THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

ENVIRONMENTAL RESOURCE MANAGEMENT PROGRAM AND
SCHOOL OF FOREST RESOURCES

EFFECTS OF ELEVATED SALINITY ON THE MORPHOLOGY AND BEHAVIOR OF GREEN FROG TADPOLES (*LITHOBATES (RANA) CLAMITANS*)

GEORGE SAMRA
Fall 2010

A thesis
submitted in partial fulfillment
of the requirements
for baccalaureate degrees
in Environmental Resource Management and Physics
with interdisciplinary honors in Environmental Resource Management and Wildlife and Fisheries Science

Reviewed and approved* by the following:

Jacqualine Grant
Senior Lecturer in Wildlife Science
Thesis Supervisor

Robert Shannon
Associate Professor of Agricultural Engineering
Honors Adviser

Margaret Brittingham
Professor of Wildlife Resources
Honors Adviser

* Signatures are on file in the Schreyer Honors College.

Abstract. Due to extensive use of road de-icers, elevated levels of NaCl have been measured in freshwater bodies associated with roads. At high enough concentrations NaCl causes mortality in anuran larvae. For example, the LC50 for *Lithobates clamitans* larvae is over 3000 mg/l Cl^-. In this study we desired to determine the sublethal effects of NaCl on *L. clamitans* larvae by measuring the growth and predator escape response of tadpoles raised in 200 and 800 mg/l NaCl. Tadpoles tested after 25 to 28 days of exposure did not differ from control tadpoles. After 148 days of exposure tadpoles raised in the control and 200 mg/l NaCl treatment had significantly smaller masses and lengths than tadpoles in the 800 mg/l NaCl treatment. No differences in escape response were observed in either time period. These results suggest that NaCl levels as low as 800 mg/l could increase growth rate of larval anurans giving them a competitive advantage in avoiding predation. Such a response to elevated salinity could be an adaptation to detect and escape drying ponds.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

Several people were essential to the completion of this project. The experiment was designed by Dr. Jacqualine Grant. Tadpoles were housed, tested and preserved using Dr. Matthew Weeg's lab, equipment and guidance. Statistical analysis was accomplished with advice from the Penn State Statistical Consulting Center and Dr. Tyler Wagner.

INTRODUCTION

Amphibian declines

The declining population of numerous species of amphibians is a well documented but relatively poorly understood occurrence. About one-third of the world's amphibians are currently threatened and extinction rates for amphibians are over 200 times greater than the background levels (IUCN et al. 2004; McCallum 2007). Most published evidence suggests that this rate will increase (McCallum 2007).

Decreasing amphibian populations are a concern for many reasons. Foremost is their innate value. In addition to the intrinsic value of any species, amphibians are exceptional animals. During their lifecycle they occupy two disparate niches by metamorphosing from completely aquatically adapted organisms into terrestrial and aquatic adults. Such a change requires an almost complete reworking of their physiology and ecology and potentially increases their ecological importance (Semlitsch 2003).

Although their intrinsic value alone warrants protection, amphibians also have significant ecological importance through their control of energy flow through the ecosystem. The consumption of primary production by anuran larvae shapes the function and composition of algal communities which form the basis of many aquatic ecosystems (Semlitsch 2003). Amphibian larvae make up a significant portion of the biomass available to secondary consumers. Predation of amphibian larvae and metamorphosis from the larval to adult stage creates an important nutrient link between the aquatic and terrestrial environments (Semlitsch 2003; Whiles et al. 2006). The consumption of many invertebrates by adult amphibians creates a pathway for the energy stored in the invertebrates to enter into the food cycle. (Semlitsch 2003). Because most amphibians occupy two distinct niches throughout their lives – one in the fully aquatic juvenile stage and another in the semi-aquatic adult stage – their absence could result in the loss of greater ecological function relative to most species which occupy only a single niche (Whiles et al. 2006). The loss of amphibian species would detrimentally alter the flow of energy to the rest of the environment (Whiles et al. 2006; Ranvestel et al. 2004).

Amphibians are more sensitive to ecological alterations due to their permeable skin, conformal thermoregulation and dual life cycle (Whiles et al. 2006). Therefore otherwise unnoticed changes in the environment may be evident in amphibians earlier than other organisms (Semlitsch 2003; Wake and Vredenburg 2008; Gallant et al. 2007). If this is the case many of the causes of amphibian declines may be the early stages of changes to the environment that will ultimately affect a wider range of life. This view has been contested with several studies indicating amphibians are actually no more sensitive to chemical pollutants than other species (Kerby et al. 2010). Regardless, it should not be assumed that the factors affecting amphibian declines will have no broader effects.

The most certain cause for the loss of amphibians is the universal occurrence of habitat destruction and alteration (Semlitsch 2003; Gallant et al. 2007; Hayes et al. 2010). Disease, invasive species, climate change and chemical pollutants are also contributing factors although the full extent of their effects is not fully known (Semlitsch 2003; Hayes et al. 2010; Alford and Richards 1999). The combined effects of two or more stressors are likely to be the most

influential in determining population alterations although single variables are often featured in research (Semlitsch 2003; Hayes et al. 2010; Linder et al. 2003).

Without a solid understanding of the causes of these declines followed by appropriate action there is no reason to expect stability in the populations of many amphibians. Research has begun to examine the causes, and the status of amphibian populations as a whole warrants continued investigation.

Sodium chloride in the environment

Chemical pollutants are one potential cause of amphibian declines. Sodium chloride (NaCl), is a pollutant with the potential to affect ecosystems because of its intense use by humans and environmental prevalence. Every year 18 million metric tons of de-icing agents, of which 97% is NaCl, are applied to roads in the northern latitudes of the United States (Jackson and Jobbagy, 2005). Sodium chloride is the most popular choice of de-icer because of its low cost and high efficiency. The complete dissolution of NaCl into its component ions, Na^+ and Cl^-, lowers the freezing temperature of water and makes it an effective de-icer. Inevitably deicing chemicals are washed from impervious surfaces and roads into the surrounding environment (National Research Council (U.S.). Committee on Ecological Impacts of Road Density. 2005).

Sodium chloride can damage environmental processes in a range of ways – it disturbs soil function, alters the function of organisms and contaminates groundwater and freshwater systems (National Research Council (U.S.). Committee on Ecological Impacts of Road Density. 2005). The habitats of pond breeding amphibians can be especially susceptible to NaCl contamination because they are often isolated and have small volumes (Semlitsch 2003). Chloride ions tend to be conserved in aquatic systems and readily move from groundwater to surface waters (Environment Canada 2001).

The background level for chloride in freshwater systems is usually under 10 mg/L but highly elevated levels of NaCl are already common, especially in environments closely associated with roads (Demers and Sage 1990). Chloride concentrations over 20 mg/l in streams have been observed and these elevated concentrations remain unchanged miles from roads (Demers and Sage 1990). Chloride concentration of snow removed from roads often ranges from 3000 to 5000 mg/l. As a result roadside ponds often reach 4000 mg/l Cl^- and 150 to 300 mg/l Cl^- in rural lakes (Environment Canada, 2001). As little as 240 mg/l Cl^- is predicted to be harmful to 10% of all aquatic species based on toxicity data reviewed by Evans and Frick (2001) for bacteria, fungi, protozoa, benthic invertebrates, fish and amphibians (Environment Canada, 2001).

Anti-predator adaptations

The most important factor in sustaining stable amphibian populations is recruitment from aquatic larvae into adults (Turner 1962). Predation is the primary cause of tadpole mortality and high predation rates are often recorded in the field (Alford and Richards 1999). Consequently, anti-predator adaptations are central to tadpole survival and maintenance of healthy frog populations. A range of adaptations from chemical deterrents, behavioral alterations and morphological changes have evolved because they decrease a tadpole's chances of being successfully hunted (Leiden 1992).

The kinds of predators that tadpoles will encounter are determined by pond type and location. Aquatic insects in particular have major effects on anuran survival (Leiden 1992). The most significant predators of tadpoles are often larval diving beetles, larval odonates and newts (Brodie and Formanowicz 1983;Caldwell 1981). When present turtles and salamander larvae can be very numerous and prevalent predators of tadpoles (Iverson 1982; Caldwell 1981).

A knowledge of the specific strategies that different predator species use in hunting and attacking tadpoles is necessary to understand how different adaptations benefit tadpoles. There are two major hunting strategies: a 'sit and wait' strategy where the predator waits for prey to come within striking distance and a strategy where predators actively pursue or chase prey. Aquatic insects are mostly sit and wait predators (Wilson et al. 2005; Pritchard 1965). Turtles, fish and larvae of certain odonates are more frequently characterized as predators which engage in pursuit (Feder 1983; Pritchard 1964).

The anti-predator adaptations of concern to this study were size (or growth rate) and escape responses.

Size

Tadpole size is often a governing factor in predation. The vast majority of common tadpole predators show decreasing predation rates with increasing tadpole size (Alford and Richards 1999). Gape limited predators - newts, salamander larvae, and fish - can only consume prey that can fit entirely inside the predator's mouth (Alford and Richards 1999; Osenburg 1994). After tadpoles reach a certain threshold size they are excluded from predation by these particular predators (Cronin and Travis 1986). Larval odonates, notonectids (backswimmers) and aquatic beetles - which kill and then consume their prey in portions are not gape limited but also have higher success preying on smaller tadpoles than larger tadpoles (Cronin and Travis 1986).

Dragonfly larvae primarily utilize tactile or optical hunting strategies depending on species (Pritchard 1965). Either method can be characterized as a sit and wait" hunting strategy (although some dragonfly larvae of the genus *Anax* have been observed to stalk prey (Pritchard 1964). Tactile hunters wait for the tadpole to make physical contact with the predator before an attack is initiated (Richards and Bull 1990a). All odonate larvae attack with a fast strike (15-20ms) of the labium followed by consumption (Pritchard 1965). For sit and wait predators strike frequency does not vary with tadpole size because size does not affect the frequency with which tadpoles inadvertently approach a predator (Richards and Bull 1990b). However strike lethality decreases for larger tadpoles because they are able to escape more frequently (Richards and Bull 1990b). Presumably for this reason, some studies have suggested that larval odonates may even preferentially attack small tadpoles over larger ones (Brodie and Formanowicz 1983). The advantage to large tadpoles may be due to an increased difficulty in handling, increased chance of injury to the predator during an attack or increased burst and sprint speed (Brodie and Formanowicz 1983; Richards and Bull 1990b; Van Buskirk et al. 2003).

Swimming speed, which is positively correlated with tail length, increases as tadpoles grow(Huey 1980). Tail length is correlated with thrust strength and swimming speed (Wu 1977). Odonates have decreasing predation success for faster swimming tadpoles (Feder 1983; Chovanec 1992). Turtles on the other hand are not sit and wait predators. They will actively

pursue tadpoles from two meters away and often for over ten seconds at a time. Again, fast tadpoles increased their chances of evading predation attempts relative to slow tadpoles (Feder 1983). High growth rates are therefore beneficial because large tadpoles are excluded as potential prey for several predator species.

Anti-predator escape response

This effort for survival does not end after a predator has initiated an attack as evidenced by the high proportion of wild tadpole populations with partially missing tails (Blair and Wassersug 2000). Up to 88% of a population of bullfrog tadpoles have been observed with some type of tail damage and such findings are common (Doherty et al. 1998).

To escape predation after an attack has been initiated amphibian larvae engage in a two-stage startle response (Webb 1975). This type of response has been most studied in teleost fish. Mauthner cells, the neurons largely responsible for the startle response, are present in most lower aquatic vertebrates including amphibians (Eaton and Nissanov 1985). Consequently the startle responses of amphibians and fish are very similar.

The first stage of the escape response is known a C-start because it initiates the startle response and moves the animal's body into the shape of a 'C.' During the C-start the body of the tadpole contracts to move the tail towards the predator and the tadpole's head and vital organs away from the predator (Hale et al. 2002). The C-start lasts about 15-20 milliseconds and is followed by the second stage return flip. The return flip is a smaller contraction of the opposite side of the body (Eaton et al. 1977). The second stage can be defined as ending after the first tail stroke following the C-start is completed (Hale et al. 2002). The return flip is more variable than the highly stereotyped C-start (Eaton et al. 1977). The second stage is followed by vigorous swimming away from the stimulus (Eaton et al. 1977).The variable return flip and subsequent swimming has been suggested to be an adaptation to prevent predators from evolving a reaction to the startle response (Eaton et al. 1977).

Neurology

The startle response is primarily regulated by two large Mauthner cell neurons (Eaton et al. 1977). It is probable that brain stem neurons other than Mauthner cells can also mediate the startle response because C-starts are still achieved without Mauthner cells but usually have less power and higher latencies (Eaton et al. 1982; Gahtan et al. 2002). The axons of the Mauthner cells run along the full length of the spine and connecting with interspinal neurons (Hale 2002). Responses can be triggered by acoustic, vibrational, visual, local water movement and tactile cues. The octavolateralis system, which consists of detectors for these cues and the lateral line system, is a significant input to the Mauthner cell (Eaton and Nissanov 1985). *L. catesbeianus* tadpoles have more than 200,000 synaptic inputs to the Mauthner cell (Cochran et al. 1980).

When a predator stimulates any of these inputs the Mauthner cell and its neuron pool located on the opposite side of the stimulus is activated by an action potential (Eaton 1977). The opposite neuron is simultaneously inhibited so that the response is controlled and directional (Fetcho and Faber 1988; Eaton and Hackett 1984). All triggers elicit an apparently identical response (Eaton et al. 1977). In teleosts the latency for the response is about 5-10 milliseconds

For attacks of odonate larvae the tadpole's startle response does not initiate until after the predator makes contact with the tadpole (Zottoli et al 2001). These strategies make a tactile stimulated startle response an important defense mechanism for tadpoles (Storch 1976). Success of an attack is determined by where the strike lands and the size of the tadpole. The probability that an attack will result in a fatality is highest when the strike lands on the head or body of the tadpole (Van Buskirk et al. 2003; Johnson and Eidietis 2005).

Tail

The posterior portion of the tadpole's tail is relatively expendable. Thirty percent of the tails of *Hyla versicolor* tadpoles can be removed without causing fatality or decreasing their maximum speed (Van Buskirk and McCollum 2000). Tail damage was also found to have little or no effect on the growth and development of *L. catesbeianus* tadpoles and no effect on predation by newts on *R. utricularia* (Wilbur and Semlitsch 1990). Tail damage of seventy five percent was required to alter predation of dragonfly naiads on *H. chryoscelis* tadpoles (Semlitsch 1990). Larger tadpoles have been shown to have larger sprint speeds and therefore better escape attacks, with or without ripped tails, than small tadpoles (Richards and Bull 1990b). The tail tends to tear because it is viscoelastic (Doherty et al. 1998). This means that the tail is relatively strong under small deformations (when swimming) but tears easily under larger deformations (an attack from a predator). This material property is necessary because the tail lacks any skeletal support (Doherty et al. 1998; Hoff and Wassersug 2000). It has been suggested that when a predator grasps the tail of a tadpole the viscoelastic properties will allow the tadpole to stretch the tail and move its head from the predator. The tail can then tear and be sacrificed which allows the tadpole to escape (Doherty et al. 1998). Therefore, in the event of contact, the C-start maximizes the chance that the predator will strike the tadpole's tail which, with its high probability of tearing under large stresses, allows an escape.

Project description

The importance of the startle response, most easily demonstrated by the prevalence of tadpoles that successfully avoided predation, motivated our study to determine how a chemical stressor might affect a tadpole's ability to escape predation attempts. The loss of all or a significant portion of tadpoles with damaged tails (the majority in many populations) from recruitment could be enough to devastate populations. Due to environmental deviations yearly anuran populations for a particular species already naturally vary greatly. Dictated by larval survival, populations commonly increase or decrease over 100 fold from year to year (Turner 1962). Because recruitment is naturally low some years, an additional unnatural obstacle to larvae creates the danger of a population missing several consecutive years of substantial recruitment. Such a situation could be too much for a population to overcome (Semlitsch 2003). A change in the ability to perform a startle response could therefore have a substantial impact on anuran populations.

In an effort to identify one potential factor that could threaten the stability of anuran populations we conducted an experiment to determine the effects NaCl may have on the tadpoles of *L. clamitans*. Portions of the experiment are modeled after the methods and motivation from Eroschenko et al. (2002) and Squires et al. (2008). Squires et al. (2008) suggest that salinity would increase tadpole's susceptibility to predation.

The objectives of this study were to determine the effects of elevated levels of NaCl on the escape response and growth rate of *L. clamitans* tadpoles. After raising *L. clamitans* tadpoles in three NaCl treatments the speed and distance of the escape response and size of the tadpoles were measured and related to salinity treatment. Thus, this project sought to establish one of the bridges between chemical and biological stressors.

Study

L. clamitans (green frogs) are one of the most common and commonly seen frogs of Pennsylvania (Serrao 2000). *L. clamitans* are also one of the largest in the same area, smaller only than the bull frog. They are mostly aquatic, spending their time in or near ponds although they can travel large distances to relocate during rain events. Green frogs usually breed in permanent or semi-permanent pools to allow for a long larval stage that lasts one to two years depending on climate (Martof 1952). This strategy produces large tadpoles and metamorphs but also allows a longer time for predation to occur (Semlitsch 2003). Their occupation of permanent pools also exposes the species to a wider range of predators (e.g. fish and certain species of insect larvae) than strictly ephemeral pool breeding amphibians would encounter.

L. clamitans tadpoles have been shown to be adversely affected by salt. The median lethal concentration of chloride Cl^- for *L. clamitans* is 3109.3 mg/l (Collins and Russell 2009). Because we were interested in the sublethal effects of salinity the maximum treatment level used in this experiment was 800 mg/L NaCl (485 mg/l Cl^-), less than one sixth the LC_{50}. Such low levels were used in order to predict the indirect effects of salinity on tadpole survival resulting from any changes to their size and anti-predator escape response (vulnerability to predation).

METHODS

General

Three clutches of *L. clamitans* eggs were collected in the field. Eggs were added to tanks of three treatments: a freshwater control, a 200 mg/l NaCl low salt treatment and an 800 g/l NaCl high salt treatment. Each clutch composed one of three replicates comprising nine experimental tanks in all (figure 1). Two additional control tanks were maintained for the purpose of obtaining mass and morphological measurements only. The volume of water kept in each tank was 30 liters. Due to background salinities in the water the control was measured to have a salinity of 100±50 ppm, the low salt treatment a salinity of 300±50 ppm and the high salt treatment a salinity of 900±50 ppm. Salinity levels were monitored weekly using a model 30-10 FT YSI 30 salinity meter. Hatching occurred within two days of adding the eggs to the treatment tanks. On day 14 each tank's population was reduced to 25 tadpoles. Salinity and partial water changes were performed weekly. Tadpoles were fed in excess every two to three days. Tadpoles were fed in excess as needed. Tanks were maintained in a controlled room with a 70°F and a 12:12 photoperiod.

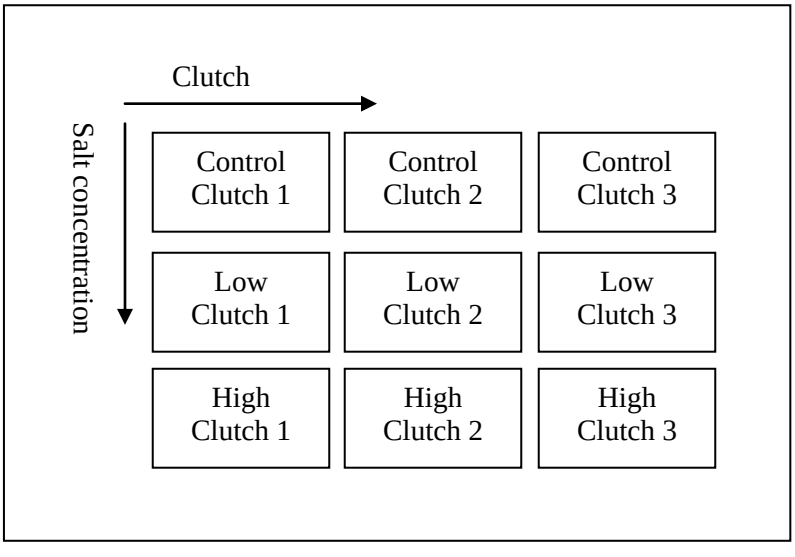

Figure 1. Experimental set up for treatment tanks

Escape response analysis

Behavioral testing began after 25 days of exposure. Tadpoles were randomly selected from the nine treatment tanks and placed in separate 87mm x 15mm Petri dishes with 50 ml of solution of equal salinity to their respective treatment tank. After a 30 minute acclimation period tadpoles were stimulated by a 150µl stream of water applied from a pipettor to the middle of the body. The investigator had no knowledge of the treatment corresponding to any of the tested tadpoles while conducting the behavioral trials. Three escape responses were elicited per tadpole with one minute rest periods after each response. Each response was recorded in a 30 frames per second video file with a Sony handycam DCR-TRV25. Behavioral trials were conducted for

177 tadpoles between days 25 and 36. Videos were analyzed frame by frame using ImageJ with the Manual_Tracking plugin modeled after the method described by (Myrick 2009). The program measured the speed and distance travelled by the tadpole between each frame. The escape response was defined to begin with the first movement of the tadpole after stimulation. The end of the escape response was defined as the moment that one of these three events occurred: (1) the tadpole stopped moving, (2) one second passed after the tadpole's last tail lateral tail movement or (3) the tadpole's tail was relaxed (straight) for two consecutive frames (67 ms) immediately before the tadpole reinitiated lateral tail movement. The total distance, speed after 33ms (speed between the first and second frame) and speed over the first 100ms of the response (average speed over the first three frames) were recorded for each escape response (table 1). The speed measurement gives an estimate of the burst speed for each tadpole.

Morphology

After behavioral trials were completed living tadpoles were photographed in order to extract dimensional data. Photoshop was used to determine the head width, length and dorsal area of each tadpole (table 1). Tadpoles were euthanized in 2mg/ml MS222 solution. After euthanasia tadpoles were gently blotted with tissue in order to remove excess moisture and then weighed and preserved. Morphological data was determined for tadpoles photographed 25 to 36 days of exposure and after 148 day of exposure.

Statistical analysis

A mixed model analysis of variance with repeated measures for the three trials per tadpole was developed in order to determine the effects of treatment and other variables on the morphological and behavioral parameters measured (Fernandez 2007). In the model treatment, trial and day were considered fixed effects and clutch and tank were considered random effects (table 1). The analysis was carried out using SAS (Statistical Analysis Software) 9.2. The SAS code for all analysis is included in appendix B.

The 33ms speed was square root transformed (sqspeed1= the square root of the 33ms speed) and distance was log transformed (lgdistance=natural log of distance) in order to achieve normality and homoscedasticity. For simplicity and consistency with the statistical code variables are referred to as their names in table 1. All other variables were normally distributed and homoscedastic. Because of the high correlation between all morphological variables head width, area and length were not tested with an analysis of variance as it would have been redundant. Mass was divided into two groups, one group from day 25 to 28 and one group from day 148 in order to avoid the interaction of exposure time with mass. Mass from day 25 through 28, mass from day 148, sqspeed1, speed2 and lgdistance were tested using the mixed model analysis of variance described above. Tadpoles tested from day 29-36 were excluded from consideration in the mixed model ANOVA to avoid detecting the interaction of exposure time with tadpole size. The critical p-value for this experiment was 0.01 using the procedure outlined by Hochberg and Bejamini (1995) for controlling the false discovery rate with a 0.05 initial alpha.

Table 1. List of response variables and explanatory variables

	Variable name	**Category of response**
Response variables		
Tadpole speed 33 ms after response began	Speed1	Behavioral
Tadpole speed 100 ms after response initiated	Speed2	Behavioral
Total distance swam in response	Distance	Behavioral
Dorsal area of tadpole	Area	Morphological
Distance from eye to eye	Head width	Morphological
Distance from nose to end of tail	Length	Morphological
Mass after tadpole was removed of excess water	Mass	Morphological
Explanatory variables		**Type of effect**
NaCl concentration	Treatment	Fixed
Trial (1-3)	Trial	Fixed
Number of days exposed in salt treatment	Day	Fixed
Clutch	Clutch	Random
Housing tank	Tank	Random

RESULTS

Four morphological parameters (mass, area, length and head width) and three behavioral variables (distance travelled, speed over the first 33ms and speed over the first 100ms) were measured for 194 and 177 tadpoles respectively. The results were checked for normality and homoscedasticity to fulfill the assumptions of an analysis of variance test (appendix C).

Correlations were determined for all tadpoles with the appropriate data available. All morphological parameters - mass, area, head width and length - were highly correlated based on 194 observations. P values for the correlation of each combination of morphological parameters were less than 0.0001. R square values were all over 0.9 (table 2).

Table 2. Pearson correlation coefficients for morphological variables

Correlation between	R-square	p
Mass and area	0.96	<0.0001
Mass and width	0.93	<0.0001
Mass and length	0.93	<0.0001
Area and width	0.93	<0.0001
Area and length	0.95	<0.0001
Width and length	0.98	<0.0001

Of the behavioral parameters the 33ms speed and 100ms speed were significantly correlated ($p < 0.0001$, r square = 0.73379) but speed was not correlated with distance. Contrary to previous findings, speed was not correlated with length (table 3).

Table 3. Pearson correlation coefficients for behavioral variables and length

Correlation between	R-square	p
Speed1 and speed2	0.73	<0.0001
Speed1 and distance	0.04	0.3887
Speed1 and length	0.09	0.0677
Speed2 and distance	0.13	0.0037
Speed2 and length	0.20	<0.0001
Distance and length	0.02	0.6386

Square root transformed 33ms escape response speed (sqspeed1)

The statistical model used showed that neither fixed effects (treatment, trial or exposure time) nor random effects (clutch or tanks) had a significant effect on the sqspeed1. This was true when the data was analyzed as a whole and for individual clutches (fig 2 and 3).

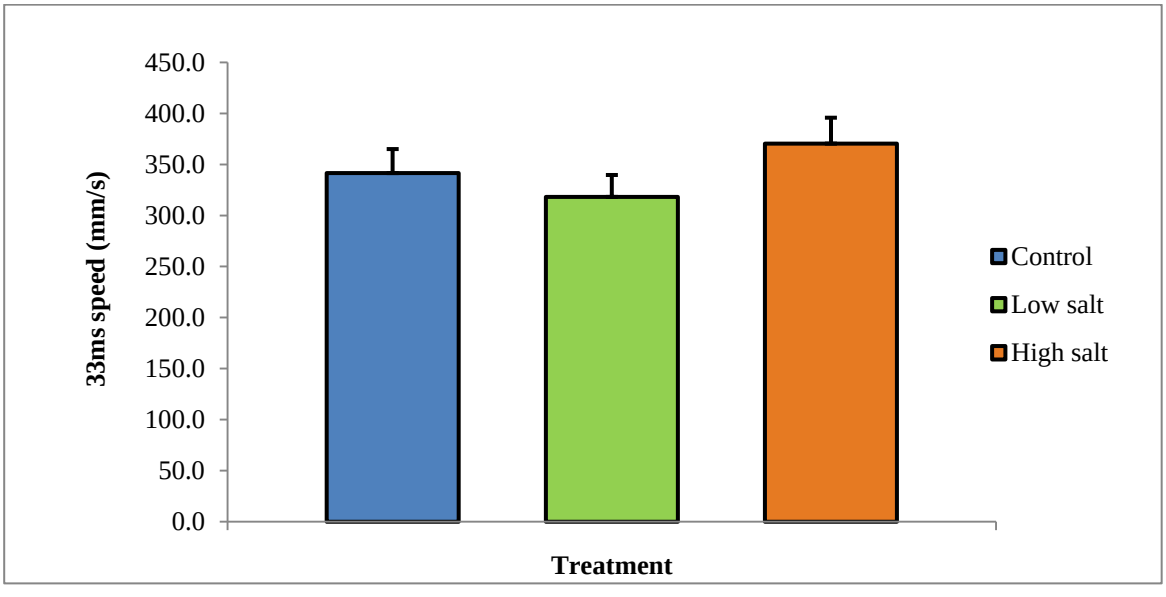

Figure 2. Comparison of 33ms burst speed (mean±SEM) over three salt treatments. Speeds varied from 342±24 mm/s for control tadpoles, 318±22 mm/s for low treatment tadpoles and 327±25 mm/s for high treatment tadpoles.

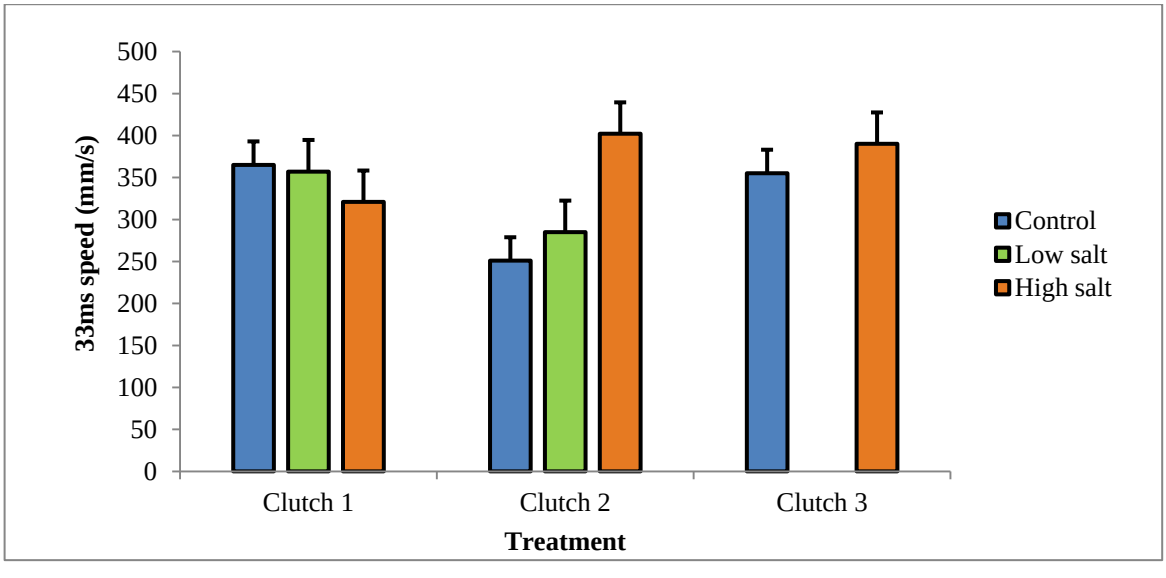

Figure 3. Thirty-three ms burst speed (mean+SEM) for individual clutches. Tadpoles from clutch 1 had 33ms speeds varying from 364±23 mm/s for the control, 357±38 mm/s for the low treatment and 321±37 mm/s for the high treatment. Tadpoles from clutch 2 had 33ms speeds varying from 251±28 mm/s for the control, 285±34 mm/s for the low treatment and 402±50 mm/s for the high treatment. Tadpoles from clutch 3 had 33ms speeds varying from 355±43 mm/s for the control and 390±45 mm/s for the high treatment. Tadpoles from the tank housing the low treatment clutch 3 tadpoles died and were therefore excluded from analysis.

Average speed over the first 100ms of the escape response

As with sqspeed1, no fixed or random effects significantly influenced speed2 overall or for individual clutches (figs. 4 and 5).

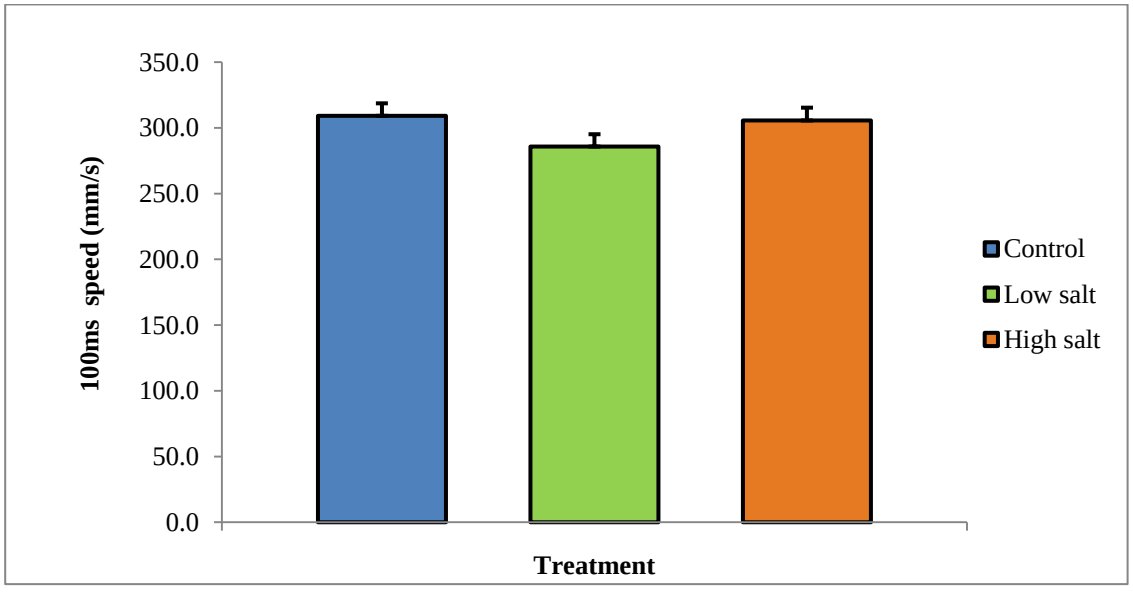

Figure 4. Comparison of 100ms speed (mean±SEM) over three salt treatments. The 100ms speed varied from 309±9 mm/s for the control, 286±9 mm/s for the low treatment and 306±10 mm/s for the high treatment.

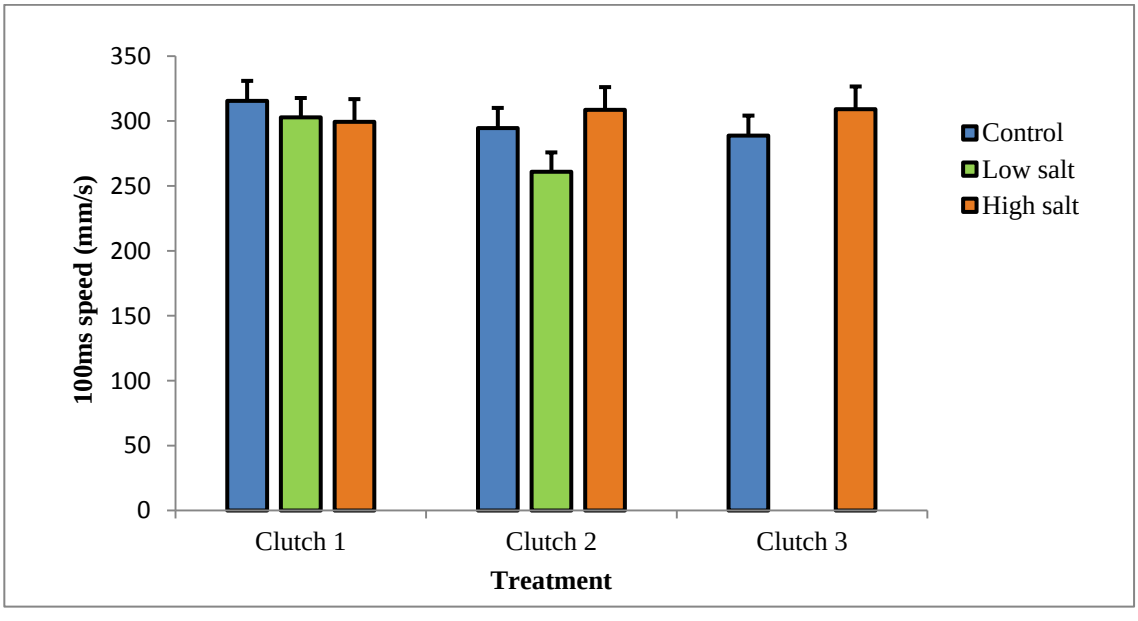

Figure 5. 100ms burst speed (mean±SEM) for individual clutches. 100ms speed for clutch 1 varied from 315±9 mm/s for the control, 303±15 mm/s for the low treatment and 299±17 mm/s for the high treatment. Clutch 2 100ms speed varied from 295±28 mm/s for the control, 261±17 for the low treatment and 309±18 mm/s for the high treatment. Clutch 3 100ms speed varied from 289±17 mm/s for the control and 309±16 mm/s for the high treatment. Tadpoles from the tank housing the low treatment clutch 3 tadpoles died and were therefore excluded from analysis.

Log transformed distance swam during escape response (lgdistance)

For combined clutches only trial had a significant effect on lgdistance (p=0.0002, fig. 7). The lgdistance swam by tadpoles in the first trial was significantly less than the lgsdistance of trial 2 and 3 (187±10 mm in trial 1 compared to 212±10 mm for trial 2 and 223±10 mm for trial 3). For clutch 3 treatment significantly affected the lgdistance (p=0.0012, fig. 8). Tadpoles from the control treatment swam 265±20 mm while tadpoles from the high treatment swam 151±10 mm.

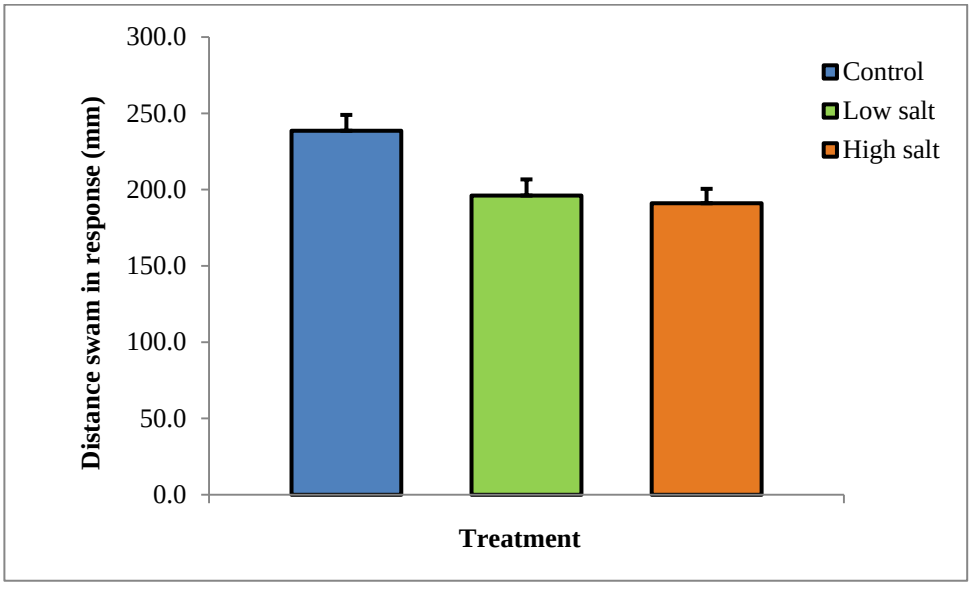

Figure 6. Comparison of the average distance covered in a startle response across three salinity treatments (mean±SEM). Response distance varied from 239±10 mm for the control, 196±11 mm for the low treatment and 191±9 mm for the high treatment.

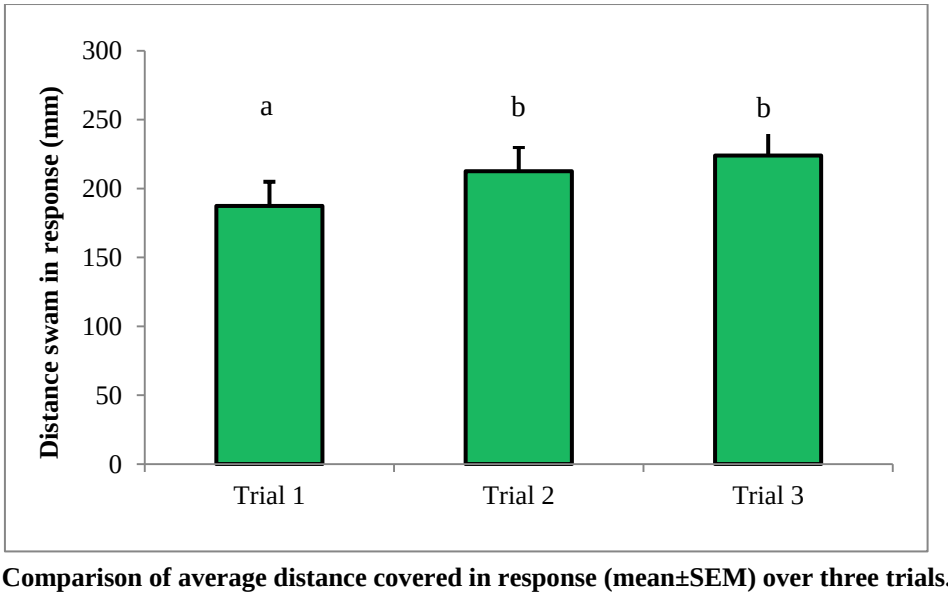

Figure 7. Comparison of average distance covered in response (mean±SEM) over three trials. The log of distance swam in the first trial (187±10 mm) was significantly shorter (p=0.0002) than the log of distance swam in trials 2 or 3 (212±10 mm and 223±10 mm respectively).

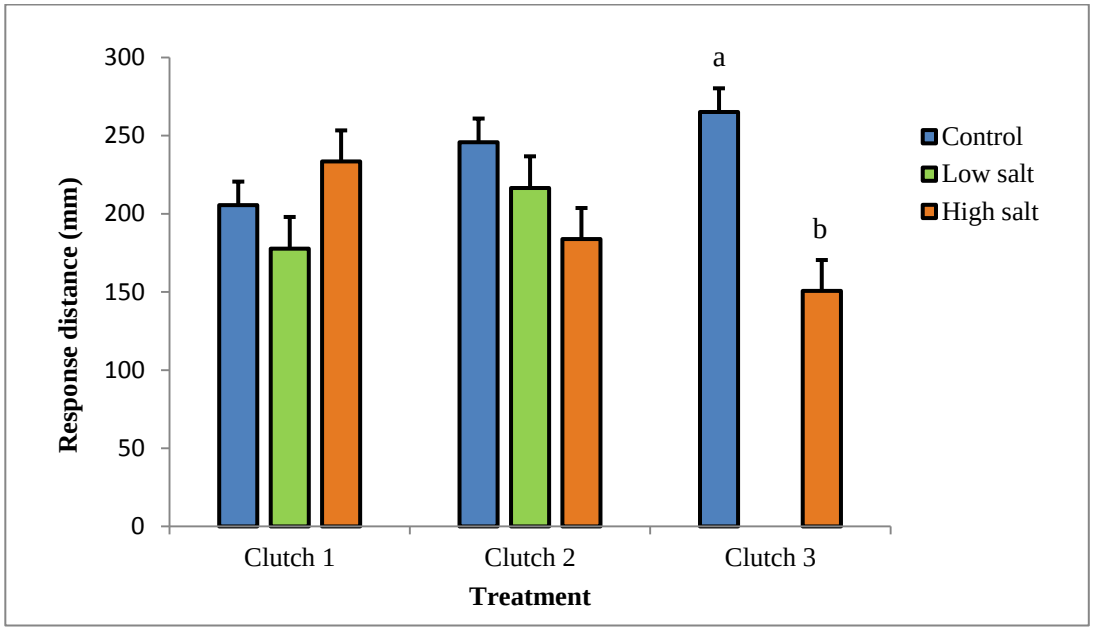

Figure 8. Average distance swam in response (mean + SEM) for individual clutches. Response distance for clutch 1 varied from 206±11 mm for control, 178±15 mm for the low treatment and 234±22 mm for the high treatment. Clutch 2 response distance varied from 247±15 mm for the control, 217±20 mm for the low treatment and 184±14 for the high treatment. Clutch 3 response distance varied from 265±20 mm for the control and 151±10 mm for the high treatment. Tadpoles from the tank housing the low treatment clutch 3 tadpoles died and were therefore excluded from analysis.

Mass, day 25 through 28

Overall tadpole mass from days 25 to 28 was not influenced by treatment, tank or clutch. Individually each clutch was affected by treatment but in varying ways (fig 9). Tadpoles raised in 800 mg/l NaCl had higher masses than tadpoles raised in control treatments for clutches 2 and 3. Tadpoles raised in 800 mg/l NaCl also had higher masses than tadpoles raised in 200 mg/l NaCl for clutches 1 and 2. For clutch 1 there was no difference between the control and 800 mg/l NaCl treatment. For clutch 2 there was no difference between the control and 200 mg/l NaCl treatment. No random effects or fixed effects other than treatment affect tadpole mass from day 25 to 28.

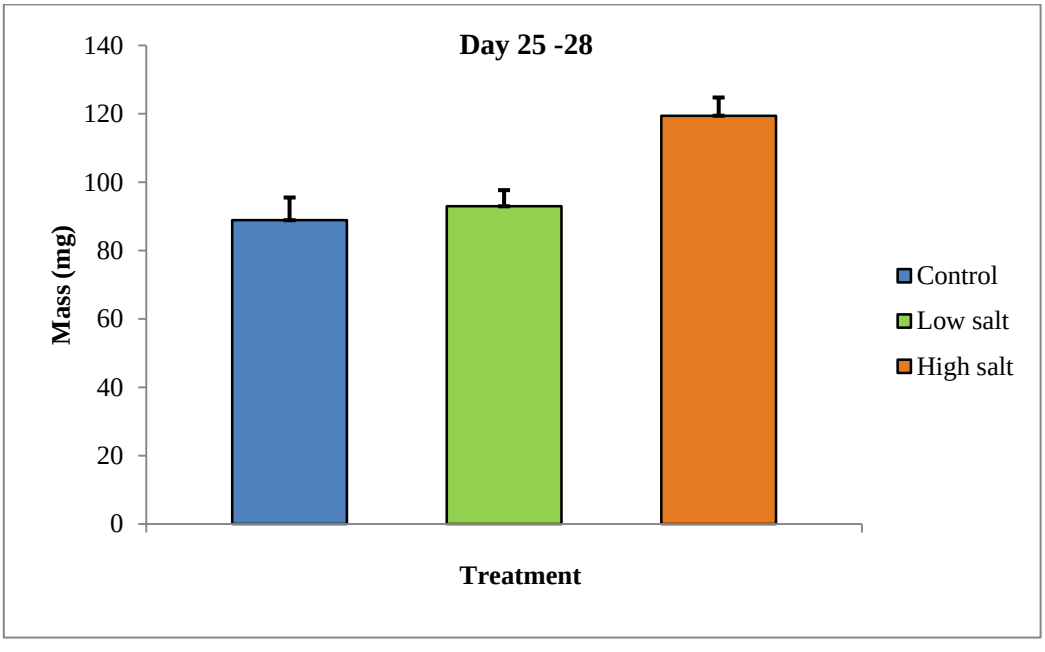

Figure 9. Differences in tadpole mass (mean±SEM) across three salt treatments for day 25-28. **Differences in tadpole mass (mean±SEM) across three salt treatments for day 25-28.** **Masses varied from 89±7 mg for the control, 93±5 mg for the low treatment and 119±5 mg for the high treatment.**

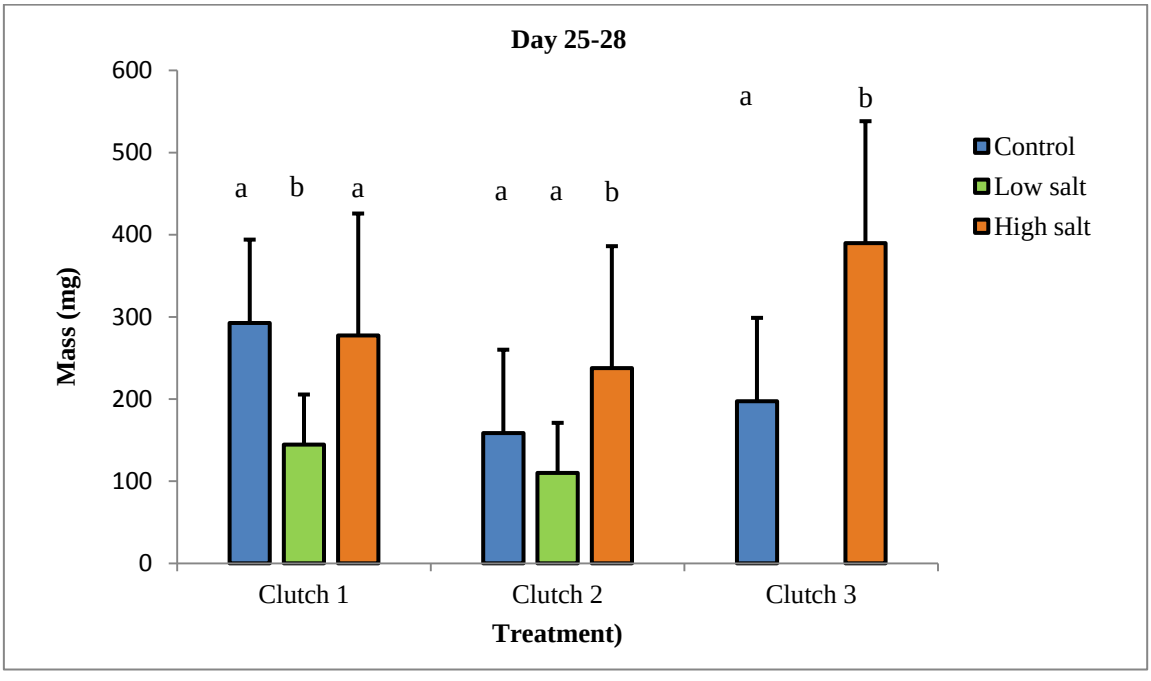

Figure 10. Differences in tadpole mass (mean±SEM) across three salt treatments for individual clutches. For clutch 1, average mass from the low treatment (145±61 mg) differed significantly (p_{CL}<0.0001 and p_{HL}<0.0001) from the control (293±115 mg) and the high treatment (277±148 mg). For clutch 2 the control (159±68 mg) and low treatment (110±26 mg) masses were significantly less (p_{CH}=0.002 and p_{HL}=0.0026) than the high treatment mass (238±120 mg). For clutch 3 the control mass (197±101 mg) was significantly smaller (p<0.0001) than the high treatment mass (390±193 mg). Tadpoles from the tank housing the low treatment clutch 3 tadpoles died and were therefore excluded from analysis.

Mass, day 148

By day 148 treatment had a significant effect on tadpole mass (p=0.0015, fig. 11). Tadpoles raised in the high salt treatment had significantly (p_{CH}=0.0061, p_{HL}=0.0013) higher masses (2510±163 mg) than those raised in the control or low) treatments (1260±202 mg 676±200 mg respectively).

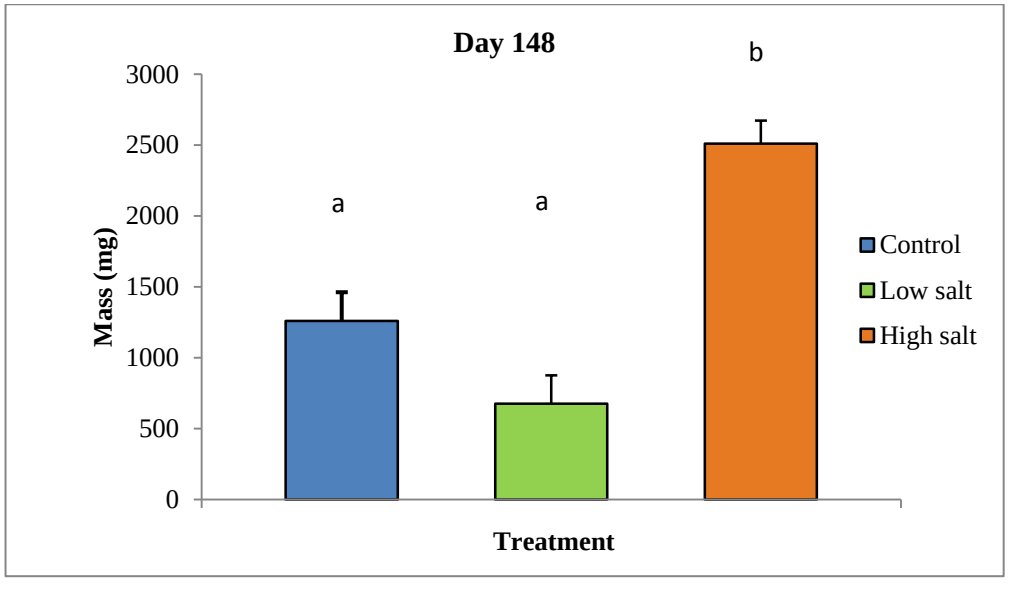

Figure 11. Differences in tadpole mass (mean±SEM) across three salt treatments for day 148. Differences in tadpole mass (mean±SEM) across three salt treatments, day 148. Tadpole masses from the control (1260±202 mg) and low treatment (676±200 mg) were significantly less (p_{CH}=0.0061, p_{HL}=0.0013) than the high treatment masses (2510±163 mg).

DISCUSSION

Anti-predator escape response

Salt level had no clear effect on any behavioral variable – 33ms speed, 100ms speed or distance swam. Slight effects of treatment were seen on the log transformed distance swam for clutch 3. Such clutch dependent results could suggest that the response to salinity of distance travelled may be a genetically variable trait but the validity of testing individual clutches is unclear. Watkins and Peek (2006) found that burst speed (in addition to growth rate and size) were highly heritable in *L. clamitans* larvae but no data on response distance was recorded.

If the difference in response distance is more than a coincidence it has the potential to significantly affect tadpole predation. Tadpoles in the control treatment of clutch 3 swam on average more than 40% (11 cm) further than tadpoles in the high salt treatment – an important difference depending on the hunting strategy of the predator and density of tadpoles. Strictly sit and wait predators will not likely see altered predation rates for an escape response shortened by 11 cm but the difference could be significant for certain dragonfly larvae species that engage in limited stalking (Pritchard 1964). Such a difference in response distance would also have an effect on predators which engage in extended pursuits of prey such as turtles.

Size

Expectedly, all morphological variables - mass, area, length and head width – were highly correlated (r square>0.9 for each combination of parameters). Therefore of the morphological variables only mass was tested using an ANOVA. Mass was considered a proxy for length, head width and area.

Treatment had no effect on mass from day 25 through 28 although fig. 9 suggests that tadpoles in the high treatments were more massive than control and low treatments. When examined by clutch, tadpoles raised in the low treatment were significantly smaller than tadpoles in the high treatments for clutches 1 and 2. The entire clutch 3 low treatment tank died so it is unknown if this trend would have extended to all clutches.

By day 148 treatment had a significant effect on tadpole mass. Tadpoles raised in high salt treatments had significantly higher masses than tadpoles raised in control or low treatments (p_{CH}=0.0061, p_{HL}=0.0013). The advantage of large tadpole size in preventing predation has been well documented. Tadpoles in the high salt treatment could therefore have higher fitness than tadpoles raised in a control or low salt environment. By day 148 though, tadpoles in the high treatment had an average length of 69.1±2.7 mm whereas tadpoles in the control and low treatments were 56.6±3.2 mm and 43.1±4.6 mm long respectively. These sizes are all above the threshold size for most predators that show a decreased predation rate for larger tadpoles (Brodie and Formanowicz 1983). At day 25-28 tadpoles ranged from 21.5 to 24.1 mm in length. For a significant ecological effect to be present the salinity would have to alter size at such a time that high treatment tadpoles are above a threshold size and low and/or control tadpoles are below the threshold size for common predators. Richards and Bull (1990b) found that the larval odonate *Hemicorduli tau* had significantly higher success hunting 10-14 mm long tadpoles opposed to tadpoles greater than 22 mm long although strike frequency was constant across sizes. For *H.*

tau then the difference in size between treatments occurs after all tadpoles are beyond the threshold size for predation. If tadpoles maintain the same growth rate that is seen up to day 28, until they are all greater than the threshold size for a predator (as was the case for *H. tau*) any effect on predation avoidance would be minimized.

Brodie and Formanowicz (1983) found that 0.5 cubic centimeter larval diving beetles *Dytiscus verticalis* preferentially consumed tadpoles with volumes between 0.3 and 0.5 cc when given the choice between tadpoles ranging from 0.05 cc and 1.0 cc. Beetles which were 1.0 cc preferentially consumed 1.0 cc *L. clamitans* tadpoles over 4.0 cc *L. clamitans* tadpole. Assuming tadpoles have a density approximately equal to that of water – 1000 mg/cc – the differences in mass (and presumably volume) seen at day 148 could affect predation rates of *D. verticalis*. Tadpoles living in 800 mg/l NaCl averaged 2510 mg or about 2.5 cc and therefore would have an advantage over the smaller control (~1260 mg or 1.3 cc) and 200 mg/l NaCl (~676 mg or 0.68 cc) treatments.

D. verticalis was the only predator (of newts and dragonfly naiads) to prey on larger tadpoles (Brodie and Formanowicz 1983). However *L. clamitans* and *L. catesbeianus* tadpoles were found to be the only two species that grew large enough to be excluded from predation by *D. verticalis*. *L. clamitans* eggs are also laid later in the season when aquatic insect populations are not as prevalent (Brodie and Formanowicz 1983). The potential for the size difference across treatments to affect predation is present but more detailed data on where the differences begin is needed. Additionally, the effects of increased salinity on the predators would also need to be determined.

Even if the increase in growth rate occurred after the threshold size is reached for all treatments, tadpoles with higher growth rate would likely have an advantage of having a larger size at metamorphosis or a shorter larval period, either of which would be beneficial to a species (Semlitsch 2003; Chinathamby et al. 2006). However, this prediction is limited when considering *L. clamitans* populations because tadpoles were not raised to metamorphosis.

Whether tadpoles simply bloated or actually had an increased growth rate in response to the 800 mg/l NaCl treatment is an important determination to make. Because tadpole length was strongly correlated with mass it is reasonable to conclude that increased masses of tadpoles in the 800 mg/l NaCl treatment (as of day 148) was, at least partially, a result of increased growth rate rather than strictly an increase in water mass.

It appears that low levels of NaCl (800 mg/L) have a stimulatory effect on tadpole growth. At some level, higher NaCl concentrations would likely cause reduced growth and emaciation as seen in salinity studies with different anuran species (Collins and Russell 2009; Chinathamby et al. 2006). Such a biphasic response of growth to a toxicant is common throughout many species and compounds (Newman 1998). At 800 mg/l *L. clamitans* is apparently capable of compensating (and possibly overcompensating depending on the degree that increased mass is a result of growth rate) for the elevated salinity by increasing their internal osmotic concentration to create a larger gradient than seen under freshwater conditions. After some point environmental salinity will be too high for the tadpole to compensate and the osmotic gradient will reverse causing water to be drawn out of the tadpole.

One explanation for increased growth rate in the presence of elevated salinity is that it is a mechanism to avoid drying ponds. As pond evaporation occurs in the summer salt is conserved and pond salinity will increase. Therefore there would be a selective pressure on tadpoles to speed up development when they encounter high salinities as it often indicates a disappearing aquatic habitat (Chinathamby et al. 2006).

Tadpoles are generally more susceptible to salinity levels than frogs. However studies on site selectivity have shown that adult anurans will avoid oviposition in ponds with elevated salinities (Karraker et al. 2008; Smith et al. 2007; Viertel 1999). Therefore tadpoles may not experience salt stress unless there is a significant influx after of salt after oviposition or if there are no other viable ponds within range of breeding adults. Early breeding species and species which stay in the larval stage for more than one year, such as *L. clamitans*, would be more susceptible to such changes.

CONCLUSIONS

We found that several months (148 days) of exposure to 800 mg/l NaCl caused an increased size and mass in *L. clamitans* tadpoles compared to tadpoles exposed freshwater and 200 mg/l NaCl. The differences in size were likely due to a combination of increased growth rate and increased internal osmotic concentration of the tadpoles in the 800 mg/l treatment compared to those in the control and 200 mg/l treatments. Differences in mass were not observed after 28 days of exposure. Increased size would likely be beneficial to tadpoles developing in waters of this concentration by increasing their chances of avoiding predation. However the effects of salinity on predators would also need to be determined to make an accurate prediction. Elevated salinity had no conclusive effect on anti-predator escape responses of tadpoles. No strong evidence was found that 200 mg/l NaCl to 800 mg/l NaCl would have a detrimental effect on survivorship of *L. clamitans* tadpoles.

APPENDIX A- Detailed materials and methods

Preliminary steps

(6/10/09) Nine glass 10-gallon tanks were washed with Alconox glass cleaner, rinsed five times, then washed with a 50% bleach solution and rinsed five times. Future mention of tank cleaning refers to the process just described. The tanks were stored in 100C Holtzinger building, Penn State University Altoona campus.

(7/21/09, 7/23/09) Three clutches of recently laid green frog eggs were collected in the field. One egg mass was collected from a human impoundment pond in Rothrock State Forest (N 40° 46' 12.5" W 77° 37' 38.8") on July 21, 2009 and two egg masses were collected from a human impoundment lake at Penn Roosevelt State Park (N 40° 43' 36.8" W 77° 42' 8.3") on July 23, 2009. Plastic bins used to collect the eggs (one bin per clutch) were previously rinsed away from the eggs five times with the pond water. Eggs were collected by sinking the bin into the pond near the clutch so that the eggs would slowly flow inside the bin. Eggs were stored in the bins indoors for one to three days before they were brought to the lab.

(7/21/09) Tap water was used to fill three plastic carboys. An aerator was submerged in each carboy in order to dechlorinate the water. The carboys were covered. Temperature equilibration with the laboratory and dechlorination continued for four days. Water taken from carboys mentioned in future steps was prepared using the same procedure described in this step.

(7/24/09) J.T. Baker sodium chloride crystal was added to the tanks to create a treated environment. Tanks 2, 4 and 9 received 6 grams of NaCl, tanks 3, 5 and 6 received 24 grams of NaCl and tanks 1,7 and 8 received no NaCl. After the addition of NaCl 30 liters of dechlorinated tap water was added to each tank. Water was removed from the carboy using a clean bucket and measured into two liter amounts in a graduated cylinder. Water was added to the tanks two liters at a time to ensure that the pouring of the water adequately dissolved the salt. There were now three treatments – a freshwater control, a 20 mg/l NaCl low treatment and a 800 mg/l NaCl high treatment – with three replicates of each treatment. The water levels were traced on each tank so that the 30 liter level would be known in the future without measuring out volumes. Salinities of each tank were measured using a model 30-10 FT YSI 30 salinity meter. Salinity measurements were always taken from the control tank first, then low treatment tanks and then high treatment tanks in order to avoid introducing extra salt into tanks.

Exposure period (begins on day 1)

Day 1 (7-24-09) - Eggs added to treatment tanks

> Eggs were transported to 100C Holtzinger building. About 40 eggs (approximately stages 15-20) were added to each tank such that each clutch comprised one replicate. Eggs were drawn out of the bin and released into the treated tanks using a cut pipette. The pipettes were cut at the tip such that the opening was wide enough to take in the egg

without constricting it. Future transfer of tadpoles using pipettes were carried out in this same manner. Extra eggs were kept in their bins in the same room as the tanks.

Day 4 (7/27/09)

All tadpoles in Tank 2 were found dead. The tank was emptied, cleaned and refilled with 200 mg/l NaCl. Forty of the remaining tadpoles left over from clutch 3 were used to restock tank 2. The rest of the tadpoles remaining in the bins were released.

Day 7 (7/30/09)

Most tadpoles had hatched by day 7. Waste and unhatched eggs were removed from a tank using a pipette and released into a graduated cylinder. Then a designated clean bucket was used to remove enough water such that the total volume in the cylinder was 1 liter. An appropriate amount of NaCl (200 mg for low treatment tanks and 800 mg for high treatment tanks) was added to a clean graduated cylinder. No salt was added to control tanks being cleaned. Water from a carboy was added slowly to the cylinder from a clean bucket such that the addition of the water dissolved the salt. The solution was slowly added to the tank in order to minimize disturbance to the tadpoles. This procedure was repeated for each tank. Salinities were measured for each tank starting with the control tanks, and ending with high treatment tanks. About two flakes of TetraMin tropical fish food were crushed and added to each tank. Aerators were turned on in tanks once the tadpoles were large enough to remain unaffected by the current. The room was then swept, mopped with neutral disinfectant and detergent and then dry mopped.

Beginning on day 8 (7/31/09) each tank was supplied with several rabbit food pellets every two to three days.

Beginning on day 14 (8/6/09) the following cleaning procedure was performed weekly:

Waste and uneaten food were syphoned from the tank until ten liters of solution were removed from each tank. Control tanks were syphoned first, then low treatment tanks then high treatment tanks to avoid introducing extra salt to tanks. Then an amount of salt equal to the amount in the removed ten liters (2 grams for the low treatment tanks and 8 grams for the high treatment tank) was added to a designated clean bucket and mixed with about five liters of dechlorinated tap water previously stored in the carboy. The solution was added to the appropriate tanks and then the water levels were brought back up to the 30 liter mark using freshwater from the carboy. More than ten liters were sometimes taken out of the freshwater tanks since salt was not required to be added to these tanks. After water changes the salinities of each tank was measured. The room was swept and mopped with neutral disinfectant and detergent and then dry mopped.

Day 14

All tadpoles in Tank 2 had died again. This tank was replaced with a new tank, and filled with 200 mg/l NaCl. The new tank (referred to as tank 2 from here on) was filled with 14 tadpoles from Tank 9 and 12 tadpoles from Tank 4 (the other two low treatment tanks). The old Tank 2 and two other tanks were cleaned and filled with water from the carboy.

These tanks are referred to as Tanks 10-12. About 25 tadpoles from the freshwater tanks (Tanks 1, 8 and 9) were moved into Tanks 10-12 using a cut pipette. Tank 10 received 26 tadpoles from Tank 8, Tank 11 received 25 tadpoles from Tank 7 and Tank 12 (old tank 2) received 25 tadpoles from Tank 1. Tadpoles were then removed from tanks 1-9 until each tank had about 25 tadpoles. Removed tadpoles were euthanized in a solution of 0.2 mg/l MS-222.

Day 21

Tadpoles in Tank 12 (old tank 2) were dying again. Survivors were moved into Tanks 10 and 11. Tank 12 was emptied and not used again.

Behavioral and morphological trials

Days 25 – 36 (8/17/09 through 8/28/09)

1. Behavioral trials

Tadpoles were randomly transferred from their tanks and placed in Petri dishes filled with 50 ml water of equal salinity to the tank in which they were raised. Petri dishes were 90 mm in diameter. The Petri dishes were placed on an index card with the tadpole number. The tank corresponding to each tadpole number was recorded. The card also contained a 13 mm piece of tape to be used as a reference scale. Twelve tadpoles, (one per dish) were arranged side by side. Tadpoles were given 30 minutes to acclimate to the Petri dish.

The set-up described above and behavioral testing were performed by different people in order to ensure that the tester was unaware of the treatments corresponding to the tadpoles. A camera was setup on a tripod so that the lens was directly over the first tadpole's dish. It was connected to a laptop to which 30 fps .wmv files were recorded using Window Movie Maker. The tadpole was given one minute to adjust after the camera was put into position. Video recording then began. Using a pipettor 150 µl of water was withdrawn from the Petri dish. The pipettor tip was moved into the water and positioned about two mm from the middle of the tadpole's head on the side of the tadpole closest to the edge of the Petri dish. This was done to ensure that the tadpole would swim away from the edge of the dish once stimulated. The pipettor was oriented so as to make an acute angle with the tadpole's tail. The water was then ejected into the side of the tadpole near the middle of the head in order to elicit the tadpole's escape response. Once the tadpole ceased swimming in response to the stimulation there was a 30 second rest period before the tadpole was stimulated again. Thirty seconds after the second escape response the tadpole was stimulated a third time. Recording ended after the end of the third escape response. If the tadpole was positioned along the edge of the Petri dish the tip of the pipettor was used to move the tail of the tadpole outward so as to expose the side of the tadpole closest to the wall. If this or any action other elicited a escape response a 30 second rest period was given before stimulating the tadpole. This process was then repeated for all twelve tadpoles. A new pipette tip was used for each tadpole.

2. Morphological data collection: After escape response trials were completed for the entire group of twelve tadpoles, each tadpole was positioned in the Petri dish so that its dorsal side was clearly visible and at least one picture was recorded.

3. Euthanasia: After each group of tadpoles was photographed they were transferred into a 2mg/ml tricaine mesylate (MS-222) solution. Tadpoles were kept in the MS-222 solution for at least 30 seconds after they stopped responding to prodding by a spatula.

4. Weighing: After each tadpole was euthanized they were removed from the MS-222 solution using a spatula and placed on a Kimwipe. The spatula was used to gently roll the tadpole over the Kimwipe to remove excess moisture. The spatula was then used to place the tadpole on a weigh boat. Mass was recorded to the one thousandth of a gram. The balance was re-calibrated for each tadpole.

5. Preservation: After each tadpole was weighed they were placed into labeled vials containing 4% paraformaldehyde in 0.1M phosphate buffer for 24 hours. After 24 hours the tadpoles were stored in 0.1 M phosphate buffer.

Behavioral trials were conducted for 36 tadpoles on day 25, 26, 27 and 28. At the end of day 28 sixteen tadpoles from each tank had been tested, photographed, weighed and preserved (144 tadpoles total). Eighteen tadpoles were tested on day 34 and 15 were tested on day 36. Tadpoles tested on days 34 and 36 were photographed but not weighed or preserved. Instead they were released back to their respective tanks.

Day 148: The remaining tadpoles were photographed, weighed, euthanized and preserved as described in steps 2-5.

Data extraction and analysis

1. Windows Movie Maker was used to cut each video into three video files each containing only one escape response. The first frame showing movement of the tadpole after the water stream was delivered was considered the beginning of the escape response. The end of the escape response was defined as the moment that one of these three events occurred first: (1) the tadpole stopped moving, (2) one second had passed after the tadpole's last lateral tail pump or (3) the tadpole's tail was relaxed (straight) for two consecutive frames (1/15 of a second) immediately before the tadpole reinitiated lateral tail movement. Video clips were ended at the end of the escape response.

2. Video analysis: Clipped .wmv were converted to .mov files using iSkysoft iMedia converter for Mac so that the files would be compatible with Image J. The following path was used to analyze the videos in Image J: File, Import, Using QuickTime, browse for .mov video file and the desired video was selected. This loaded the video of the escape response as an image stack. Then the path Plugins, Compile and Run, plugins, Manual_Tracking.class was selected. The Manual Tracking plugin was previously downloaded and added to Image J's plugin folder. In the Tracking window that subsequently appeared the Time Interval was set to 0.03333 seconds (corresponding to 30 frames per second). The x/y calibration was determined by using the Analyze,

Measure function to measure the how wide in pixels a known scale on the image (a 13 mm wide piece of tape for this experiment). The x/y scale was then entered as 13 mm/ x pixels where x is the number of pixels across the width of the tape. Scales were adjusted for each tadpole but assumed to be constant for the three trials of the same tadpole. Once the x/y scale was entered Add Track was selected on the Manual Tracking window. Tracking was accomplished by clicking on the outermost tip of the tadpole's head throughout the stack of images. This produced a spreadsheet containing the distance traveled and corresponding speed of the tadpole between the each frame. If over the course of the image tracking the tadpole was seen to perform one of the three definitions of an end to a escape response before the end of the image stack was reached then the tracking was ended.

3. Results were compiled into one spreadsheet. From there the total distance, speed travelled in the first frame, and average speed over the first three frames (100ms average speed) were recorded for each escape response.

Sometimes two or more adjacent frames were identical. As a result the distance and speed between the frames was recorded by ImageJ was zero. These repeated frames were followed or preceded by an abnormally large increase in tadpole displacement indicating that the repeated frame had taken the place of a frame showing intermediate displacement. When determining the speed travelled in the first frame and average speed over the first three frames the following procedure was used: A particular trial was discarded if second and third frames had a speed of zero as a result of repeated frames. As long as the first and second frames had nonzero speeds the first frame speed was recorded. If the second and third frame speed were zero then the first frame speed was not recorded for that trial. If the third frame speed was zero the average speed of the first four frames was used for the 100ms average speed. If the third and fourth frame speeds were zero the average of the first two frames was used. If the distance moved between the first and second frame was less than 1 mm it was not considered part of the escape response and frames 2-4 were treated as frames 1-3. These procedures were used in order to reduce the effects of repeated frames on the data.

4. Image analysis: Photoshop to extract the surface area, head width and length of each tadpole from digital images.

APPENDIX B – SAS 9.2 code

Table 4 shows the code used to introduce a data set spread sheet saved as a comma delimited file (.csv) into the SAS program. All lines of code are ended with a semicolon. The first line of code titles the dataset 'tadpole.' The second line specifies the file path of the data set. 'firstobs2' signifies that the first row of the dataset are column titles. The column titles are included in the fourth line. Non-numeric titles are followed by a $. Pond (from which the eggs were collected was a category recorded in the dataset but not considered a variable in the analysis. The lines 'sqspeed1=sqrt(speed1)' and 'lgdistance=log(distance)' define the new transformed variables described in the Materials and Methods. The last line displays the dataset just created in SAS.

Table 4. SAS code used to create and display data set

```
DATA tadpole;
INFILE "C:\Documents and Settings\George\Desktop\SAS Data.csv" dsd missover
delimiter=',' firstobs=2;
INPUT Sample Area Length Width Mass Tank Treatment$ Clutch Distance Speed1 Speed2 Trial
Pond Day Latency;
sqspeed1= sqrt(speed1);
lgdistance=log(distance);
proc print data=tadpole (obs=200); run;
```

In order to remove the redundant measures of trials 2 and 3 when analyzing morphological parameters a new dataset was created from the original set, tadpole (Table 5). The line 'IF trial > = 2 THEN Delete' removes trials 2 and 3 from the original dataset. This dataset, titled tadpole2, was used when performing analysis on morphological variables.

Table 5. SAS code used to create and display morphology dataset

```
Data tadpole2;
SET tadpole;
IF trial > = 2 THEN Delete;
Run;
proc print data=tadpole2(obs=10); run;
```

Table 6 alters the morphological dataset coded for in Table 5 by removing all data corresponding to a day later than 28. This is the dataset analyzed when mass, day 25-28 is considered.

Table 6. SAS code used to create the dataset of morphological variables for day 28 and before

```
Data TM25;
SET tadpole2;
IF day>28 THEN Delete;
Run;
proc print data=TM144(obs=10); run;
```

Table 7 alters the morphological dataset coded for in Table 6 by removing all data that was not measured on day 148.

Table 7. SAS code used to create the dataset of morphological variables for day 148

```
Data TM148;
SET tadpole2;
IF day < 148 THEN Delete;
Run;
proc print data=TM148 (obs=10); run;
```

Table 8 shows the code to create datasets for individual clutches. 'IF clutch > = 2 THEN Delete' could be replaced with 'IF clutch = 1 THEN Delete; IF clutch = 3 THEN Delete' and 'IF clutch <3 THEN Delete' to create datasets for clutches 2 and 3 respectively. To make clutch specific datasets for morphological analysis the set specified in line 2 is tadpole2, the dataset without trials 1 and 2.

Table 8. SAS code used to create clutch specific datasets

```
Data clutch1;
SET tadpole2;
IF clutch > = 2THEN Delete;
Run;
proc print data=clutch1(obs=10); run;
```

Correlations of variables are determined using the code in Table 9. Variables tested for correlation are including after the 'VAR' cue. The code from Table 9 produces the information seen in tables 2 and 3.

Table 9. SAS code used to produce Pearson correlation coefficients

```
proc corr data=tadpole2;
VAR Area Length Width Mass;
run;

proc corr data=tadpole;
VAR speed1 speed2 distance length;
run;
```

The analysis of variance is performed using the code in Table 10. The two first and two last lines of the code are for aesthetic purposes of the output display. All variables to be considered by the model are listed after 'class' in the fourth line of code. In the fifth line, after the 'model' the response variable (in this case speed1) is listed. After the response variable and an '=' the fixed effects and interaction terms are listed. Every two way and three way combination of fixed effect are listed and separated by an '*.' Random effects are listed after the word 'Random' in the sixth line. The 'Random' line defines variation between tadpoles. The seventh line specifies that trial is a repeated measure and 'type=cs' defines that the covariance between trials is compound symmetry (see Table 12 and Table 13). This defines the covariation within tadpoles (Littell et al. 1998).

The code in Table 10 will also display the Studentized residual graphs seen in Appendix C which are used to check that the normality and homoscedasticity conditions for the ANOVA are met.

Table 10. SAS code for performing ANOVA on speed1

```
ODS html;
ODS graphics on;
proc mixed data=tadpole covtest;
        class sample tank treatment clutch trial day;
        model speed1=treatment trial day treatment*trial treatment*day trial*day
treatment*trial*day/residual;
        random tank clutch;
        repeated trial/subject=sample type=cs;
run;
ODS graphics off;
```

The code in Table 10 will output Table 11. When the p value (last column in table 11) of the three way interaction (treatment*trial*day) was above the critical α the interaction term was deleted from the code and the analysis was performed again. Then two way interactions were deleted from the code, beginning with the interaction with the highest p value above the critical α, until no interaction terms remained or the interaction term with the highest p was below α. For all the data analyzed in this experiment no interactions were significant.

Table 11. Fixed effects table produced by code from table 10

Type 3 Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Treatment	2	390	0.43	0.6529
Trial	2	390	2.16	0.1166
Day	5	390	1.69	0.1348
Treatment*Trial	4	390	1.7	0.1492
Treatment*Day	10	390	0.83	0.6037
Trial*Day	10	390	1.16	0.3194
Treatment*Trial*Day	20	390	1.55	0.0631

Trial was the repeated measure and an appropriate covariance structure between trials was determined by replacing the seventh line from Table 10 with each of the lines from Table 12 individually. Three covariance structure types are possible: unstructured (un), compound symmetry (cs) and autoregressive (ar(1)). The option with the smallest Bayesian information criterion (BIC),compound symmetry for this dataset, gives the best covariance structure for the repeated measures. The BIC is given as an output under the title 'Fit Statistics' (Table 13).

Table 12. SAS code to determine the goodness of fit for three covariance structures

```
repeated trial/subject=sample type=cs;

repeated trial/subject=sample type=un;

repeated trial/subject=sample type=ar(1);
```

In this case compound symmetry (cs) gives the lowest BIC and was therefore chosen as the covariance structure for trial.

Table 13. Goodness of fit for three covariance structures

Autoregressive	-2 Res Log Likelihood	3096.6
	AIC (smaller is better)	3102.6
	AICC (smaller is better)	3102.6
	BIC (smaller is better)	3102.8
Compound symmetry	-2 Res Log Likelihood	3095.8
	AIC (smaller is better)	3101.8
	AICC (smaller is better)	3101.9
	BIC (smaller is better)	3102.1
Unstructured	-2 Res Log Likelihood	3092.1
	AIC (smaller is better)	3106.1
	AICC (smaller is better)	3106.3
	BIC (smaller is better)	3106.6

The SAS output for significance of random effects (also produced by the code in Table 10) is given in a table titled 'Covariance parameter estimates.' The values given can be made more accurate using the -2 Res Log Likelihood (given in Table 13) of the complete mixed model and then of the mixed model with the random effect of concern removed from the 'class' and 'random' line in Table 10. Table 14 gives the code to perform a likelihood ratio test on random effects. The code will output a table with a title specified by the purple text after 'title1' and a corrected p value for the random variable tested. LLfull is the 2 Res Log Likelihood of the complete model (given in Table 13) and LLreduced is the 2 Res Log Likelihood of the model when the random effect of concern is removed from the model (given in the equivalent of table 53 when the model is run without the random effect for which a p value is being determined). '%let' defines the variables LLfull and LLreduced which must be manually input for each random effect and explanatory variable. This method assumes the test statistic (tst1 in Table 14) follows a chi-square distribution and corrects for the boundary problem for variances by dividing the determined probability by two, corresponding to the two sides of the boundary. Table 14 must be used separately for every random variable of every ANOVA.

Table 14. SAS code to determine significance of random effects

```
%let LLfull = 3102.1;
%let LLreduced = 3484.1;

data LRT;
tst1 = (&LLreduced - &LLfull);
df = (1);
prob1 = 1 - probchi(tst1,df);
Pvalue = (prob1/2);
proc print; title1 'LRT for sqspeed1 clutch'; run;
```

Table 15 through Table 20 are the code to perform an analysis of variance on the rest of the explanatory variables. When morphological variables are tested the dataset is changed to the appropriate morphological dataset. Clutch specific code is left out. These would just include using the clutch specific dataset in the 'data= ' line and removing clutch as a variable in the 'class' line and 'random' line. Interactions were already removed because none of the terms were significant for this particular dataset.

Table 15. SAS code for performing ANOVA on the square root transformed 33ms speed

```
ODS html;
ODS graphics on;
proc mixed data=tadpole cov test;
        class sample tank treatment clutch trial day;
        model sqspeed1=treatment trial day/residual;
        random tank clutch;
        repeated trial/subject=sample type=cs;
run;
ODS graphics off;
ODS html close;
```

Table 16. SAS code for performing ANOVA on the 100ms speed

```
ODS html;
ODS graphics on;
Proc mixed data=tadpole cov test;
        class sample tank treatment clutch trial day;
        model speed2=treatment trial day/residual;
        random tank clutch;
        repeated trial/subject=sample type=cs;
run;
ODS graphics off;
ODS html close;
```

Table 17. SAS code for performing ANOVA on distance

```
ODS html;
ODS graphics on;
proc mixed data=tadpole cov test;
        class sample tank treatment clutch trial  day;
        model distance=treatment trial day/residual;
        random tank clutch;
run;
ODS graphics off;
```

Table 18. SAS code for performing ANOVA on the log transformed distance

```
ODS html;
ODS graphics on;
proc mixed data=tadpole cov test;
        class sample tank treatment clutch trial  day;
        model lgdistance=treatment trial day/residual;
        random tank;
        repeated trial/subject=sample type=cs;
        lsmeans trial / adjust=tukey;
run;
ODS graphics off;
ODS html close;
```

Table 19. SAS code for performing ANOVA on mass, day 25-28

```
ODS html;
ODS graphics on;
proc mixed data=TM25 cov test;
        class sample tank treatment clutch  day;
        model mass=treatment day/residual;
        random tank clutch;
run;
ODS graphics off;
ODS html close;
```

Table 20. SAS code for performing ANOVA on mass, day 148

```
ODS html;
ODS graphics on;
proc mixed data=TM148 cov test;
        class sample tank treatment clutch  day;
        model mass=treatment day/residual;
        random tank clutch;
run;
ODS graphics off;
ODS html close;
```

When a fixed effect is significant the line in Table 21 is included in the ANOVA code before the line 'ODS graphics off;'. 'Treatment' in Table 21 would be replaced with the significant fixed effect. Adding this line will perform a multiple comparison test with a Tukey adjustment to determine which of the means of the particular explanatory variable significantly differ for different fixed effect levels. Table in Appendix D is an example of the output the code from Table 21 will produce.

Table 21. SAS code for performing multiple comparison test

```
lsmeans treatment / adjust=tukey;
```

APPENDIX C – Preliminary statistics

Graphical studentized residuals were created in SAS using the code from table 10 with the appropriate variable include after 'model' in the fifth line (appendix B) in order to check that each response variable had normally distributed residuals and variances of the different groups were equal (data was homscedastic). Distributions were considered normal when the data points of the residual vs quantile plot (lower left corner of figures) fell along the diagonal line. Data was considered homoscedastic when no clear pattern could be seen along the residual versus predicted means plot (upper left corner of the figures).

Speed1 (33ms speed) did not meet the conditions of normality and homoscedasticity. The residual versus predicted mean data spreads as the predicted mean increases. The residual normality plot also significantly deviate from a perfectly normal distribution (Figure 12).

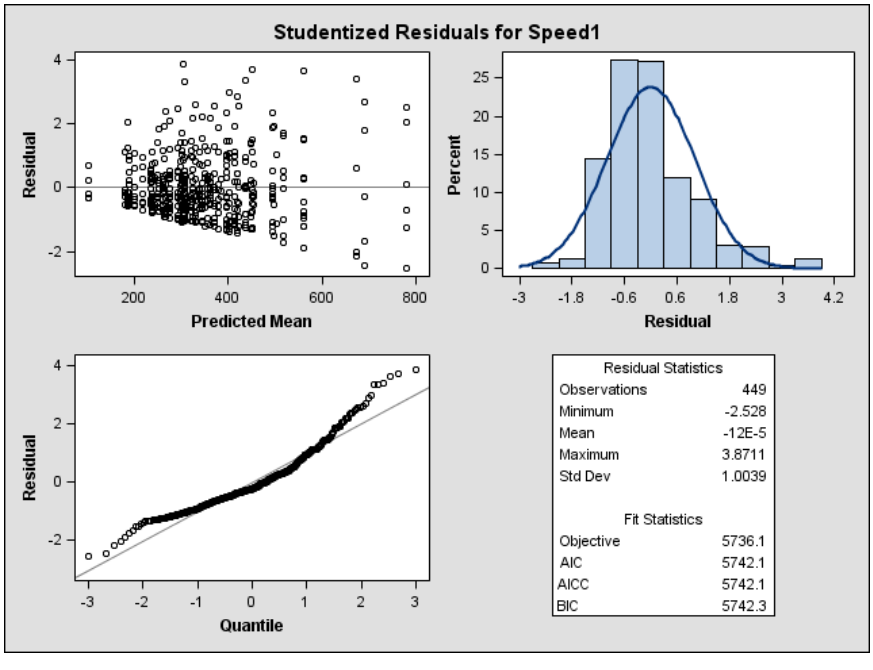

Figure 12. Studentized residuals for 33ms burst speed

After performing a square root transformation on speed1 the transformed data fit the conditions of normality and homoscedasticity (Figure 13).

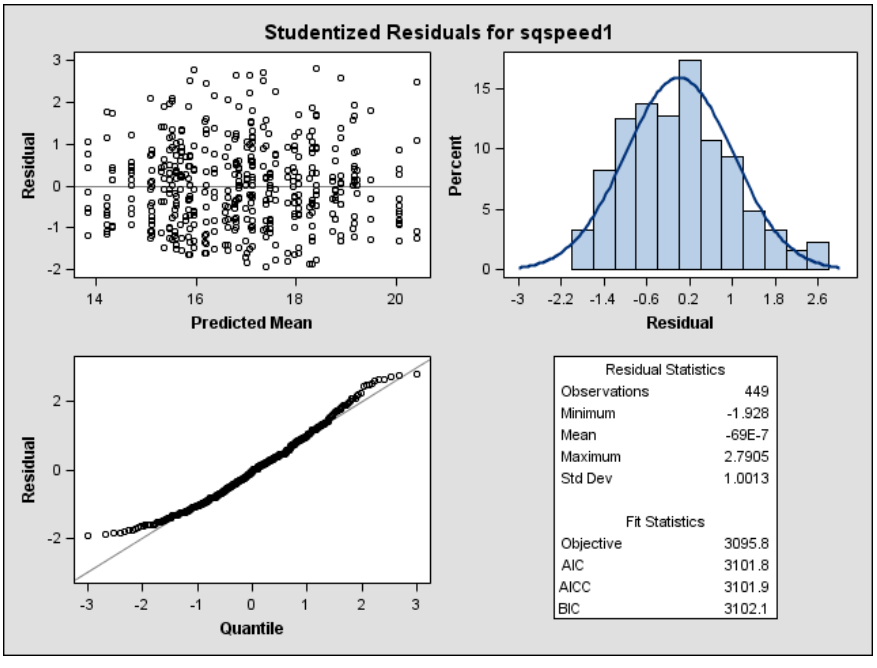

Figure 13. Studentized residuals for square root transformed 33ms speed (sqspeed1)

Speed2 (100ms speed) fulfilled the conditions of normality and homoscedasticity without the need of a transformation (Figure 14).

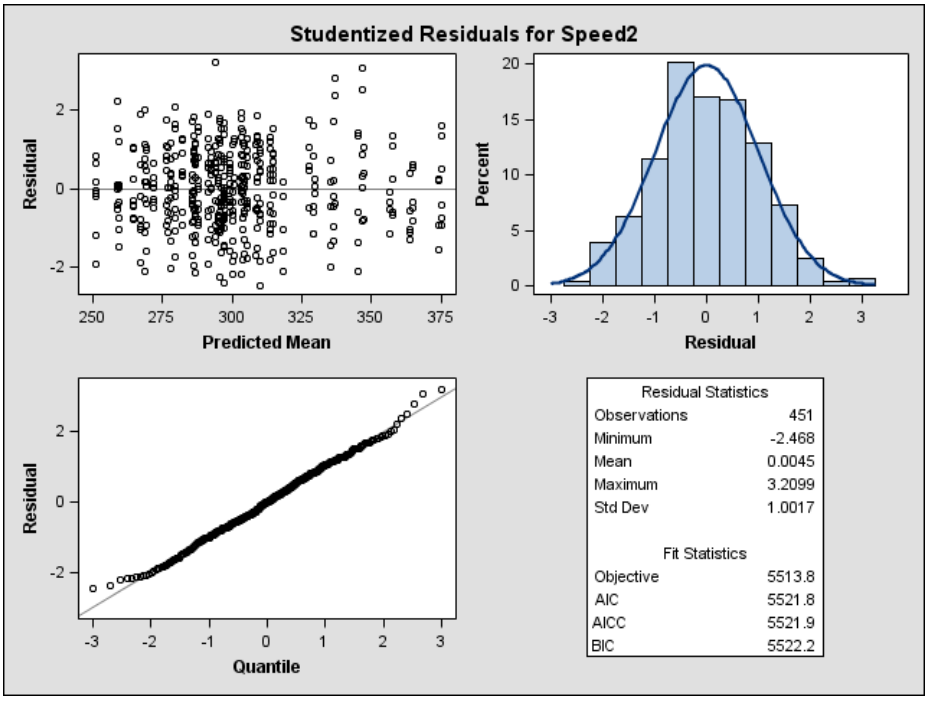

Figure 14. Studentized residuals for 100ms speed

Like speed2, distance was neither normal nor homoscedastic (Figure 15) until it was log transformed (Figure 16).

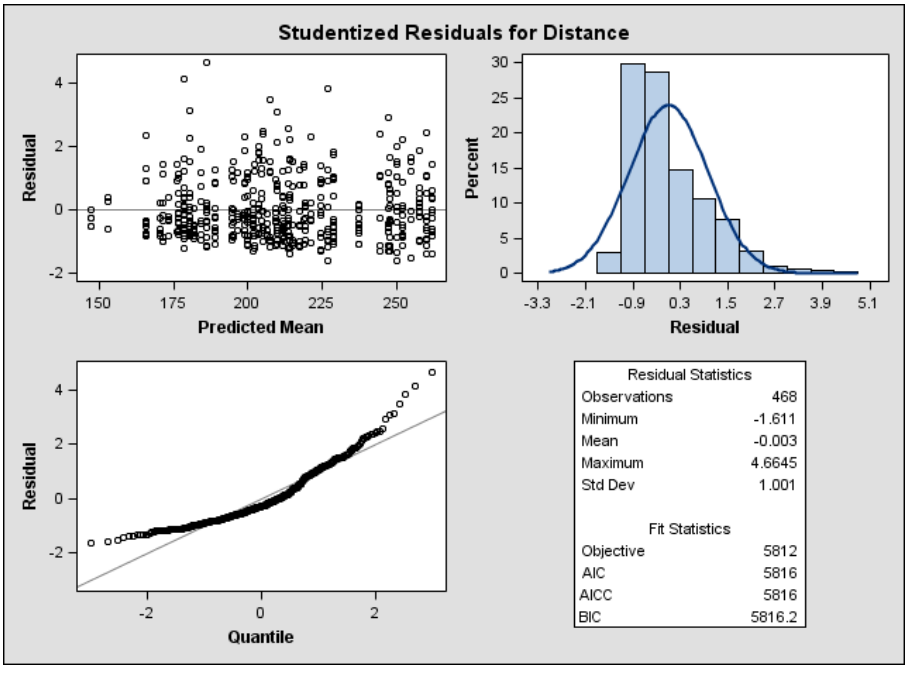

Figure 15. Studentized residuals for distance

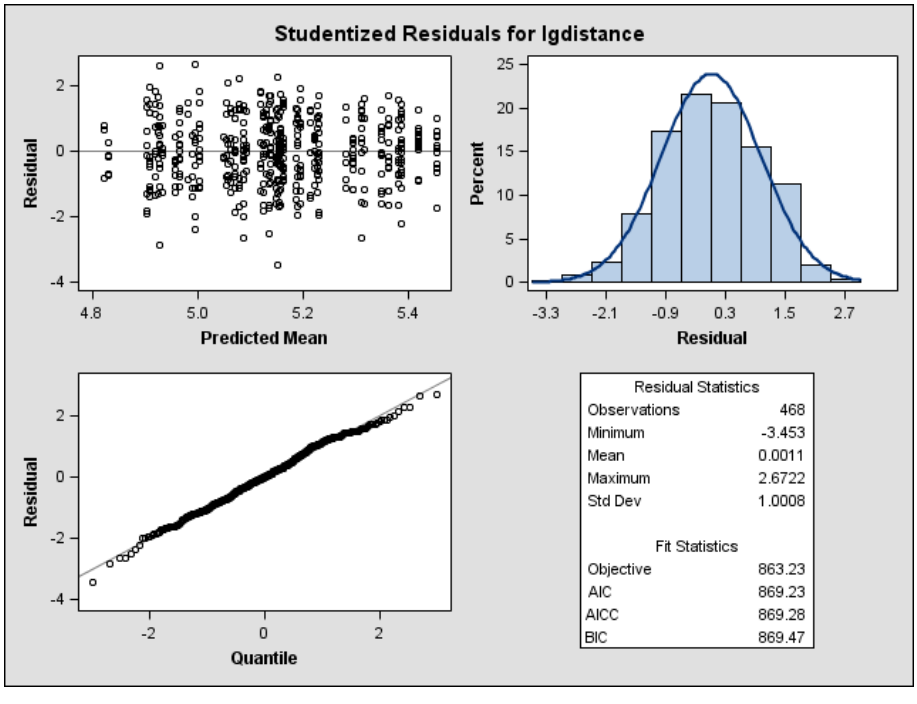

Figure 16. Studentized residuals for log transformed distance (lgdistance)

When considered as a whole, the mass measurements were highly non normal and heteroscedastic because of the 100+ day period in between measurements in which the tadpoles grew significantly (Figure 17). Consequently the decision was made to consider mass measurements in two groups: one consisting of measurements taken from day 25 through 28 (Figure 18) and one consisting of measurements taken on day 148 (Figure 19). When considered in this manner both groups were normal and homoscedastic. Mass measurements taken on day 148 deviate more than other variables because only 16 observations are considered. Because the ANOVA is tolerant of deviations from its assumptions of normality and homoscedasticty these are considered acceptable distributions.

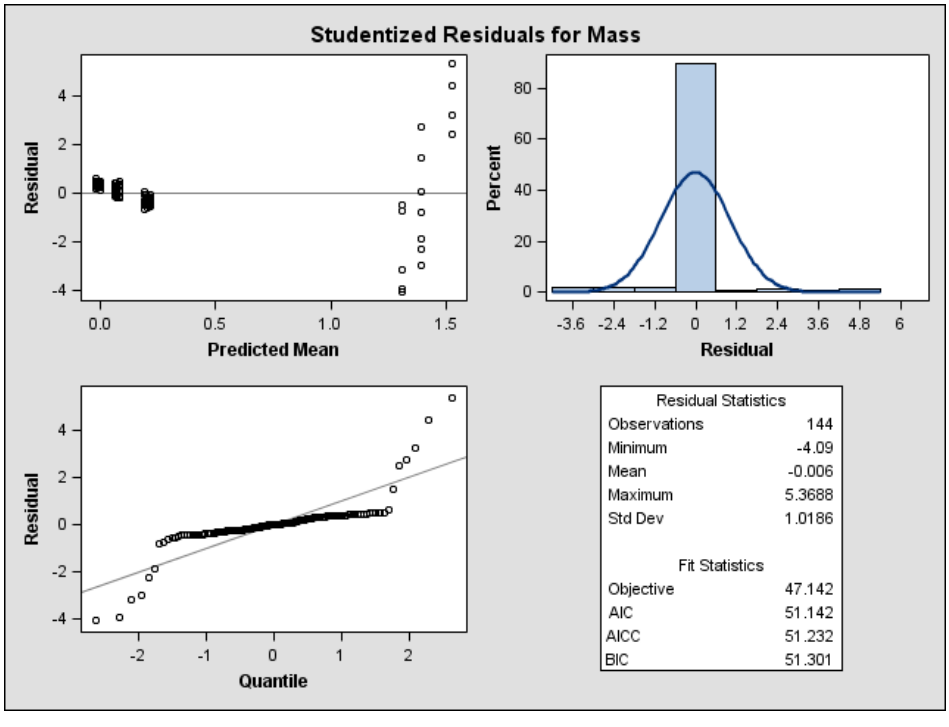

Figure 17. Studentized residuals for all mass

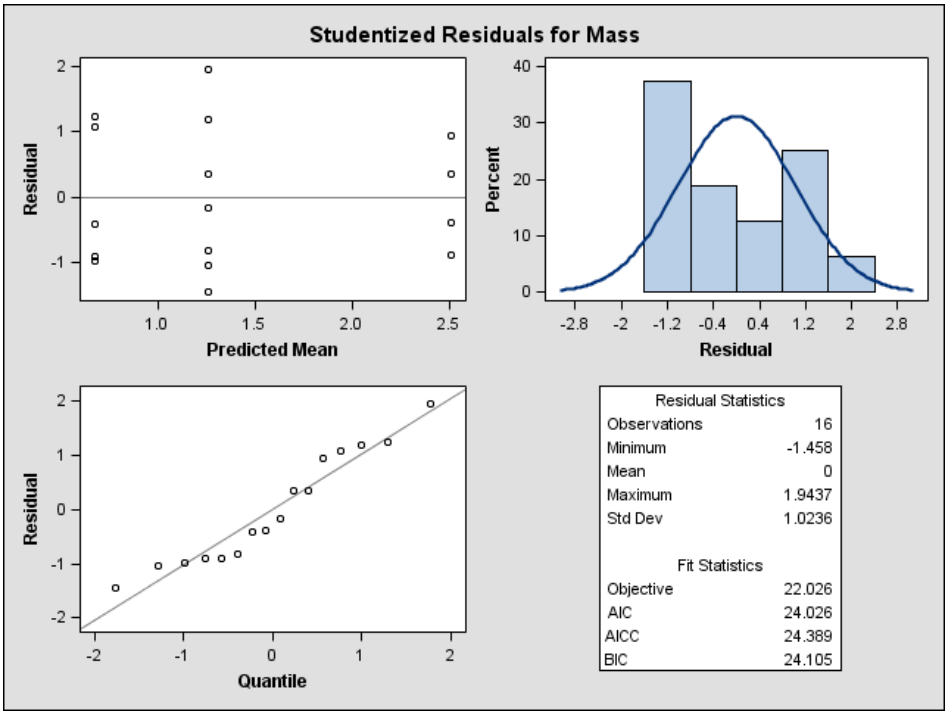

Figure 18. Studentized residuals for mass, day 25-28

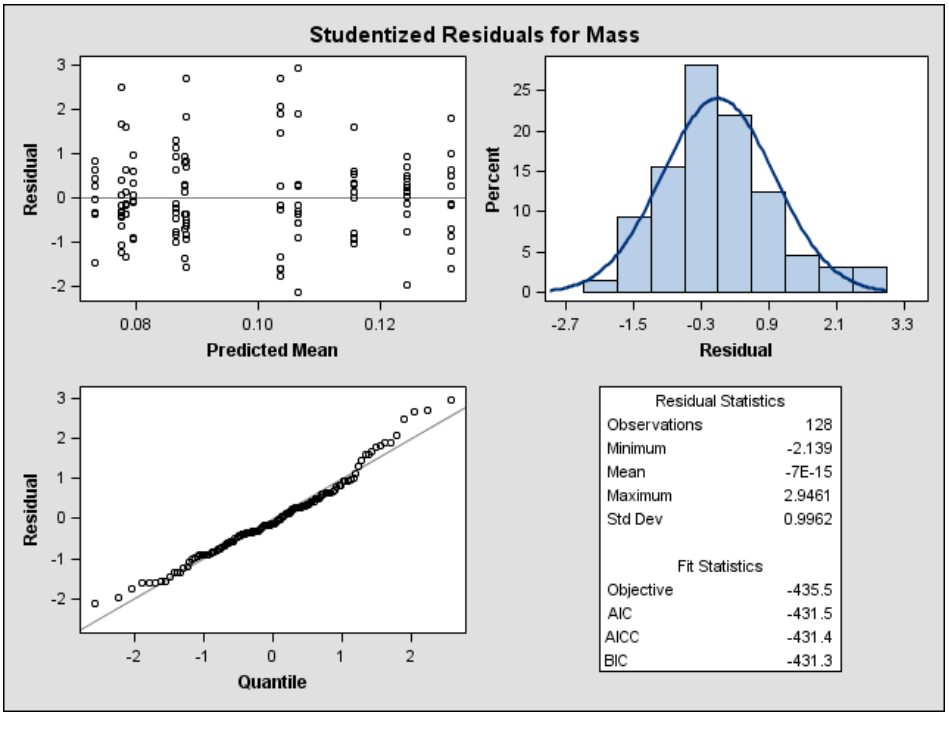

Figure 19. Studentized residuals for mass, day 148

Figure 20 through Figure 31 show the studentized residuals for the square root transformed 33ms speed, 100ms speed, log transformed distance and mass from day 25 through 28 for individual clutches. Mass data taken on day 148 was not analyzed by clutch due to inadequate sample size.

Clutch 1

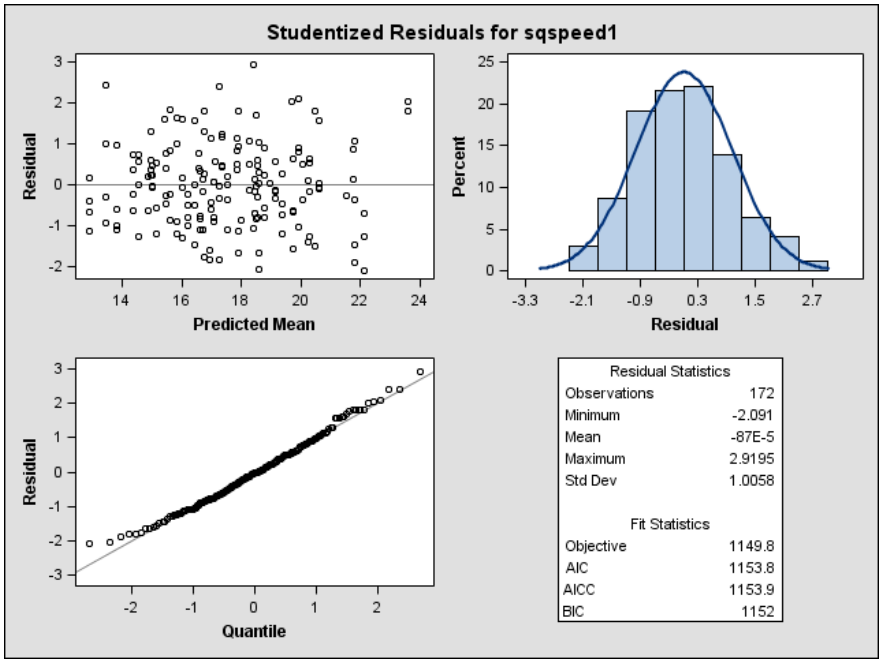

Figure 20. Studentized residuals for square root transformed 33ms speed, clutch 1

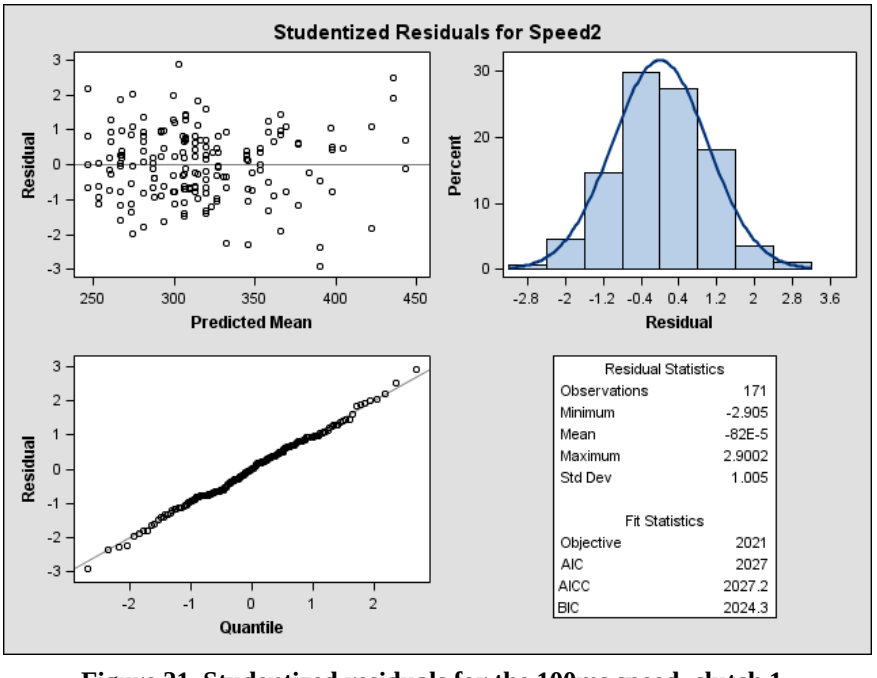

Figure 21. Studentized residuals for the 100ms speed, clutch 1

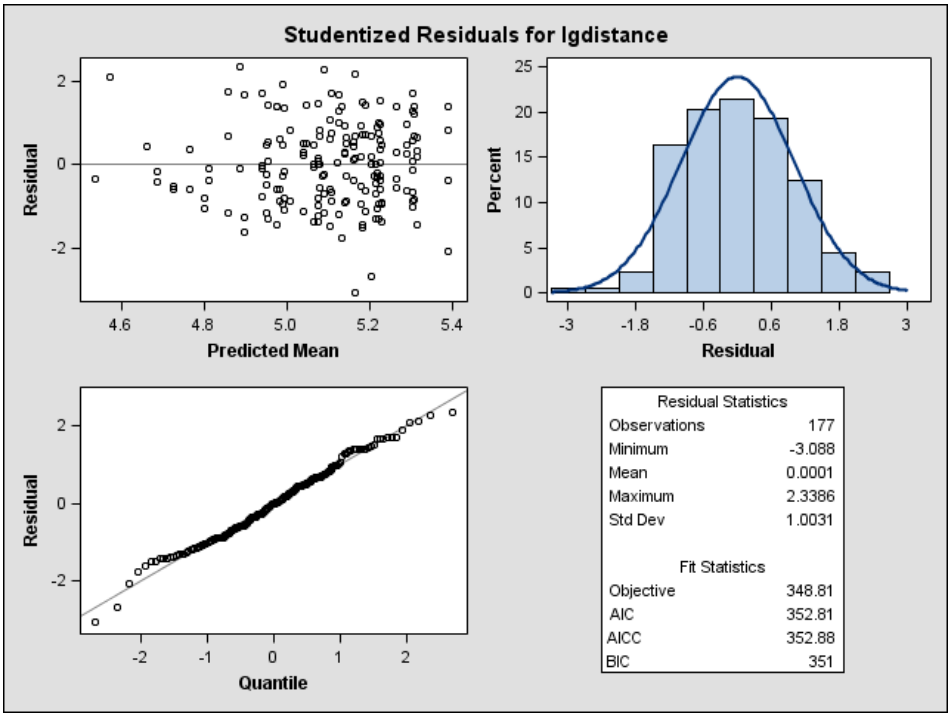

Figure 22. Studentized residuals for the log transformed distance swam, clutch 1

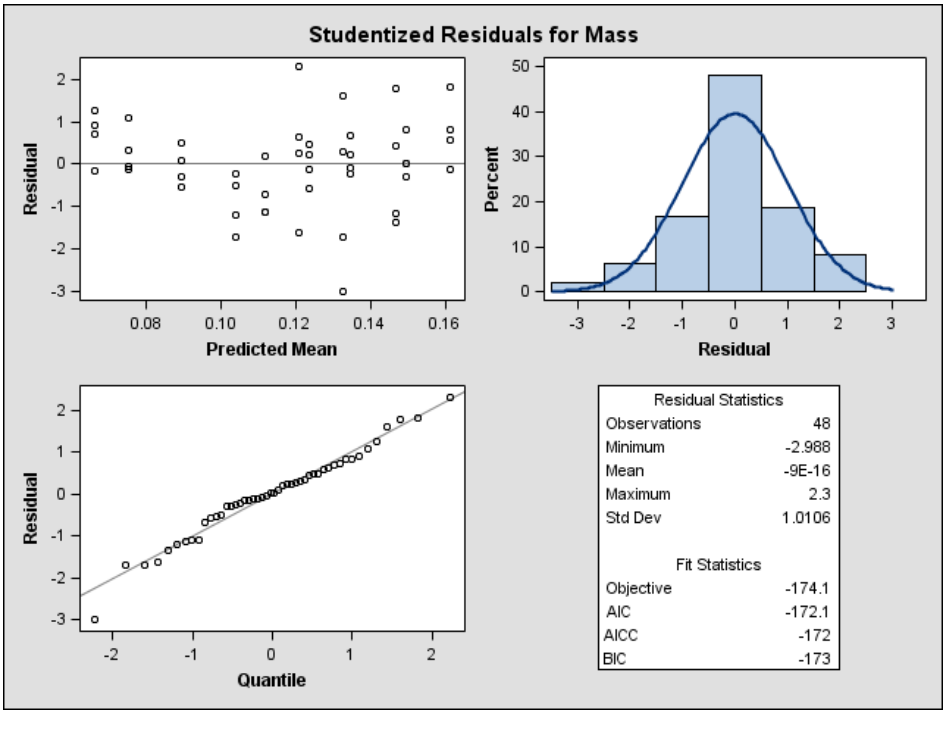

Figure 23. Studentized residuals for mass day 25-28, clutch 1

Clutch 2

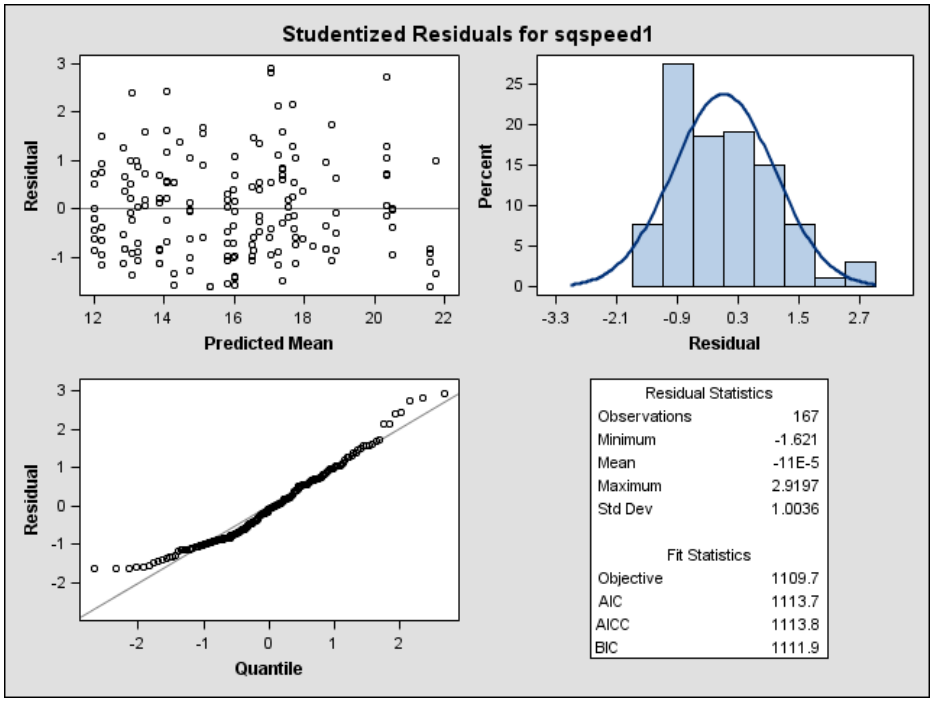

Figure 24. Studentized residuals for square root transformed 33ms speed, clutch 2

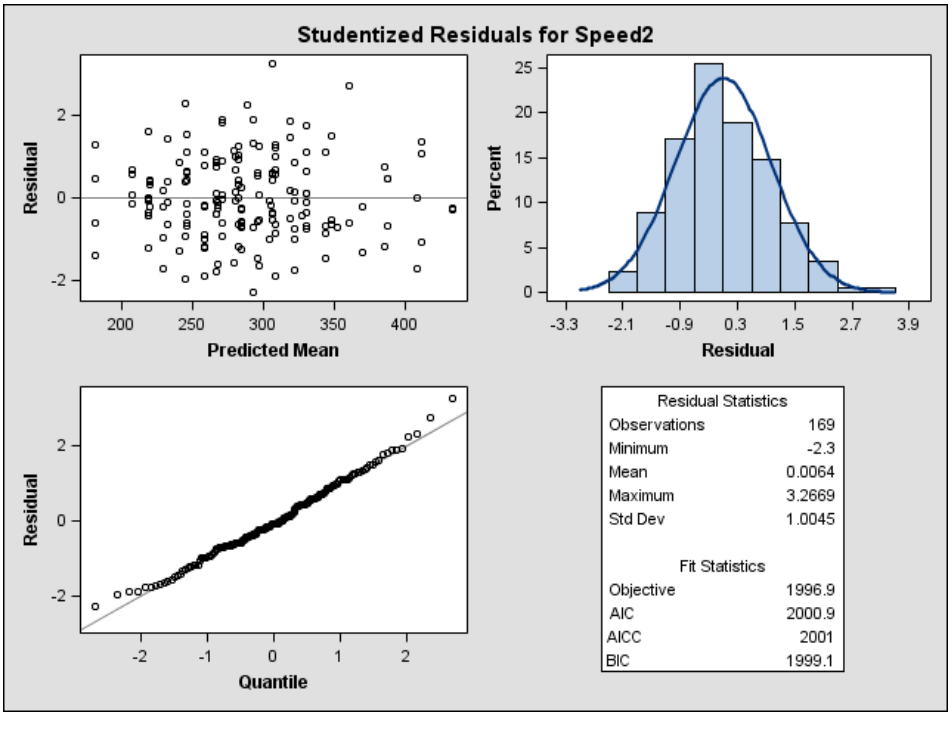

Figure 25. Studentized residuals for 100ms speed, clutch 2

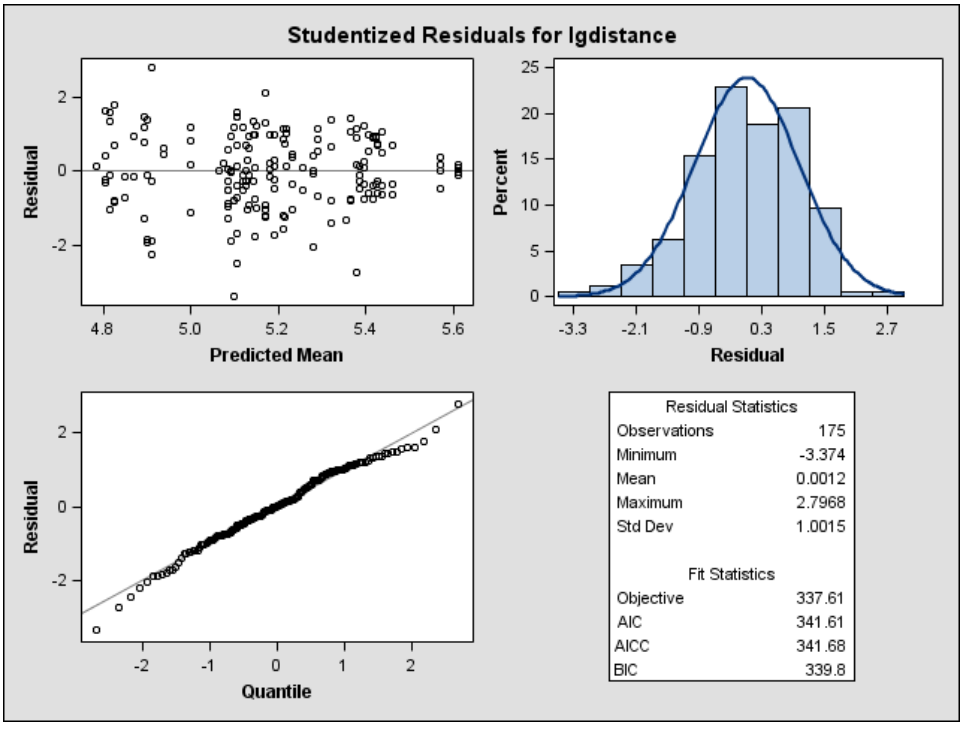

Figure 26. Studentized residuals for log transformed distance swam, clutch 2

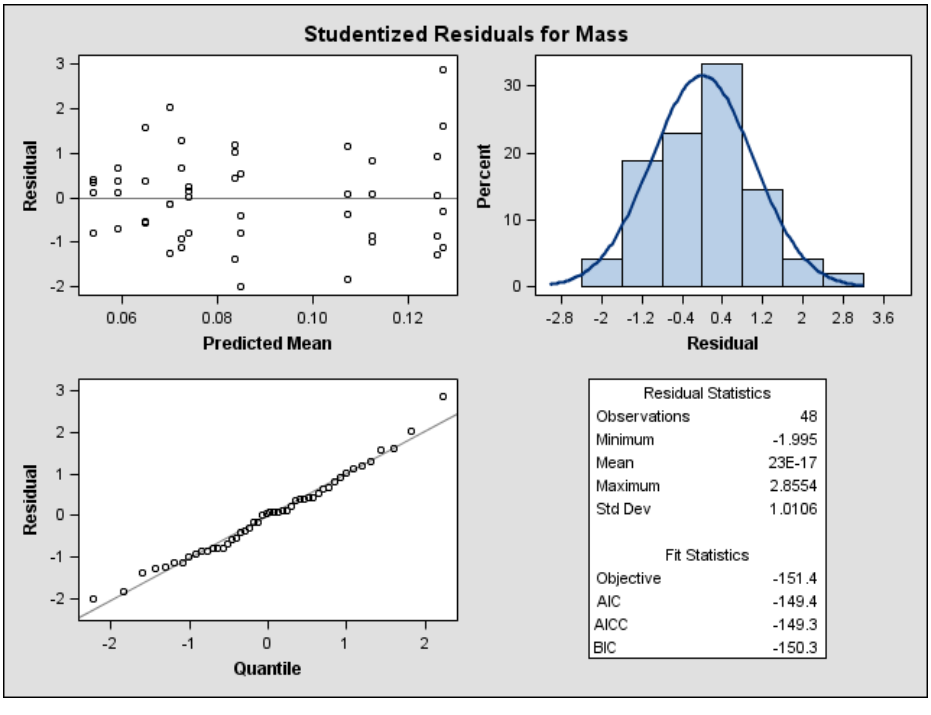

Figure 27. Studentized residuals for mass, day 25-28, clutch 2

Clutch 3

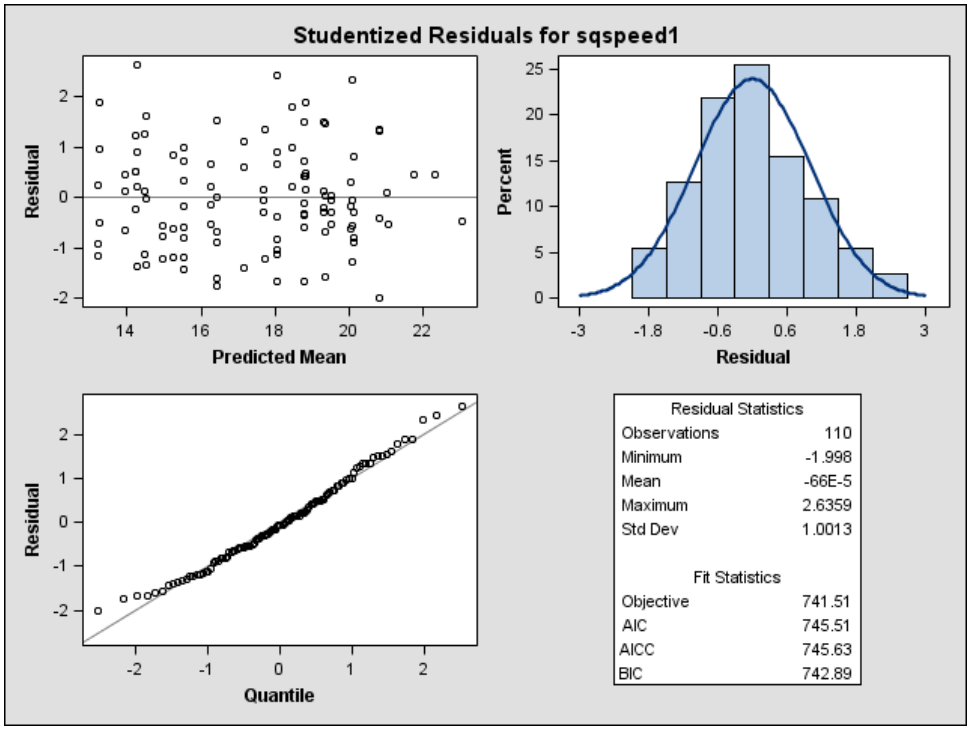

Figure 28. Studentized residuals for square root transformed 33ms speed, clutch 3

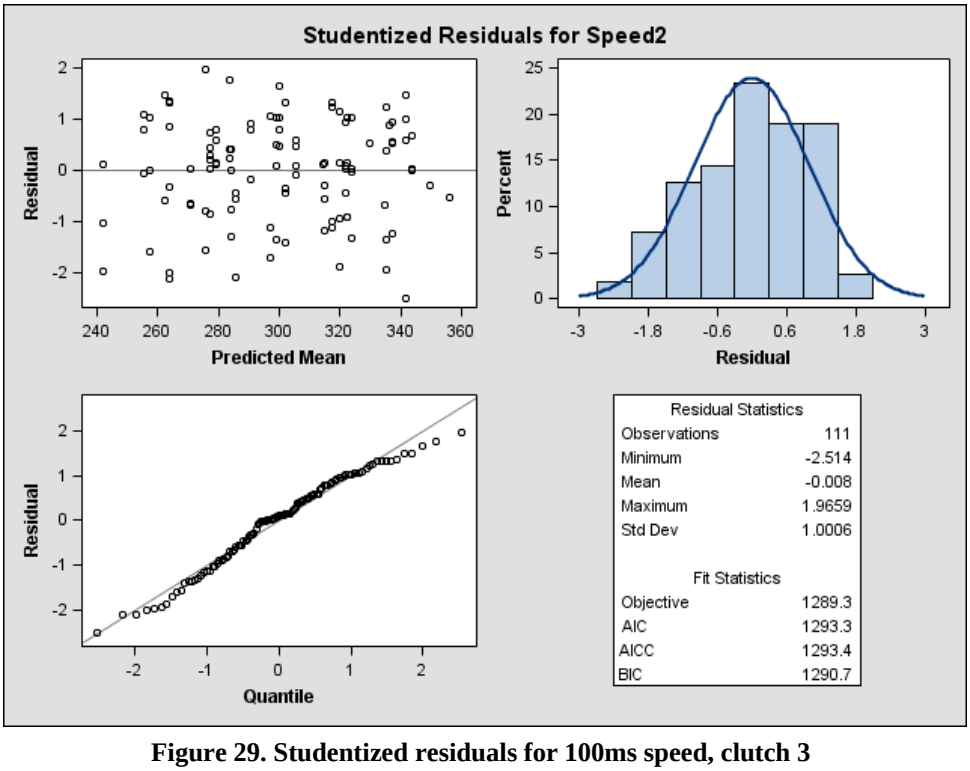

Figure 29. Studentized residuals for 100ms speed, clutch 3

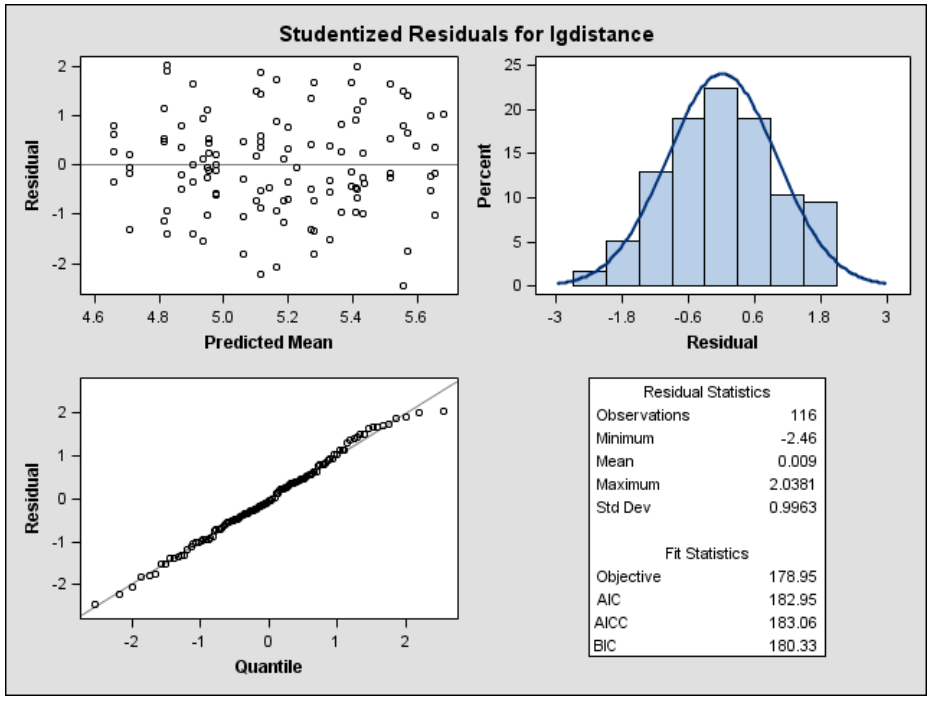

Figure 30. Studentized residuals for log transformed distance swam, clutch 3

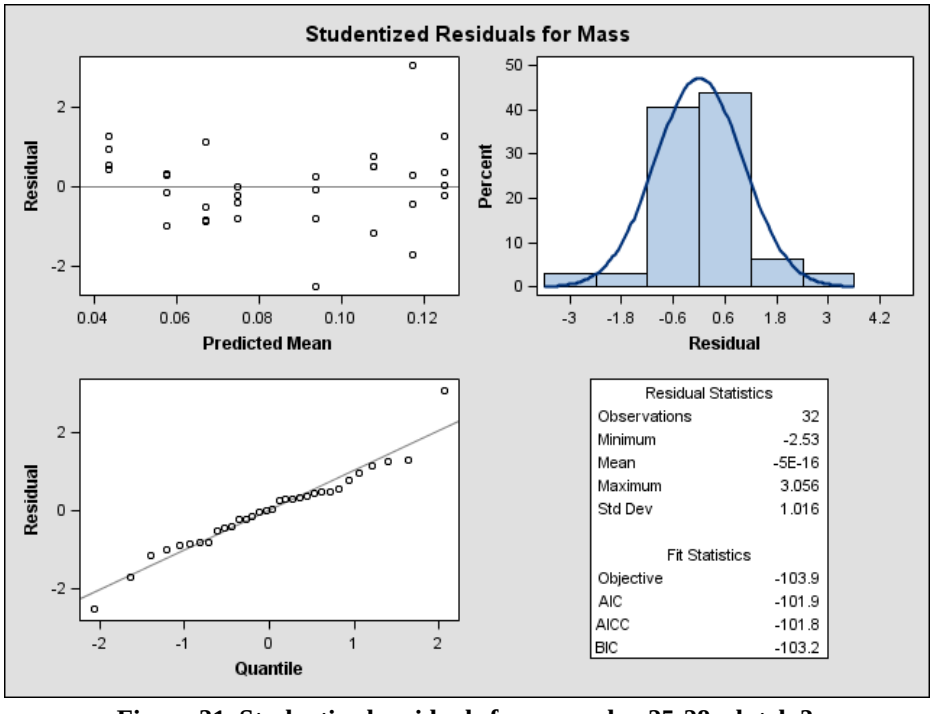

Figure 31. Studentized residuals for mass, day 25-28, clutch 3

APPENDIX D – Results tables

Table 22. Mean measurements for morphological variables from day 25 through 28

Treatment	Area (mean±SEM)	Width (mean±SEM)	Length (mean±SEM)	Mass (mean±SEM)
Control	42±2 mm^2	4.7±0.1 mm	21.6±0.5 mm	89±7 mg
200 mg/l NaCl	42±1 mm^2	4.9±0.1 mm	21.5±0.4 mm	93±5 mg
800 mg/l NaCl	51±2 mm^2	5.3±0.1 mm	24.1±0.4 mm	119±5 mg

Table 23. Mean measurements for morphological variables from day 148

Treatment	Area (mean±SEM)	Width (mean±SEM)	Length (mean±SEM)	Mass (mean±SEM)
Control	310±38 mm^2	10.3±0.6 mm	56.6±3.2 mm	1260±202 mg
200 mg/l NaCl	188±59 mm^2	8.1±0.9 mm	43.1±4.6 mm	676±200 mg
800 mg/l NaCl	468±55 mm^2	13.6±0.2 mm	69.1±2.7 mm	2510±0.163 mg

Table 24. Covariance Parameter Estimates for the square root transformed 33ms speed

Cov Parm	Subject	Estimate	Standard Error	Z Value	Pr Z
Tank		1.5902	1.7677	0.9	0.0899
Clutch		0	.	.	.
CS	**Sample**	3.3552	3.172	1.06	0.2902
Residual		57.6116	4.7491	12.13	<.0001

Table 25. Type 3 Tests of Fixed Effects for the square root transformed 33ms speed

Effect	Num DF	Den DF	F Value	Pr > F
Treatment	2	434	0.41	0.6657
Trial	2	434	1.91	0.1489
Day	5	434	1.97	0.0817

Table 26. Covariance Parameter Estimates for the square root transformed 33ms speed, clutch 1

Effect	Num DF	Den DF	F Value	Pr > F
Treatment	2	162	0.95	0.3883
Trial	2	162	3.38	0.0366
Day	5	162	1.17	0.3263

Table 27. Type 3 Tests of Fixed Effects for the square root transformed 33ms speed, clutch 1

Cov Parm	Subject	Estimate	Standard Error	Z Value	Pr Z
Tank		0	.	.	.
CS	**Sample**	5.1515	5.1602	1	0.3181
Residual		53.1641	7.0956	7.49	<.0001

Table 28. Covariance Parameter Estimates the square root transformed 33ms speed, clutch 2

Cov Parm	Subject	Estimate	Standard Error	Z Value	Pr Z
Tank		0	.	.	.
CS	**Sample**	-2.9145	4.4393	-0.66	0.5115
Residual		58.3695	7.9753	7.32	<.0001

Table 29. Type 3 Tests of Fixed Effects the square root transformed 33ms speed, clutch 2

Effect	Num DF	Den DF	F Value	Pr > F
Treatment	2	157	4.43	0.0135
Trial	2	157	0.49	0.6125
Day	5	157	2.46	0.0353

Table 30. Covariance Parameter Estimates the square root transformed 33ms speed, clutch 3

Cov Parm	Subject	Estimate	Standard Error	Z Value	Pr Z
Tank		7.54E-30	.	.	.
CS	**Sample**	6.5355	8.1059	0.81	0.4201
Residual		64.4267	10.8349	5.95	<.0001

Table 31. Type 3 Tests of Fixed Effects the square root transformed 33ms speed, clutch 3

Effect	Num DF	Den DF	F Value	Pr > F
Treatment	1	101	0.54	0.4633
Trial	2	101	0.1	0.9063
Day	5	101	1.43	0.2206

Table 32. Covariance Parameter Estimates for the 100ms average speed

Cov Parm	Subject	Estimate	Standard Error	Z Value	Pr Z
Tank		64.562	354.46	0.18	0.3759
Clutch		143.62	323.25	0.44	.
CS	**Sample**	3445.22	934.75	3.69	0.0002
Residual		11549	951.73	12.13	<.0001

Table 33. Type 3 Tests of Fixed Effects for the 100ms speed

Effect	Num DF	Den DF	F Value	Pr > F
Treatment	2	436	1.22	0.2952
Trial	2	436	1.02	0.3632
Day	5	436	2.12	0.0625

Table 34. Covariance Parameter Estimates for the 100ms speed, clutch 1

Cov Parm	Subject	Estimate	Standard Error	Z Value	Pr Z
Tank		995.51	8.45E+10	0	0.5
CS	**Sample**	1757.14	1283.28	1.37	0.1709
Residual		12024	1612.92	7.45	<.0001

Table 35. Type 3 Tests of Fixed Effects for the 100ms average speed, clutch 1

Effect	Num DF	Den DF	F Value	Pr > F
Treatment	2	161	0.47	0.6275
Trial	2	161	0.52	0.5927
Day	5	161	2.69	0.0229

Table 36. Covariance Parameter Estimates for the 100ms speed, clutch 2

Cov Parm	Subject	Estimate	Standard Error	Z Value	Pr Z
Tank		0	.	.	.
CS	**Sample**	3394.26	1512.53	2.24	0.0248
Residual		11036	1493.45	7.39	<.0001

Table 37. Type 3 Tests of Fixed Effects for the 100ms speed, clutch 2

Effect	Num DF	Den DF	F Value	Pr > F
Treatment	2	159	1.53	0.2195
Trial	2	159	5.24	0.0062
Day	5	159	2.53	0.0312

Table 38. Covariance Parameter Estimates for the 100ms speed, clutch 3

Cov Parm	Subject	Estimate	Standard Error	Z Value	Pr Z
Tank		0	.	.	.
CS	**Sample**	5464.4	2390.48	2.29	0.0223
Residual		10560	1771.55	5.96	<.0001

Table 39. Type 3 Tests of Fixed Effects for the 100ms speed, clutch 3

Effect	Num DF	Den DF	F Value	Pr > F
Treatment	1	102	0.42	0.5193
Trial	2	102	0.42	0.657
Day	5	102	0.51	0.7646

Table 40. Covariance Parameter Estimates for log transformed distance swam

Cov Parm	Subject	Estimate	Standard Error	Z Value	Pr Z
Tank		0.00541	0.01148	0.47	0.2635
Clutch		0	.	.	.
CS	**Sample**	0.1648	0.02987	5.52	<.0001
Residual		0.2438	0.01962	12.43	<.0001

Table 41. Type 3 Tests of Fixed Effects for log transformed distance swam

Effect	Num DF	Den DF	F Value	Pr > F
Treatment	2	453	2.69	0.0692
Trial	2	453	8.7	0.0002
Day	5	453	0.28	0.9253

Table 42. Differences of Least Squares Means for log transformed distance swam, trial

Effect	Trial	Trial	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
Trial	**1**	**2**	-0.1595	0.05573	453	-2.86	0.0044	Tukey-Kramer	0.0122
Trial	**1**	**3**	-0.2275	0.05608	453	-4.06	<.0001	Tukey-Kramer	0.0002
Trial	**2**	**3**	-0.06802	0.05608	453	-1.21	0.2258	Tukey-Kramer	0.4461

Table 43. Covariance Parameter Estimates for log transformed distance swam, clutch 1

Cov Parm	Subject	Estimate	Standard Error	Z Value	Pr Z
Tank		0	.	.	.
CS	**Sample**	0.2105	0.06038	3.49	0.0005
Residual		0.266	0.03492	7.62	<.0001

Table 44. Type 3 Tests of Fixed Effects for log transformed distance swam, clutch 1

Effect	Num DF	Den DF	F Value	Pr > F
Treatment	2	167	0.85	0.4273
Trial	2	167	0.9	0.4084
Day	5	167	0.64	0.6715

Table 45. Covariance Parameter Estimates for log transformed distance swam, clutch 2

Cov Parm	Subject	Estimate	Standard Error	Z Value	Pr Z
Tank		0	.	.	.
CS	**Sample**	0.1638	0.05154	3.18	0.0015
Residual		0.2682	0.03546	7.56	<.0001

Table 46. Type 3 Tests of Fixed Effects for log transformed distance swam, clutch 2

Effect	Num DF	Den DF	F Value	Pr > F
Treatment	2	165	1.73	0.181
Trial	2	165	6.59	0.0018
Day	5	165	0.26	0.9356

Table 47. Covariance Parameter Estimates for log transformed distance swam, clutch 3

Cov Parm	Subject	Estimate	Standard Error	Z Value	Pr Z
Tank		0	.	.	.
CS	**Sample**	0.1246	0.04736	2.63	0.0085
Residual		0.1723	0.02828	6.09	<.0001

Table 48. Type 3 Tests of Fixed Effects for the log transformed distance swam, clutch 3

Effect	Num DF	Den DF	F Value	Pr > F
Treatment	1	107	11.13	0.0012
Trial	2	107	3.54	0.0326
Day	5	107	0.61	0.693

Table 49. Covariance Parameter Estimates for mass, day 25-28

Cov Parm	Estimate	Standard Error	Z Value	Pr > Z
Tank	0.00053	0.000477	1.12	<.0001
Clutch	0.00027	0.000531	0.5	0.3091
Residual	0.00092	0.000121	7.65	<.0001

Table 50. Covariance Parameter Estimates for mass, day 25-28, clutch 1

Cov Parm	Estimate	Standard Error	Z Value	Pr > Z
Tank	0	.	.	.
Residual	0.00066	0.000144	4.58	<.0001

Table 51. Type 3 Tests of Fixed Effects for mass, day 25-28

Effect	Num DF	Den DF	F Value	Pr > F
Treatment	2	117	2.18	0.1172
Day	3	117	2.78	0.0444

Table 52. Type 3 Tests of Fixed Effects for mass, day 25-28, clutch 1

Effect	Num DF	Den DF	F Value	Pr > F
Treatment	2	42	22.37	<.0001
Day	3	42	4.96	0.0049

Table 53. Differences of Least Squares Means for mass, day 25-28, clutch 1

Effect	Treatment	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
Treatment	**C**	**H**	0.01194	0.009073	42	1.32	0.1954	Tukey	0.3945
Treatment	**C**	**L**	0.0575	0.009073	42	6.34	<.0001	Tukey	<.0001
Treatment	**H**	**L**	0.04556	0.009073	42	5.02	<.0001	Tukey	<.0001

Table 54. Covariance Parameter Estimates for mass, day 25-28, clutch 2

Cov Parm	Estimate	Standard Error	Z Value	Pr > Z
Tank	0	.	.	.
Residual	0.00057	0.000134	4.24	<.0001

Table 55. Type 3 Tests of Fixed Effects for mass, day 25-28, clutch 2

Effect	Num DF	Den DF	F Value	Pr > F
Treatment	2	42	11.25	0.0001
Day	3	42	1.05	0.382

Table 56. Differences of Least Squares Means for mass, day 25-28, clutch 2

Effect	Treatment	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
Treatment	**C**	**H**	-0.05338	0.01189	42	-4.49	<.0001	Tukey	0.0002
Treatment	**C**	**L**	-0.01094	0.01189	42	-0.92	0.3628	Tukey	0.6308
Treatment	**H**	**L**	0.04244	0.01189	42	3.57	0.0009	Tukey	0.0026

Table 57. Covariance Parameter Estimates for mass, day 25-28, clutch 3

Cov Parm	Estimate	Standard Error	Z Value	Pr > Z
Tank	0	.	.	.
Residual	0.00075	0.000216	3.46	0.0003

Table 58. Type 3 Tests of Fixed Effects for mass, day 25-28, clutch 3

Effect	Num DF	Den DF	F Value	Pr > F
Treatment	1	27	23.74	<.0001
Day	3	27	1.71	0.188

Table 59. Differences of Least Squares Means for mass, day 25-28, clutch 3

Effect	Treatment	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
Treatment	**C**	**H**	-0.05019	0.0103	27	-4.87	<.0001	Tukey	<.0001

Table 60. Covariance Parameter Estimates for mass. dav 148

Cov Parm	Estimate	Standard Error	Z Value	Pr > Z
Tank	0	.	.	0.5
Clutch	0.02681	0.06821	0.39	<.0001
Residual	0.199	0.08359	2.38	0.0086

Table 61. Type 3 Tests of Fixed Effects for mass, day 148

Effect	Num DF	Den DF	F Value	Pr > F
Treatment	2	8	16.4	0.0015

Table 62. Differences of Least Squares Means for mass, day 148

Effect	Treatment	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
Treatment	**C**	**H**	-1.2288	0.2821	8	-4.36	0.0024	Tukey-Kramer	0.0061
Treatment	**C**	**L**	0.5298	0.2684	8	1.97	0.0838	Tukey-Kramer	0.1807
Treatment	**H**	**L**	1.7586	0.3146	8	5.59	0.0005	Tukey-Kramer	0.0013

LITERATURE CITED

Alford, R. A. and S. J. Richards (1999). "Global amphibian declines: A problem in applied ecology." Annual Review of Ecology and Systematics **30**: 133-165.

Benjamini Y, Hochberg Y. 1995. Controlling the False Discovery Rate - a Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society Series B-Methodological 57(1): 289-300.

Blair, J. and R. J. Wassersug (2000). "Variation in the pattern of predator-induced damage to tadpole tails." Copeia(2): 390-401.

Brodie, E. D. and D. R. Formanowicz (1983). "Prey Size Preference of Predators - Differential Vulnerability of Larval Anurans." Herpetologica **39**(1): 67-75.

Bullock, T.H. 1981. Comparisons of the electric and acoustic sense and central processing. pp. 525-570. /n: W.N. Tavolga, A.N. Popper & R.R. Fay (ed.) Hearing and Sound Communication in Fishes. Springer-Verlag. New York.

Chinathamby, K., R. D. Reina, et al. (2006). "Effects of salinity on the survival, growth and development of tadpoles of the brown tree frog, Litoria ewingii." Australian Journal of Zoology **54**(2): 97-105.

Caldwell, J. P. (1981) "Disruptive selection: a tail color polymorphism in *Acris*tadpoles in reponse to differential predation." Journal of Zoology. **60**: 2818-2827

Chovanec, A. (1992). "The influence of tadpole swimming behaviour on predation by dragonfly nymphs." Amphibia-Reptilia **13**: 341-349.

Cochran, S. L., J. T. Hackett, et al. (1980). "The Anuran Mauthner Cell and Its Synaptic Bed." Neuroscience **5**(9): 1629-&.

Collins, S. J. and R. W. Russell (2009). "Toxicity of road salt to Nova Scotia amphibians." Environmental Pollution **157**(1): 320-324.

Cronin, J. T. and J. Travis (1986). "Size-Limited Predation on Larva Rana-Areolata (Anura, Ranidae) by 2 Species of Backswimmer (Insecta, Hemiptera, Notonectidae)." Herpetologica **42**(2): 171-174.

Demers, C. L. and R. W. Sage (1990). "Effects of Road Deicing Salt on Chloride Levels in 4 Adirondack Streams." Water Air and Soil Pollution **49**(3-4): 369-373.

Doherty, P. A., R. J. Wassersug, et al. (1998). "Mechanical properties of the tadpole tail fin." Journal of Experimental Biology **201**(19): 2691-2699.

Eaton, R. C., R. A. Bombardieri, et al. (1977). "Mauthner-Initiated Startle Response in Teleost Fish." Journal of Experimental Biology **66**(Feb): 65-81.

Eaton R. C., Lavender, W. A. and Wieland, C. M. Alternative neural pathways initiate fast start responses following lesions of the Mauthner neuron in goldfish. *J Comp Physiol* 145: 485–496, 1982.

Eaton, R. C. and J. Nissanov (1985). "A Review of Mauthner-Initiated Escape Behavior and Its Possible Role in Hatching in the Immature Zebrafish, Brachydanio-Rerio." Environmental Biology of Fishes **12**(4): 265-279.

Environment Canada, 2001. Canadian Environmental Protection Act, 1999, Priority Substances List Assessment Report – Road Salt, Hull, Quebec.

Eroschenko VP, Amstislavsky SY, Schwabel H, Ingermann RL. 2002. Altered behaviors in male mice, male quail, and salamander larvae following early exposures to the estrogenic pesticide methoxychlor. Neurotoxicology and Teratology 24(1): 29-36.

Evans, M. and C. Frick. 2001. The effects of road salts in stream, lake and wetland ecosystems. National Water Research Institute, Saskatoon, Saskatchewan.

Feder, M. E. (1983). "The Relation of Air Breathing and Locomotion to Predation on Tadpoles, Rana-Berlandieri, by Turtles." Physiological Zoology **56**(4): 522-531.
Fernandez, G., 2007. Model Selection in PROC MIXED—A User-Friendly SAS® Macro Application Proceedings of 2007 SAS Global Forum April 16-18 Orlando FL paper 191-2007.
Fetcho, J. R. and D. S. Faber (1988). "Synapse Location and the Selection of Motor Behaviors." American Zoologist **28**(4): A91-A91.
Foreman, M. B., and R. C. Eaton. 1993. The direction change concept for reticulospinal control of the goldfish escape.J . Neurosci. 13:4101-4113.
Gahtan, E., N. Sankrithi, et al. (2002). "Evidence for a widespread brain stem escape network in larval zebrafish." Journal of Neurophysiology **87**(1): 608-614.
Gallant, A. L., R. W. Klaver, et al. (2007). "Global rates of habitat loss and implications for amphibian conservation." Copeia(4): 967-979.
Hale, M. E., J. H. Long, et al. (2002). "Evolution of behavior and neural control of the fast-start escape response." Evolution **56**(5): 993-1007.
Hayes, T. B., P. Falso, et al. (2010). "The cause of global amphibian declines: a developmental endocrinologist's perspective." Journal of Experimental Biology **213**(6): 921-933.
Hoff, K. V. and R. J. Wassersug (2000). "Tadpole locomotion: Axial movement and tail functions in a largely vertebraeless vertebrate." American Zoologist **40**(1): 62-76.
Huey, R. B. (1980). "Sprint Velocity of Tadpoles (Bufo-Boreas) through Metamorphosis." Copeia(3): 537-540.
IUCN, NatureServe and Conservation International. Global Amphibian Assessment. 2004. Available: www.globalamphibians.org
Iverson, J. B. (1982). "Biomass in Turtle Populations - a Neglected Subject." Oecologia **55**(1): 69-76.
Johnson, K. E. and L. Eidietis (2005). "Tadpole body zones differ with regard to strike frequencies and kill rates by dragonfly naiads." Copeia(4): 909-913.
Karraker, N. E., J. P. Gibbs, et al. (2008). "Impacts of road deicing salt on the demography of vernal pool-breeding amphibians." Ecological Applications **18**(3): 724-734.
Kerby, J. L., K. L. Richards-Hrdlicka, et al. (2010). "An examination of amphibian sensitivity to environmental contaminants: are amphibians poor canaries?" Ecology Letters **13**(1): 60-67.
Linder, G., S. K. Krest, et al. (2003). Amphibian decline : an integrated analysis of multiple stressor effects. Pensacola, Fla., SETAC Press.
Littell RC, Henry PR, Ammerman CB. 1998. Statistical analysis of repeated measures data using SAS procedures. Journal of Animal Science 76(4): 1216-1231.
Martof, B. 1952. Early Transformation of the Greenfrog, Rana-Clamitans Latreille. Copeia(2): 115-116.
McCallum, M. L. (2007). "Amphibian decline or extinction? Current declines dwarf background extinction rate." Journal of Herpetology **41**(3): 483-491.
Myrick, C. A. (2009). "A low-cost system for capturing and analyzing the motion of aquatic organisms." Journal of the North American Benthological Society **28**(1): 101-109.
National Research Council (U.S.). Committee on Ecological Impacts of Road Density. (2005). Assessing and managing the ecological impacts of paved roads. Washington, D.C., National Academies Press.
Newman, M. C. (1998). Fundamentals of ecotoxicology. Chelsea, MI, Ann Arbor Press.
Osenburg, G. G. M. a. C. W. (1994). "Using foraging theory to study trophic interactions." Theory and Application in Fish Feeding Ecology: 45-59.

Pritchard (1965). "Prey Capture by Dragonfly Larvae (Odonata - Anisoptera)." Canadian Journal of Zoology **43**(2): 271-&.
Pritchard, G. (1964). "Prey of Dragonfly Larvae (Odonata Anisoptera) in Ponds in Northern Alberta." Canadian Journal of Zoology **42**(5): 785-&.
Ranvestel, A. W., K. R. Lips, et al. (2004). "Neotropical tadpoles influence stream benthos: evidence for the ecological consequences of decline in amphibian populations." Freshwater Biology **49**(3): 274-285.
Richards, S. J. and C. M. Bull (1990). "Nonvisual Detection of Anuran Tadpoles by Odonate Larvae." Journal of Herpetology **24**(3): 311-313.
Richards, S. J. and C. M. Bull (1990). "Size-Limited Predation on Tadpoles of 3 Australian Frogs." Copeia(4): 1041-1046.
Semlitsch, R. D. (2003). Amphibian conservation. Washington, D.C., Smithsonian Books.
Semlitsch, R.D. 1990. Effects of body size, sibship, and tail injury on the susceptibility of tadpoles to dragonfly predation. Can. J. Zool. 68: 1027-1030.
Serrao, J. (2000). The reptiles and amphibians of the Poconos and Northeastern Pennsylvania : covering Monroe and Pike Counties, parts of Wayne, Lackawanna, Luzerne, Carbon and Northampton Counties, and the adjacent Delaware River Valley of Pennsylvania, New York and New Jersey. Wilkes-Barre, PA, Llewellyn & McKane.
Smith, M. J., E. S. G. Schreiber, et al. (2007). "Associations between anuran tadpoles and salinity in a landscape mosaic of wetlands impacted by secondary salinisation." Freshwater Biology **52**(1): 75-84.
Squires ZE, Bailey PCE, Reina RD, Wong BBM. 2008. Environmental deterioration increases tadpole vulnerability to predation. Biology Letters 4(4): 392-394.
Storch, R. H. (1976). "Prey Detection by 4th Stage Coccinella-Transversoguttata Larvae (Coleoptera - Coccinellidae)." Animal Behaviour **24**(Aug): 690-693.
Turner, F. B. (1962). "The Demography of Frogs and Toads." Quarterly Review of Biology **37**(4): 303-314.
Van Buskirk, J., P. Anderwald, et al. (2003). "The lure effect, tadpole tail shape, and the target of dragonfly strikes." Journal of Herpetology **37**(2): 420-424.
Van Buskirk, J. and S. A. McCollum (2000). "Influence of tail shape on tadpole swimming performance." Journal of Experimental Biology **203**(14): 2149-2158.
Viertel, B. (1999). "Salt tolerance of Rana temporaria: spawning site selection and survival during embryonic development (Amphibia, Anura)." Amphibia-Reptilia **20**(2): 161-171.
Wake, D. B. and V. T. Vredenburg (2008). "Are we in the midst of the sixth mass extinction? A view from the world of amphibians." Proceedings of the National Academy of Sciences of the United States of America **105**: 11466-11473.
Watkins TB, McPeek MA. 2006. Growth and predation risk in green frog tadpoles (Rana clamitans): A quantitative genetic analysis. Copeia(3): 478-488
Webb, P. W. (1975). Acceleration performance of rainbow trout Salmo gcarneri and green sunfish Lepomis cyanellus. J. exp. Biol. 63, 451-65
Whiles, M. R., K. R. Lips, et al. (2006). "The effects of amphibian population declines on the structure and function of Neotropical stream ecosystems." Frontiers in Ecology and the Environment **4**(1): 27-34.
Wilbur, H.M., and R.D. Semlitsch. 1990. Ecological consequences of tail injury in Rana tadpoles. Copeia 1990: 18-24.
Wilson, R. S., P. G. Kraft, et al. (2005). "Predator-specific changes in the morphology and swimming performance of larval Rana lessonae." Functional Ecology **19**(2): 238-244.

Wu, T. Y. 1977. Introduction to the scaling of aquatic animal locomotion, p. 203-232. In: Scale effects in animal locomotion. T. J. Pedley (ed.). Academic Press.

Zottoli, S. J., D. T. Walfish, D. A. Westbrooks and D. C. Smith, 2001. Small tadpoles do not initiate a startle response before being struck by the labium of dragonfly larvae. Society for Neuroscience Abstracts 27:1984.

ACADEMIC VITA OF GEORGE A. SAMRA

George Samra
52 Deerfield Way
Scotrun, PA 18355
george.a.samra@gmail.com

Education

The Pennsylvania State University
Schreyer Honors College, Graduation Fall 2010
BS in Environmental Resource Management
BS in Physics
Minor in Biology and Mathematics

Scholarships and awards:

- Second place poster presentation in Health and Life Sciences at 2010 Undergraduate Research Exhibition
- Schreyer Honors College scholarship
- Pre-eminence in Honors Education scholarship
- University Trustee scholarship
- Gerald & Leoda Excellence in Agriculture scholarship

Work experience

Undergraduate researcher – The Pennsylvania State University (2009-2010)

- Conducted experiment to test the effects of salinity on tadpole behavior and development
- Performed statistical analysis of data using SAS
- Presented posters based on findings at the 2010 Joint Meeting of Ichthyologists and Herpetologists and the 2010 Ecological Society of America meeting

Peer tutor – The Pennsylvania State University

- January 2009 – May 2009: Comprehensive Studies Program tutor - biology, chemistry and physics
- January 2010 – Summer 2010: Penn State Learning group subject tutor - Fluids and thermal physics and wave motion and quantum Physics