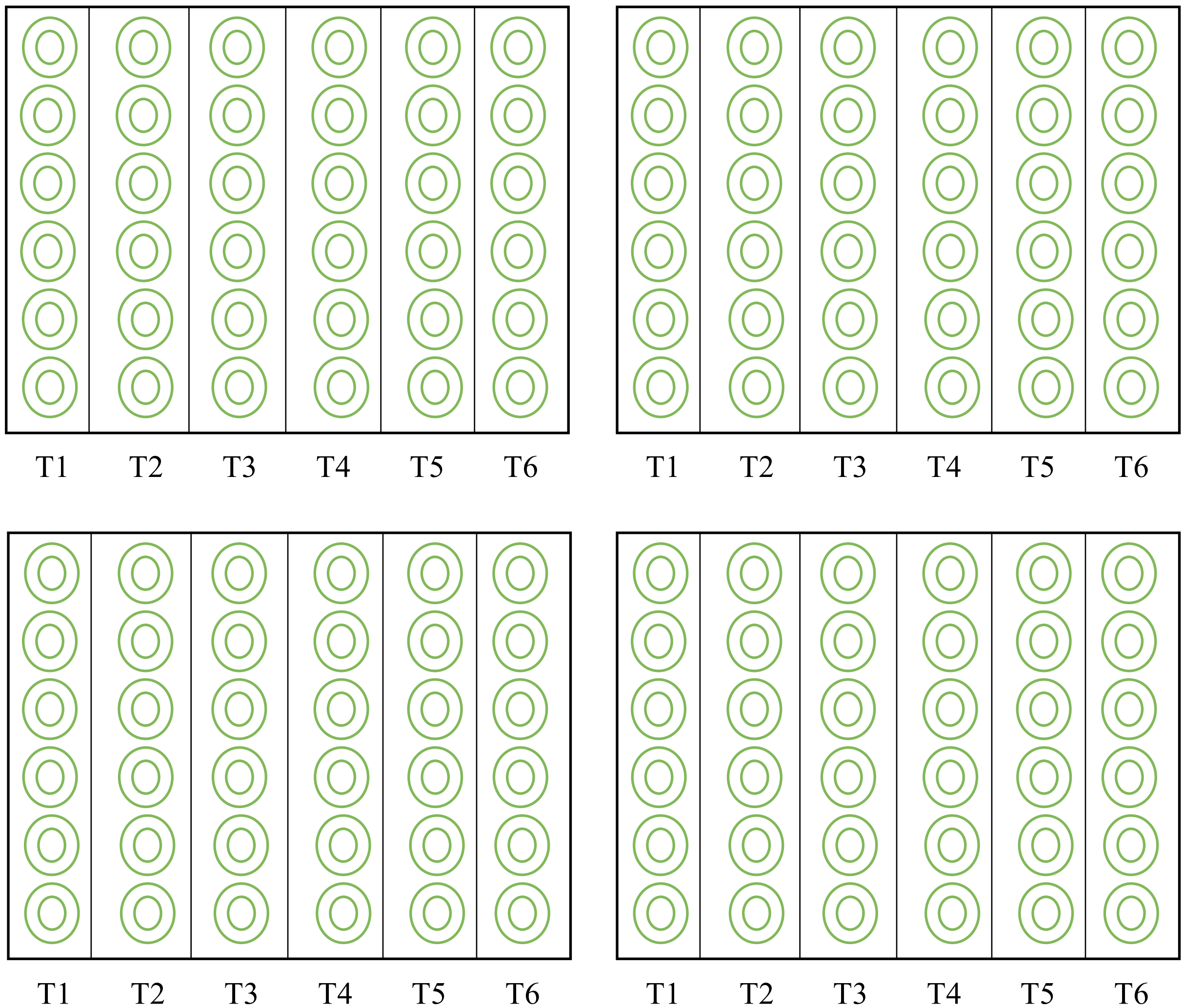

Figure 4. Survival of axolotl larvae exposed to each treatment. Mortality was recorded on a daily basis. Aquamaster and Aquaneat had a 100% survival rate, while Helosate Plus had a 90% survival rate, the control group had about a 75% survival rate, and Roundup Pro had the lowest survival with 0% of larvae surviving the trials. Dashed vertical lines indicate timing of increased dosages of herbicide. No mortality was experienced in any treatments subsequent to day 10, even as concentrations were increased to 18 mg/L a.e. (data not shown).

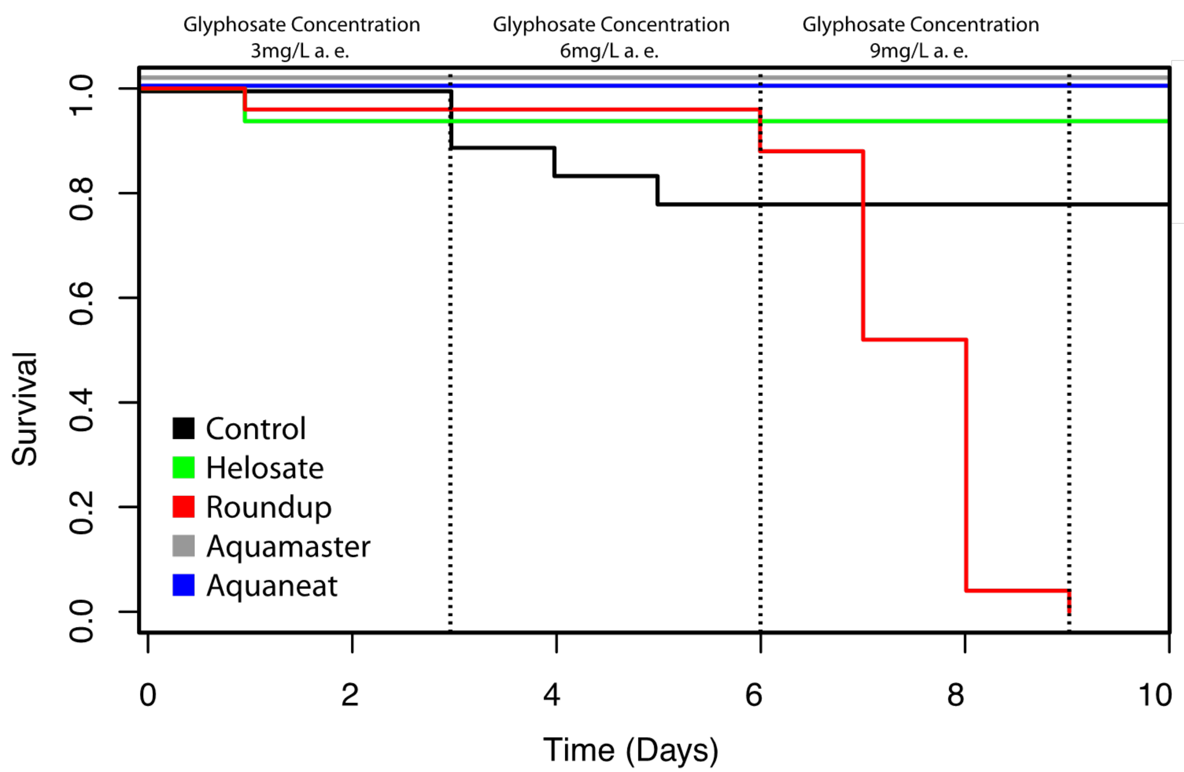

Figure 5. Total length of larvae surviving all concentrations of herbicide. There was an increased total length found in those larvae surviving the Aquaneat treatment. The larvae surviving the Helosate and Aquamaster treatments, as well as the control group, did not significantly differ in their total length (see text). Boxplots depict the median value, bounded by the 1st and 3rd quartile extent and the whiskers represent the maximum and minimum values up to 1.5X the interquartile range (IQR). Values exceeding 1.5X IQR are denoted by open circles.

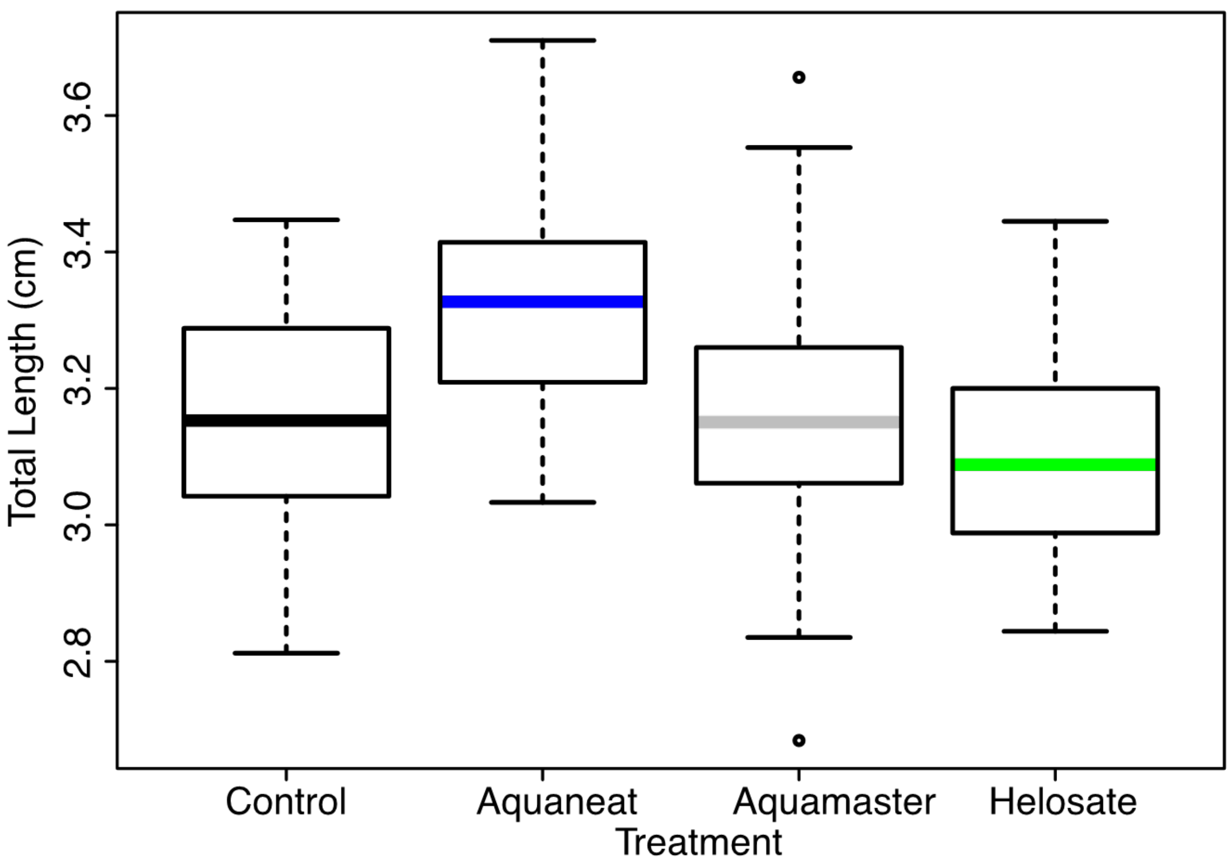

Figure 6. Boxplots demonstrating lack of effects of crossing family on total length in Experiment 1. Boxplots depict the median value, bounded by the 1st and 3rd quartile extent and the whiskers represent the maximum and minimum values up to 1.5X the interquartile range (IQR). Values exceeding 1.5X IQR are denoted by open circles.

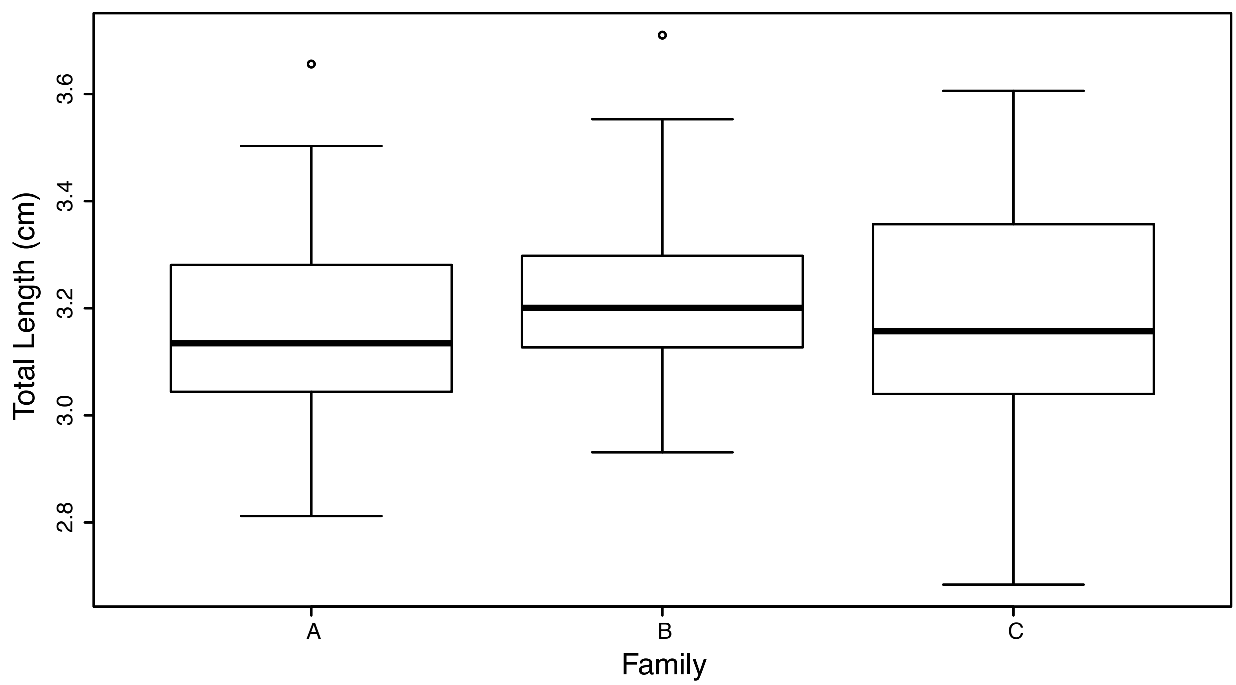

Figure 7. A normal probability plot showing that total length data were not significantly non-normal, and met the normality assumption of parametric statistics (*e.g.* ANOVA).

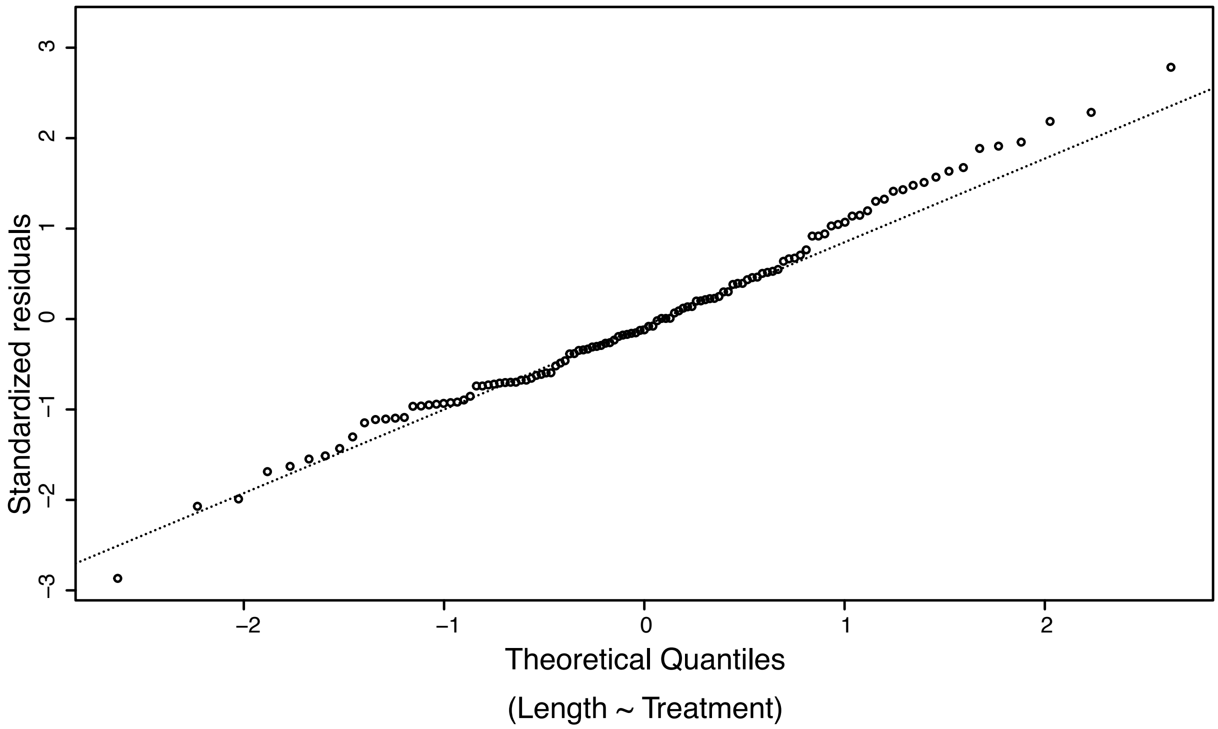

Figure 8. Plot demonstrating homogeneity of variances for total length data, indicating data satisfy the homoscedasticity assumption of ANOVA.

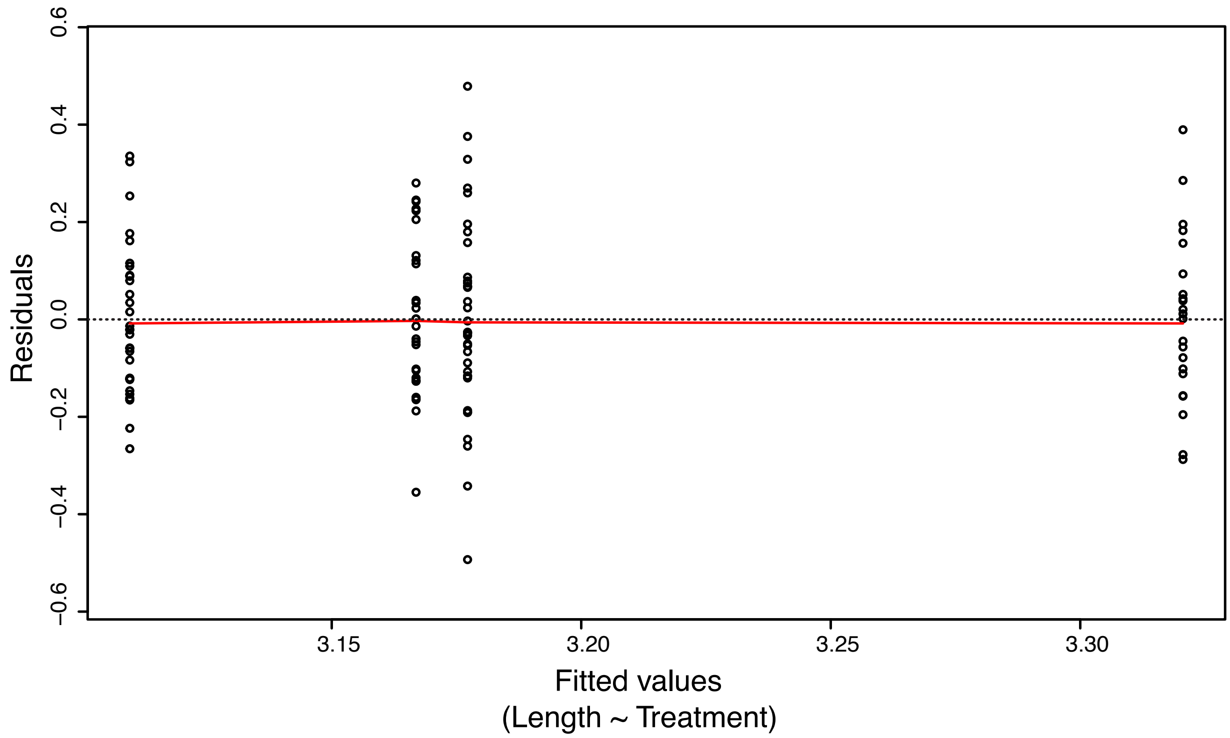

Figure 9. Head width of larvae surviving all concentrations of herbicides. The head width of the larvae surviving Aquaneat, Aquamaster, and Helosate treatments, as well as the control group, were measured and recorded. The head widths between these groups did not significantly differ from one another. Boxplots depict the median value, bounded by the 1st and 3rd quartile extent and the whiskers represent the maximum and minimum values up to 1.5X the interquartile range (IQR). Values exceeding 1.5X IQR are denoted by open circles.

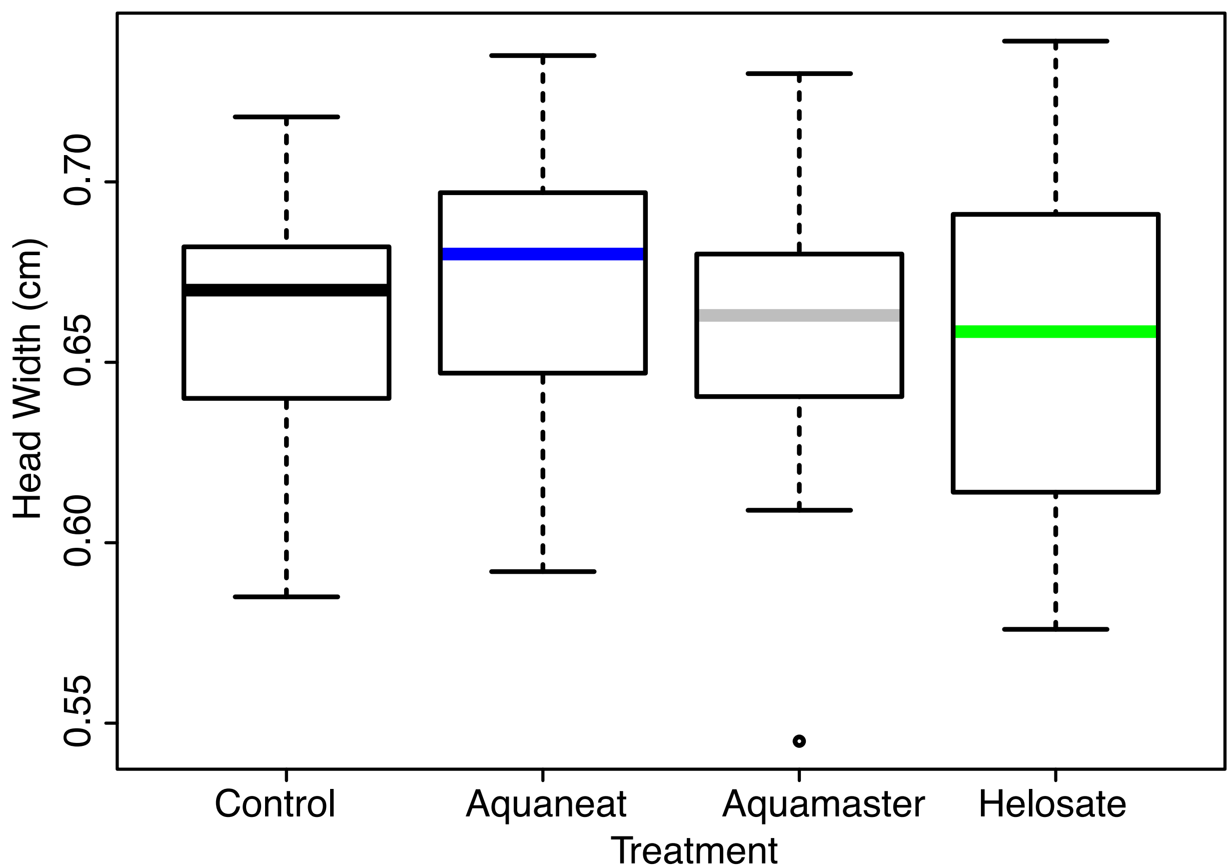

Figure 10. Boxplot demonstrating lack of effects of crossing family on head width in Experiment 1. Boxplots depict the median value, bounded by the 1st and 3rd quartile extent and the whiskers represent the maximum and minimum values up to 1.5X the interquartile range (IQR). Values exceeding 1.5X IQR are denoted by open circles.

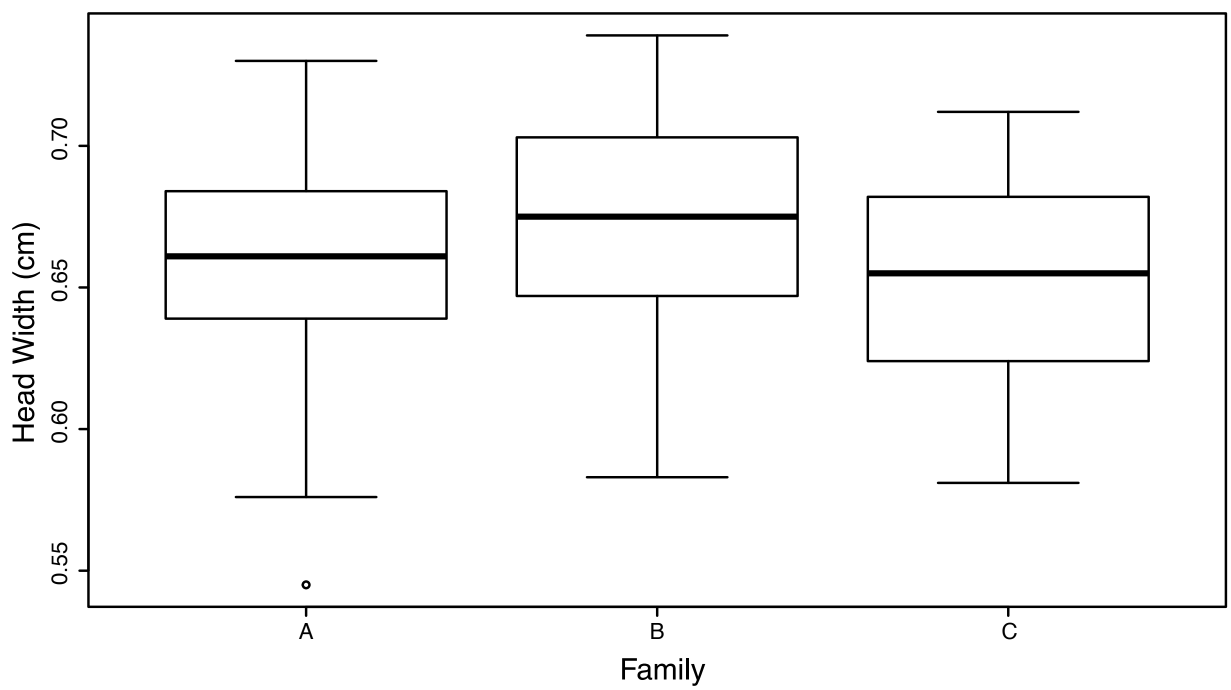

Figure 11. A normal probability plot showing that head width data were not significantly non-normal, and met the normality assumption of parametric statistics (*e.g.* ANOVA).

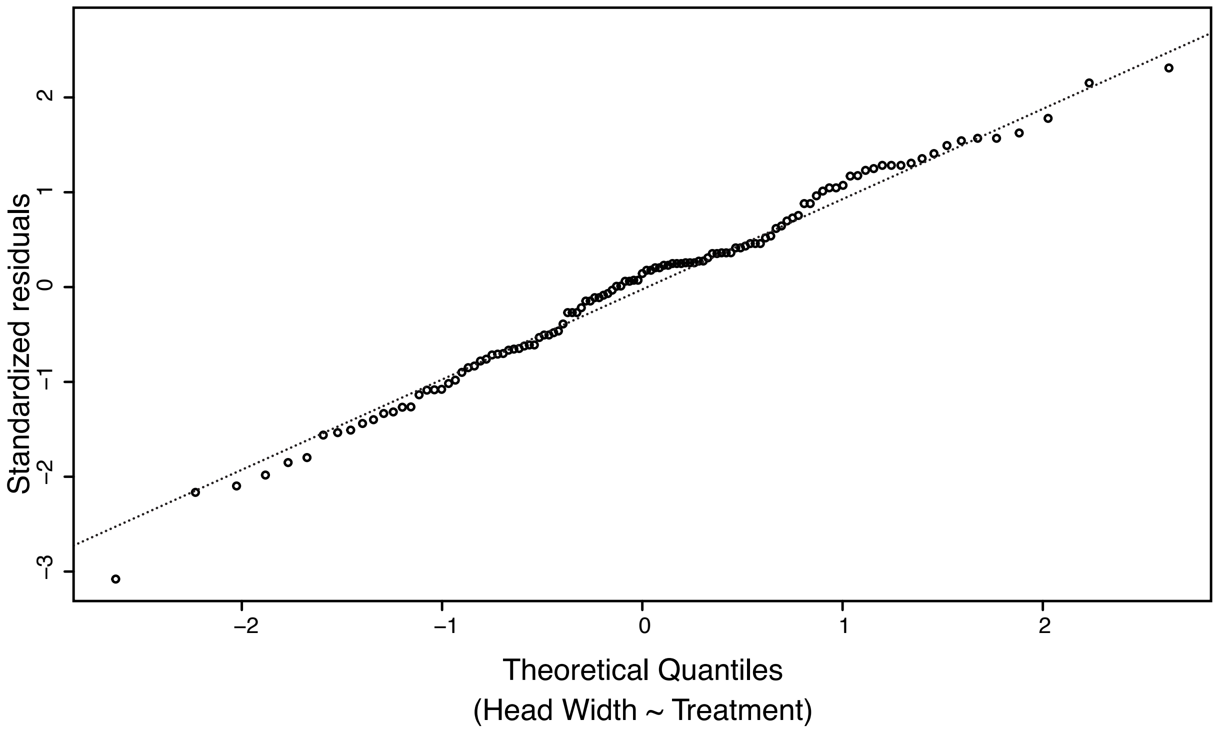

Figure 12. Plot demonstrating homogeneity of variances for head width data, indicating data satisfy the homoscedasticity assumption of ANOVA.

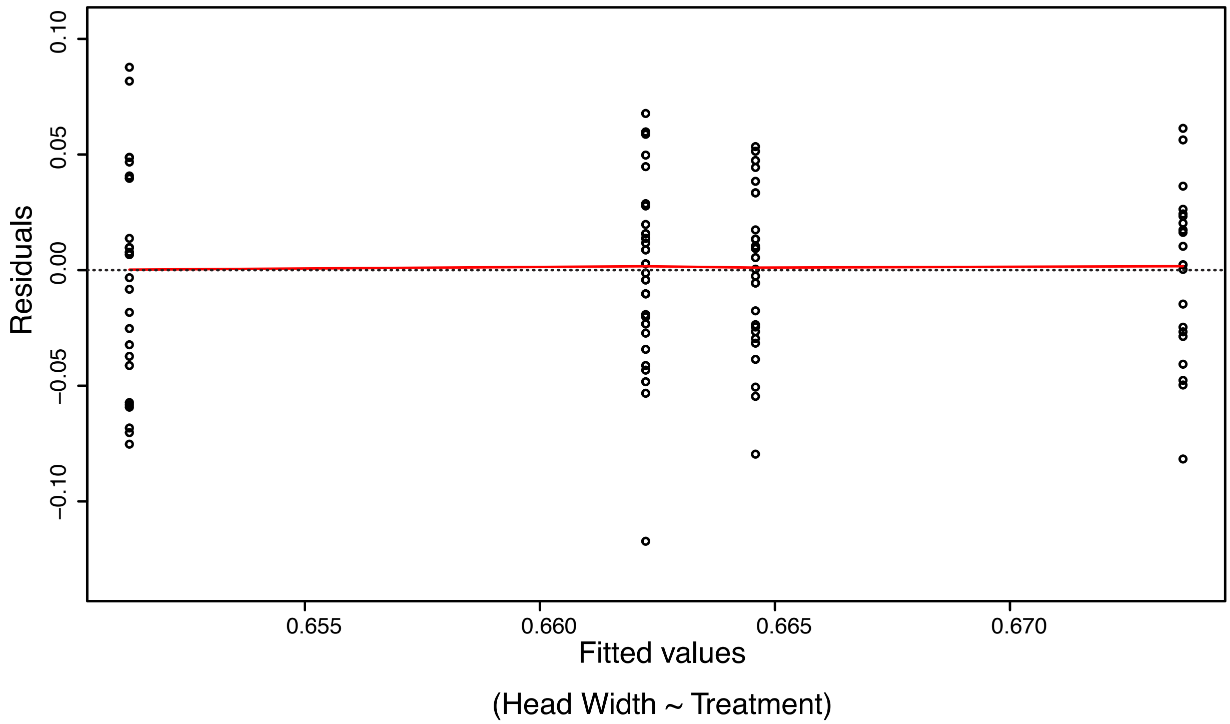

Figure 13. Survival of larvae exposed to Aquamaster herbicide and three added surfactants. Mortality was recorded daily for larvae in each surfactant treatment. Roundup Pro had a 0% survival rate after 0.0003% surfactant concentration. Vertical dashed lines depict start and endpoints for the four 3-day exposures to surfactants at the labeled concentrations.

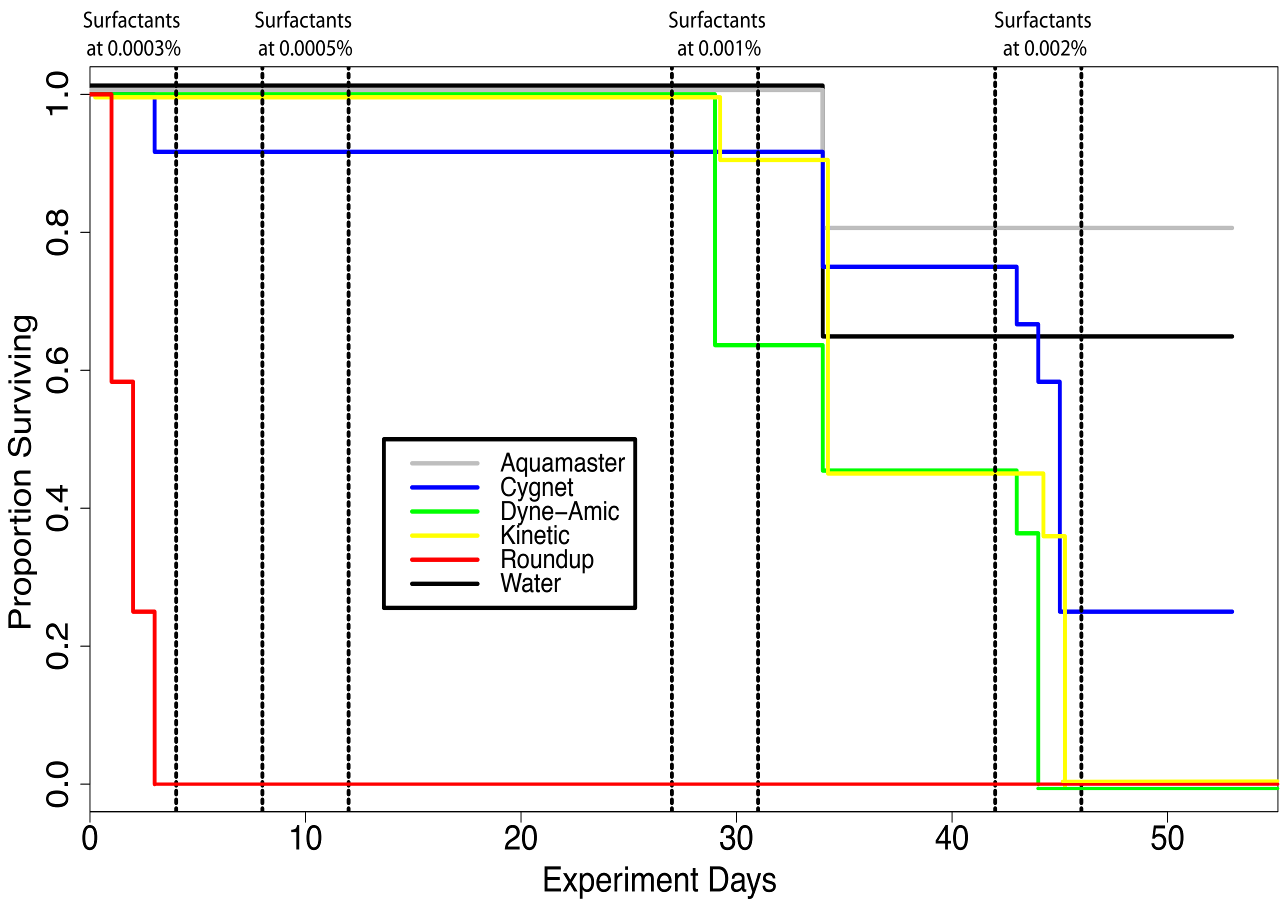

Figure 14. Length boxplot Experiment 2. No statistically significant differences among treatment groups were found. Boxplots depict the median value, bounded by the 1st and 3rd quartile extent and the whiskers represent the maximum and minimum values up to 1.5X the interquartile range (IQR). Values exceeding 1.5X IQR are denoted by open circles.

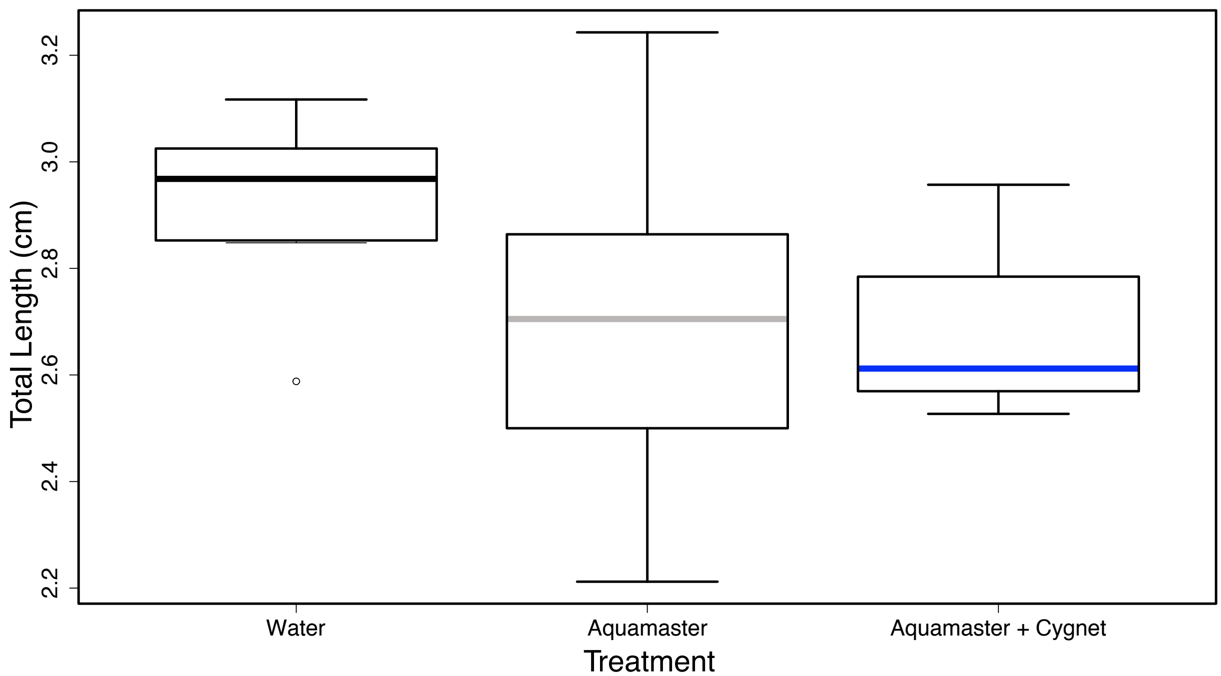

Figure 15. Boxplots showing the lack of family effects on total length for Experiment 2. Boxplots depict the median value, bounded by the 1st and 3rd quartile extent and the whiskers represent the maximum and minimum values up to 1.5X the interquartile range.

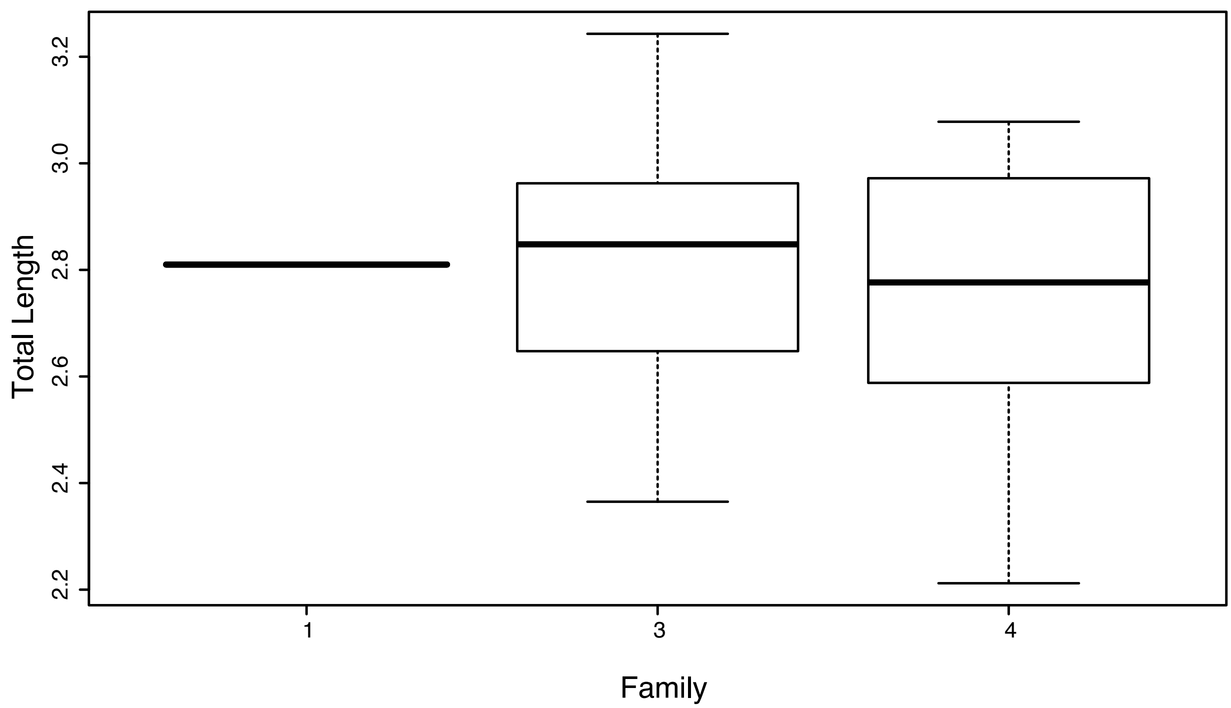

Figure 16. A normal probability plot showing that data were not significantly non-normal for total length in Experiment 2, matching the normality assumption of ANOVA.

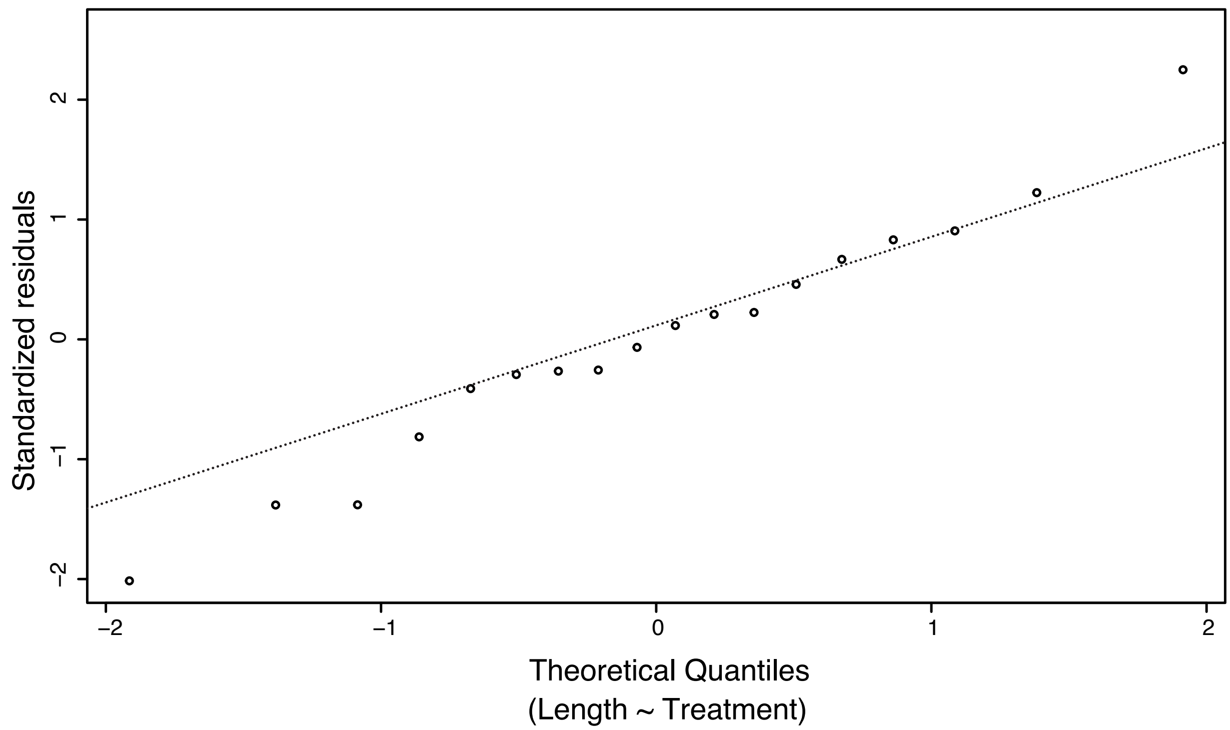

Figure 17. Plot demonstrating homogeneity of variances for total length data in Experiment 2, indicating data satisfy the homoscedasticity assumption of ANOVA.

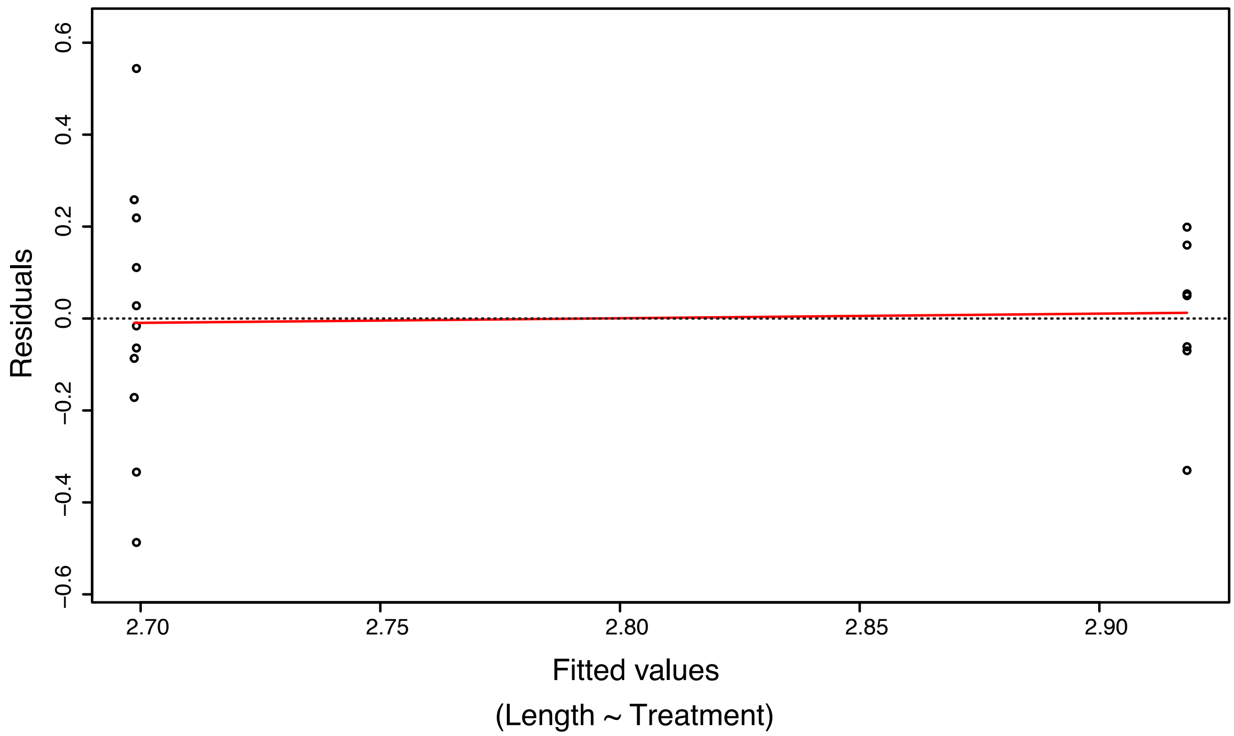

Figure 18. Head Width boxplot for Experiment 2. No statistically significant differences among treatment groups were found. Boxplots depict the median value, bounded by the 1st and 3rd quartile extent and the whiskers represent the maximum and minimum values up to 1.5X the interquartile range.

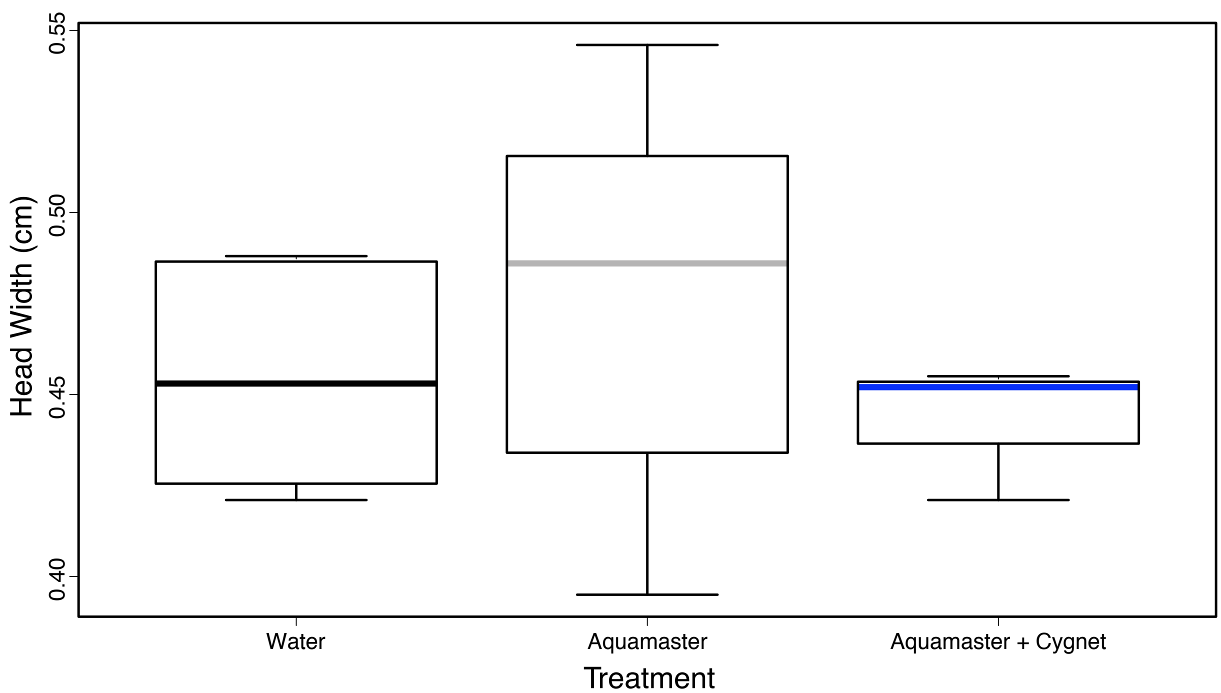

Figure 19. Boxplots showing the lack of family effects on head width in Experiment 2. Boxplots depict the median value, bounded by the 1st and 3rd quartile extent and the whiskers represent the maximum and minimum values up to 1.5X the interquartile range (IQR).

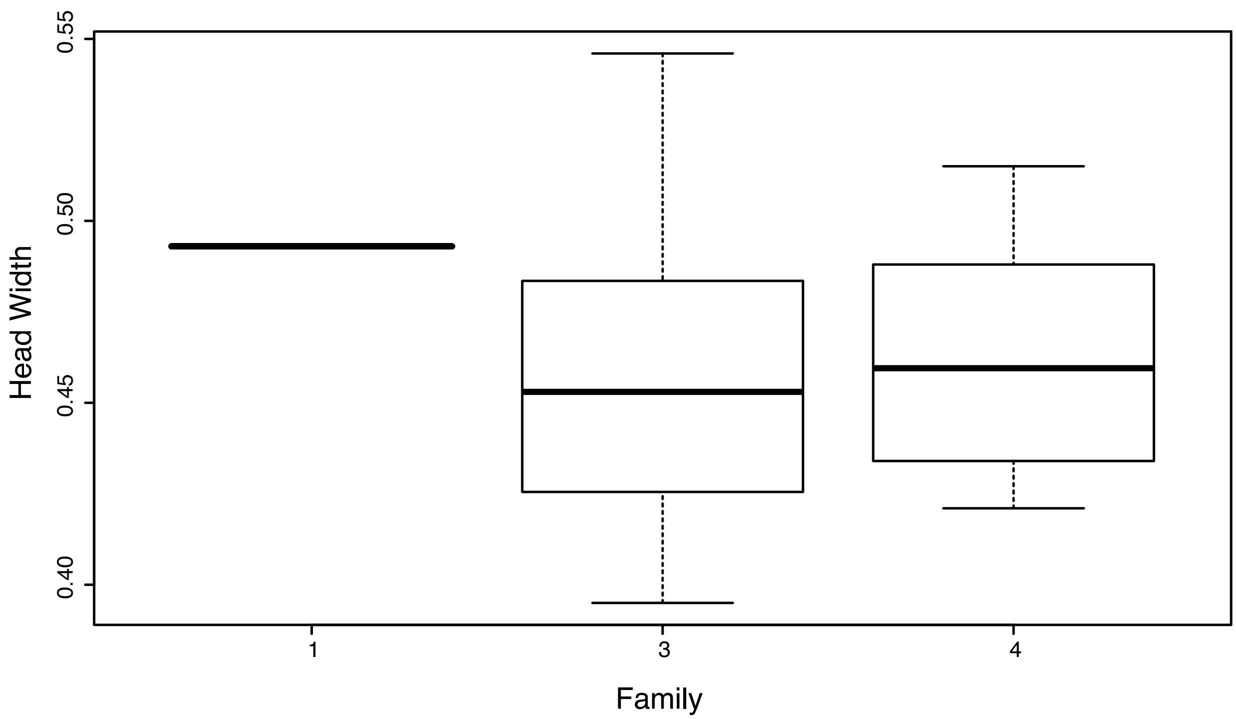

Figure 20. A normal probability plot showing that data were not significantly non-normal for head width in Experiment 2, matching the normality assumption of ANOVA.

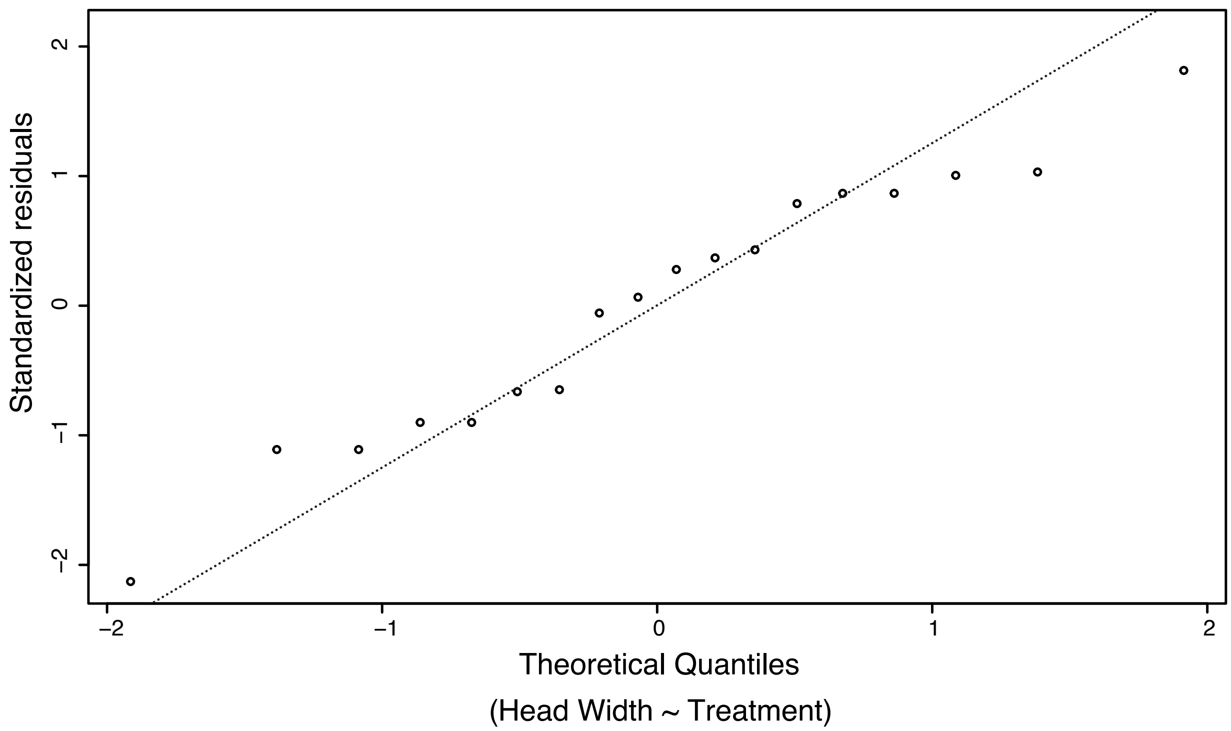

Figure 21. Plot demonstrating homogeneity of variances for head width data in Experiment 2, indicating data satisfy the homoscedasticity assumption of ANOVA.