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Turning Tadpoles: Effects of Salinity and Lateralization on Hyla versicolor Escape Behaviors

Victor LoPiccolo

Abstract

Road salt, which is used as a roadway deicer in the winter time, is a major contributor to
the salinization of freshwater habitats. Amphibians are particularly vulnerable to the salinization
of aquatic environments due to the reliance of many species on pond, lake, and stream habitat in
their aquatic larval stage. This study examined how Northern Gray Treefrog (*Hyla versicolor*)
tadpole behavior is affected by salinity in a multiple t-maze. Alternate turning is sequential
turning in opposite directions. Alternating turns lead the animal further from the starting point,
and are innate behaviors associated with foraging, exploration, and escape. A multiple t-maze is
a complex maze environment where an individual can make zero to three alternating turns. At
elevated salinity *H. versicolor* displayed more alternating turn behavior than expected if turns
were made randomly. However, there were no differences among salinity levels in alternating
turn frequency, which suggests that tadpoles will continue to move directionally straight
regardless of salt concentration. At elevated salinities, tadpoles were more likely to complete the
maze within five minutes, spent less time completing the maze, and were more likely to make
180° turns (u-turns). Prior salt exposure from earlier pilot studies significantly reduced the
frequency of alternating turns and reduced the rate of maze completion. Further studies are
warranted to determine how brief prior exposure affected turning behavior. In a t-maze binary
turning bias test, tadpoles exhibited a trend of lateralized turning bias that was also evident in the
multiple t-maze trials. These effects could have implications for how *H. versicolor* tadpoles
forage, explore and evade predators in their natural habitat. This protocol for exploring escape
behaviors in a multiple t-maze can be applicable for studies of other amphibian species or to
determine the effects of other chemical stimuli such as predator cues and pesticides on behavior.

Keywords: road salt, alternating turns, tadpoles, multiple t-maze, lateralization

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Turning Tadpoles: Effects of Salinity and Lateralization on *Hyla versicolor* Escape Behaviors

by

Victor LoPiccolo

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TURNING TADPOLES: EFFECTS OF SALINITY AND LATERALIZATION ON *HYLA*

VERSICOLOR ESCAPE BEHAVIORS

A THESIS

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Introduction

Overview of the Problem

Road salt, which is used as a roadway deicer in the winter time, is a major contributor to the salinization of freshwater habit. Salts carried by water runoff pollute waterways can temporarily spike salt concentrations. Road salt pollution threatens drinking water supply (Kelly et al., 2018; Parker and Tatum, 2021; Pieper et al., 2018; Soper et al., 2021) and represents a danger to freshwater aquatic life and ecological systems (Lawson and Jackson, 2021; Tiwari and Rachlin, 2018). Salinity can be toxic to organisms and can also have more subtle effects (Gibbons et al., 2018; Sanzo and Hecnar, 2006). Rising salt concentrations disrupt food-chains and lead to trophic cascades (Hintz et al., 2017). In animals, they can lead to changes in gene expression and alter anti-predator behavior (Gibbons et al., 2017; Hall et al., 2017). These consequences are being felt at the local, regional, and national levels in the United States (Corsi et al., 2010; Mazumder et al., 2021).

There has been a massive increase in the use of road salts since the middle of the 20th century (Corsi et al., 2010; Fay et al., 2013). A 2015 study conducted by the USGS found that the increased chloride concentrations found in streams directly correlated with the increased use of road salts over a two decades span, from 1990-2011 (Corsi et al., 2010). Long term trend projections predict steady salinization of freshwater lakes throughout the northeast and midwest North American lakes (Dugan et al., 2017). Upward trends in urbanization will only exacerbate current road salt usage concerns and runoff pollution of freshwater bodies (Mazumder et al., 2021).

Salinization and Amphibians

Among other factors, the salinization of freshwater habitat has led to declines of amphibian populations (Green et al., 2020). Amphibians are particularly vulnerable to changes in salinity of terrestrial and aquatic environments caused by road salt surface runoff. This point source pollution can cause brief but consequential moments of elevated salinity. Many species of amphibians have an aquatic larval stage and use vernal pools and other freshwater habitats to reproduce. (Semlitsch and Skelly, 2008). Amphibian susceptibility to salt pollution stems from permeable, unshelled aquatic eggs, larval gills, and porous skin. These morphological features, present at various stages of development, play key roles in their osmoregulation (Szeligowski et al., 2021).

Freshwater salinization leads to physiological consequences for amphibians,due to disruptions in osmoregulation (Collins and Russell, 2009; Jones et al., 2015). Elevated salt concentrations can be toxic to larval amphibians and lead to reduced survival (Hall et al., 2017; Jones et al., 2015; Tornabene et al., 2021a). Exposure to elevated salt concentrations can lead to adverse developmental effects including damaged gills and various deformities (Sanzo and Hecnar, 2006; Szeligowski et al., 2021; Tornabene et al., 2021a; Tornabene et al. 2021b). As a stressor, long term exposure to elevated salinity reduces locomotor performance and overall activity in amphibians. This has been directly linked impaired predator escape responses and anti parasite behavior (Denoël et al., 2010; Kearney et al., 2016; Milotic et al., 2017; Sanzo and Hecnar, 2006). Some amphibian species are more salt tolerant than others and physiological effects of salinity may vary greatly from species to species (Collins and Russell, 2009).

Oviposition habitat selection behavior is affected by the presence of low pH, high heavy metal concentrations and pesticides in pond habitats. Amphibians have demonstrated preference

for uncontaminated ponds for oviposition and there is reduced oviposition activity in

contaminated ponds (Skelly, 2001; Takahashi, 2007; Vonesh and Buck, 2007). Oviposition

habitat selection behavior can also be affected by salinity. Increased salinity may reduce

oviposition activity (Haramura, 2008; Karraker et al., 2008; Wilder and Welch, 2014). Habitat

selection behavior mitigates the impacts of pollutants and salinization. However, with continued

habitat degradation and fragmentation, there are less overall breeding sites for amphibians

(Hocking and Semlitsch, 2007). During their development, larval *H. versicolor* are isolated to

freshwater bodies and vulnerable to changes within that habitat. Runoff pollution may

contaminate this habitat after habitat selection behavior has already led to the same habitat being

chosen as a breeding site.

Amphibian Behavior

Chemical stimuli such as salt and pesticides have been shown to alter amphibian

behavior. Elevated salinity can affect frogs foraging and antipredator behavior (Hall et al., 2017).

Salinity also reduces overall locomotor performance including most amphibian avoidance

behavior (Denoël et al. 2010; Kearney et al. 2016). Pesticide exposure similarly affects

amphibian behavior by reducing both avoidance behavior and overall locomotion in amphibians

(Denoël et al., 2013; Leeb et al., 2020). Other stimuli such as predator cues can cause amphibian

tadpoles to reduce their activity levels (Babbitt and Tanner, 1998; Lawler, 1989). Short term

exposure to these chemical stimuli may also affect amphibian turning behavior.

Alternating Turns

Alternating turns or alternate turning is sequential turning in opposite directions (Dember

and Richman, 1989). Alternating turn behaviors that allow individuals to efficiently move

towards or away from stimuli are innate behaviors (Carbines et al., 1992; Hughes, 1967;

Moriyama et al., 2015). In some instances, this has been described as a correcting behavior

where individuals compensate for a prior turn in one direction by changing direction in a

sequential turn (Carbines et al., 1992). This allows an animal to move directionally straight.

Examples of animals that demonstrate this behavior are woodlice, *Porcellio scaber,* and pill

bugs, *Belostoma flumineum,* They both make alternating turns in response to predator cues

(Hughes, 1967, Paradis, 2020). Alternating turn behavior is also seen as advantageous for

exploration and foraging. Rats, *Rattus norvegicus domestica*, have carried out alternating turns

to explore a complex t-maze environment and in foraging behaviors in a plus-maze (Estes and

Schoeffler, 1955).

Lateralized Turning Behavior

Lateralization or laterality is a result of lateralized differences in the brain hemispheres

that lead to biases in behavior (Wiper, 2017). Lateralization may occur at the population level

when biases become coordinated in a social group setting or just in single individuals (Bisazza et

al., 2000; Frasnelli and Vallortigara, 2018). Laterality is common among both vertebrates and

invertebrates (Walker, 1980). This includes amphibians (Malashichev and Robins, 2018;

Malashichev and Wassersug, 2004; Rogers, 2002; Wassersug and Yamashita, 2002).

The asymmetrical emergence of forelimbs at metamorphosis often leads to forearm limb

bias in adult amphibians (Malashichev, 2002; Malashichev, 2006; Malashichev and Nikitina,

2002; Robins et al., 1998). Hemispheric lateralization results in many other lateralized behaviors.

The right hemisphere is associated with rapid responses including predator escape behaviors in

anurans and vertebrates more broadly (Malashichev and Wassersug, 2004). Limb bias in adults

can be established by observing how anurans right themselves after being turned and when they use a limb to wipe something off of their snout (Malashichev, 2006; Malashichev and Nikitina, 2002). Multiple species of toads demonstrate lateralized jumping escape mechanisms in response to predators (Lippolis et al., 2002). In tadpoles, researchers have observed lateralized biases in startle responses (Malashichev and Robins, 2018).

There are factors that are known to affect lateralization in amphibians. Predation has been shown to impact behavioral asymmetries in wood frog, *Lithobates sylvaticus*, tadpoles. Tadpoles exposed prenatally to predator cues were more likely to develop more intense lateralization demonstrated by bias in swimming directional preference (Lucon-Xiccato et al., 2016). The pesticide, Roundup® Power 2.0, impacted lateralization in developmental marsh frog, *Pelophylax ridibundus*, tadpoles (Bolis et al., 2020). This exposure resulted in a lower intensity of lateralization demonstrated by less rotational preference in the tadpoles. Less is understood about how other noxious stimuli, including salinity, may affect lateralization in amphibians. While not involving amphibians, a study found that short-term exposure to elevated salinity caused the freshwater fish, Iberian barbels, *Luciobarbus bocagei*, to increase lateralization at higher salinity (Leite et al., 2019).

T-Mazes, Multiple T-Mazes and Alternating Turns

T-mazes force individuals to make a turn towards one of two alternate directions as opposed to continuing to travel in the same direction. They offer a binary choice and can be useful in determining turn preferences in individuals or populations (Figure 1; Wassersug and Yamashita, 2002). A multiple t-maze is a complex environment whereby an individual must make multiple consecutive turning decisions. The multiple t-maze allows for individuals to make

three decisions at t-junctions to turn left or right after beginning from the start in the center to completion at one of eight endpoints (Figure 2). An individual may start with a right turn and continue to turn right at each of the subsequent three t-junctions. That would result in an individual making zero alternating turns. If an individual makes an initial right turn but then turns left at the first t-junction that would represent one alternating turn. From the start of the maze to one of eight endpoints an individual could make zero, one, two, or three alternating turns.

Figure 1. T-Maze Design to Assess Turning Bias in Tadpoles
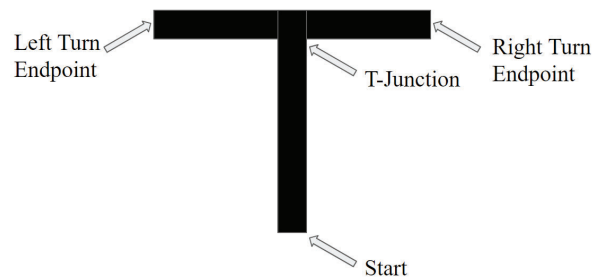
Note. Tadpoles begin where "start" is noted and must make either a left or right turn at the t-junction.

Figure 2. Multiple T-Maze Used to Assess Alternating Turn Behavior in Response to Salinity
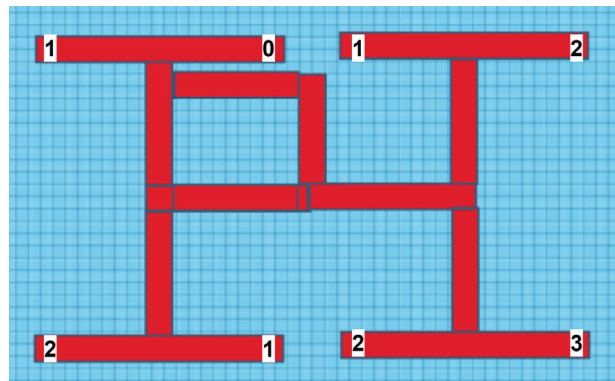
Note. Multiple t-maze 3D model designed using TINKERCAD (© 2022 Autodesk, Inc.) from earlier multiple t-maze devices used by Hughes (1967) and Paradis (2020). The number of alternating turns completed to reach an endpoint is noted on the maze endpoints.

Earlier designs of multiple t-mazes fail to account for handedness biases (or turn preferences) that result from laterality (Hughes, 1967; Paradis, 2020). Original multiple t-maze designs force individuals to make an initial right turn (Figure 2). Depending on the lateralized turning preference of an individual, or more broadly, a population, this can impact subsequent turning behavior at junctions in the multiple t-maze. In multiple t-maze trials, the turning bias of a population can be compensated for through the use of two separate mazes. The mazes should be identical except for the initial forced turn that an individual must make; either a forced right or left turn.

Hyla versicolor

H. versicolor, also known as the northern gray treefrog, is a species that is native to a broad swath of North America ranging from southern Quebec to regions of eastern Texas (Dodd,

2013). *H. versicolor* prefers forest habitat and doesn't range far from breeding ponds. Typical

breeding and larvae habitat includes temporary and permanent wetland that is adjacent to

woodlands. Breeding activity typically occurs from spring to early summer. They have clutch

sizes that range from 1,000 to 2,600. Developmentally, *H. versicolor* tadpoles take up to 60 days

to reach metamorphosis under laboratory conditions but up to 20 days less (40-60 days) in

natural conditions. *H. versicolor* diet consists mostly of insects such as beetles, crickets, moths,

roaches, true bugs, and spiders. Common predators include spiders (predate upon juveniles),

birds, garter snakes, water snakes, American bullfrogs, and meso-mammals (Dodd, 2013).

        H. versicolor have a lower salinity tolerance than other amphibian species. In choice

trials for salinity aversion, adult *H. versicolor* showed aversion for salinity at an EC_{50} threshold,

the threshold for half of the individuals to show aversion, of 0.155 M NaCl (Jamieson, 2012).

This threshold is lower than reported thresholds for other amphibian species including leopard

frogs, *Rana pipiens* (EC_{50} = 0.40 M), green frogs, *Rana clamitans melanota* (EC_{50} = 0.417 M),

and eastern newts, *Notophthalmus viridescens* (EC_{50} = 0.205 M) (Gonzalez-Abreu, 2011;

Jamieson, 2012; Koelmel, 2011; Kwasek, 2011). Road salts have explicitly been linked to

reduced survival of *H. versicolor* embryos in stormwater ponds (Brand et al., 2010).

        While *H. versicolor* are listed as a species of least concern, they are still affected by

pollution and habitat degradation resulting from the increasing use of road salts (New Jersey

Department of Environmental Protection, 2021). Additionally, other species of amphibians such

as the New Jersey native Southern Gray Treefrogs, *Hyla chrysoscelis*, are endangered in the state

(New Jersey Department of Environmental Protection, 2021). Understanding this aspect of

amphibian behavior can help conservation managers to better predict amphibian behavioral

changes in response to changes in salinity of vital vernal pool habitat.

Purpose of the Study

Studies of behavior in conjunction with studies of physiology can aid conservation strategies for amphibians (Walls and Gabor, 2019). This enables researchers to parse the complexity of amphibian population declines and their underlying causal factors. The purpose of this study is to determine how amphibian behavior will be impacted at varying salt concentrations. Specifically this study examines how alternating turn behavior, exhibited in foraging, exploration, and anti-predator responses, is affected in a population of *H. versicolor* tadpoles. The New Jersey native *H. versicolor* serves as an indicator species for ecological monitoring. It alsos is closely related to and shares habitat with the New Jersey endangered, *H. chrysoscelis* (Estes-Zumpf et al., 2022; New Jersey Department of Environmental Protection, 2021). This study of amphibian behavior improves our understanding of behavioral responses to salinity and can inform conservation efforts. The purpose of this study is to determine:

1) To what degree is there motor lateralization in the population of *H. versicolor* tadpoles demonstrated by turning bias in a t-maze,

2) To what degree will *H. versicolor* tadpoles exhibit alternate turning behaviors when in a multiple t-maze, and

3) To what degree will salinity, at environmentally relevant concentrations, affect those alternate turning behaviors.

It is hypothesized that amphibian tadpoles will demonstrate increased alternating turn behavior when introduced to the elevated salt concentrations. Increased alternating turns behavior in response to a chemical stimulus would be consistent with avoidance and escape behaviors demonstrated by amphibians (Carbines et al., 1992; Moriyama et al., 2015) and other animals (Hughes, 1967; Paradis, 2020).

There are reasons to suspect that alternating turn behavior will decrease as salinity increases. Both salinity and predator cue exposure can lead to reduced motor activity in amphibian tadpoles (Babbitt and Tanner, 1998; Lawler, 1989). Less motor activity could reduce the amount of alternating turn behavior that tadpoles exhibit at higher salt concentrations. This alternative explanation is not hypothesized due to the fact that this experiment will assess a brief acute exposure to salinity. Reduced motor activity is more so associated with prolonged exposure to elevated salinity and a physiological response (Babbitt and Tanner, 1998; Lawler, 1989).

Methods

Field Collection and Care

      H. versicolor tadpoles were collected from a residential swimming pool cover in West Milford, Passaic County, New Jersey during the spring of 2021. The approximately 800 tadpoles were collected in one 19 L bucket. The tadpoles were then divided evenly and kept in six separate 19 L buckets filled with dechlorinated tap water (18.9-21.1°C) in an animal holding room at Montclair State University. Partial water changes with additional dechlorinated tap were completed two to four times a week. Tadpoles were fed crumbled Hikari® Tropical Algae Wafers (Kyorin Co., Ltd., Japan) on a biweekly basis. During the trials the tadpoles' developmental stages ranged between Gosner 26 and Gosner 38 over eight consecutive days (Figure 3; Gosner, 1960).

Figure 3. Tadpole Gosner Developmental Stages
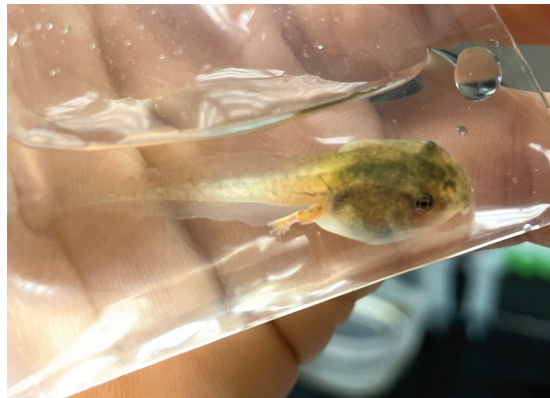
Note. This tadpole was at the developmental stage Gosner 38 during the experimental trials. The *H. versicolor* tadpoles used in the multiple t-maze trials were all within developmental stages ranging from when their hindlimbs were beginning to develop at Gosner 26 to the development of the metatarsal tubercle at Gosner 38 (Gosner, 1960).

T-Maze Trials

T-maze trials were conducted to determine the potential lateralized turning bias of the

tadpole population. A t-maze was carved into a 2.5 cm thick extruded polystyrene (XPS) panel

using an electric hot foam wire cutter. XPS was chosen for the maze because it is not water

absorbent. The first channel, 1 cm deep and 1 cm wide, was 10 cm long and perpendicular to a

second 10 cm channel that is perpendicular to the first channel. The maze was filled with

dechlorinated tap water at room temperature. Tadpoles ($N = 50$) were introduced into the first

channel of the t-maze and allowed to swim until they reached the t-junction, where they turned

either right or left. The t-maze was rotated on the benchtop by 180º so that it faced towards or

away from the researcher. This allowed for statistical testing to rule out potential bias that may

be caused by either the position of the researcher in front of the bench or some other unforeseen

confounding aspect of the lab environment (e.g. lighting, ambient noise).

Multiple T-Maze Design and Development

The multiple t-mazes (Figure 4) were designed on the online 3D design platform,

TINKERCAD (© 2022 Autodesk, Inc.), based on the multiple t-maze used by Hughes (1967)

and Paradis (2020). The multiple t-maze design was carved into a 2.5 cm thick XPS panel using

an electric hot foam wire cutter. The maze channels are approximately 1 cm deep and 1 cm wide,

and pathways are 10 cm long. The generic design can be scaled for trials with larger or smaller

sized specimens. The channel width and depth for gray tree frog tadpoles was determined

through trial and error. An appropriately sized multiple t-maze and single t-maze allowed enough

room for an individual to travel forward and around turns but was small enough that an

individual was less likely to be able to turn around 180º and go in the opposite direction (make a

u-turn).

Figure 4. Multiple T-Maze 3D Design
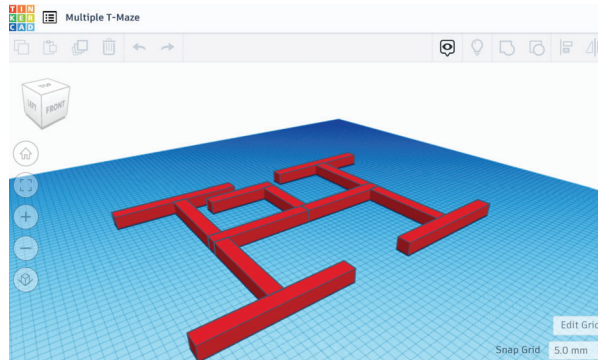
Note. Scalable multiple t-maze 3D design constructed using TINKERCAD (© 2022 Autodesk,

Inc.) derived from (right) the multiple t-maze used by Hughes (1967) and Paradis (2020).

The multiple t-maze contained three sequential t-junctions where an individual can turn

left or right. An individual begins in the center of the maze and can travel to one of eight

endpoints (Figure 4). For example, an individual may start with a forced right turn and continue

to turn right at each of the subsequent three t-junctions, which would result in zero alternating

turns. As another example, if an individual makes an initial right turn but then turns left at the

first t-junction that would represent one alternating turn. From the start of the maze to one of

eight endpoints, zero alternating turns (one possible endpoint), one alternating turn (three

possible endpoints), two alternating turns (three possible endpoints), or three alternating turns

(one possible endpoint) could be completed (Figure 2).

Maze Types and Orientations

Two mazes were used for the trials that are identical but mirror each other (Figure 5). The separate mazes either start the tadpole with an initial forced left turn or an initial forced right turn; otherwise the dimensions of the mazes are identical. This is meant to control for possible lateralized biases present in the population of tadpoles.

Figure 5. Forced Right and Left Turn Maze Types

Note. Multiple t-maze XPS panels with 1 cm x 1 cm x 10 cm channels that allow for a tadpole to make either an initial forced right turn or initial forced left turn.

Additionally, the mazes are marked "F" and "B" on the top and bottom so that they may be rotated on the benchtop by 180° into a forwards "F" or backwards "B" orientation that faces towards or away from the researcher (Figure 3). This allows for statistical testing to rule out potential bias that may be caused by either the position of the researcher in front of the bench or some other unforeseen confounding aspect of the lab environment (e.g. lighting, ambient noise).

Multiple T-Maze Trials

For each trial, the orientation and forced turn (maze type) were randomly assigned by coin toss. 50 mL of solution was added to the maze using a graduated cylinder. Salinity was assigned sequentially starting with 0.0 M then 0.1 M, 0.15 M, and 0.20 M NaCl. Tadpoles were used in a single trial at the randomly assigned orientation and maze type and at the sequentially assigned salinity. Once tadpoles completed a trial they were set aside in a labeled 19 L bucket.

Tadpoles ($N = 468$) were taken from one of six holding buckets using a turkey baster, and were placed into the center channel (Figure 6). The tadpoles were blocked from advancing into the maze for approximately five seconds by a 7 mm stainless steel spatula held at the end of the first channel before the tool was removed and the timer began (Figure 6). The intention of this initial gating period with a spatula is to allow for the tadpole to acclimate to the solution after being placed in the maze. This gating also reduces the possibility of a startle response that is caused purely by being transported to and placed into the maze. The largest tadpoles were chosen on initial days in an effort to capture tadpoles at roughly similar Gosner developmental stages as on later days smaller tadpoles would be further developed. Trials ended when a tadpole reached a maze end point, or after five minutes maximum. This time limit was chosen to limit how long tadpoles would spend in a hypertonic environment. Between each trial, the maze, spatula, graduated cylinder and turkey baster were thoroughly rinsed with tap water.

Figure 6. Multiple T-Maze Trial Setup and Tools
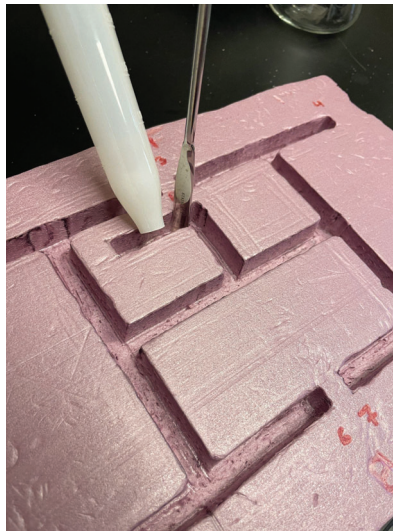
Note. A turkey baster was used to transport the tadpole from a holding bucket to the start

channel of the multiple t-maze. The spatula is held at the end of the start channel for a five

second count before being removed from the maze and the trial timer started.

Data Collection

Before trials, date, time, lab temperature, lab humidity, bucket water temperature, and

prior salt exposure from pilot studies were recorded. During each trial, latency time, which is the

time that it took for a tadpole to make an initial turn after being placed in the maze and a five

second gated period passed, was recorded. At the end of each trial, the endpoint, total time, and

number of u-turns was recorded. A u-turn was considered to be any full $180°$ turn after the

latency period. The tadpole body length, which was measured as the distance from the snout to

the point where their tail begins, and total length were recorded. All data were entered into a

google sheet spreadsheet.

These data were used to calculate the maze time (latency time subtracted from total time in maze), tail length (body length subtracted from total length), and number of alternating turns completed. From the recorded endpoints, it was determined whether tadpoles made an initial alternating turn, whether they made the first two consecutive alternating turns in a row, or whether they made any two or more alternating turns in the trial (Figure 7).

Figure 7. Turn Alternation Data Determined From Trial Endpoints
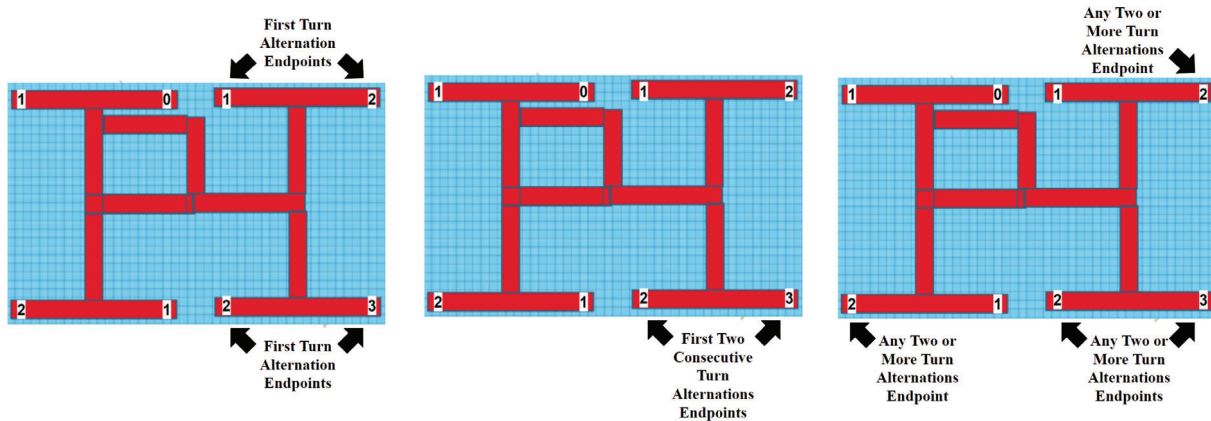
Note. Recorded endpoints per trial were used to determine whether tadpoles made an initial alternating turn (left), whether they made the first two consecutive alternating turns in a row (middle), or whether they made any two or more alternating turns in the trial (right).

Statistical Analysis

All statistical analyses were conducted using JMP® TM (SAS) Pro 14.2.0 for Microsoft Windows. The t-maze test for lateralized turning biases was analyzed using a binomial probability test and an Agresit-Coull exact test to calculate power and the generalizability of the result (Agresti and Coull, 1998).

Multiple T-Maze Behavior Analysis

Regression models were used to determine the effect of salinity, body size, forced turn, maze orientation, prior salt exposure, salinity*prior salt exposure, and salinity*body size on maze completion, alternating turns, latency time, maze time, and total time. Water temperature was excluded from the model due to the small range of recorded temperature measurements (18.9-21.1°C). Due to the deliberate selection of larger tadpoles on earlier days of the experiment, body size was correlated with date. For this reason, the date of trial completion was excluded as an explanatory variable from the model.

Maze Completion

Maze completion was defined as a tadpole reaching one of eight endpoints within five minutes. Maze completion was analyzed using a nominal logistic fit regression model to determine the effect of salinity and other factors on the likelihood of completion. The number of u-turns completed per trial was analyzed using a standard least squares fit regression model to determine the effect of salinity and other factors. If the tadpole completed the maze and did not make any u-turns ("clean trial") then that trial was included in an analysis to determine the effect of salinity and other factors on alternating turn behavior, maze time, latency time, and total time.

Alternating Turns

Alternating turn distributions were analyzed using a chi-square test to determine whether observed frequency distributions differed from the expected frequency distribution for making zero alternating turns (one possible endpoint), one alternating turn (three possible endpoints), two alternating turns (three possible endpoints), or three alternating turns (one possible

endpoint). Assuming an equal chance of making a right or left turn at each maze junction, the

expected distribution is 1:3:3:1 respectively. A logistic fit regression model of observed

alternating turn distributions was used to determine whether there was a significant difference in

the observed alternating turn distributions at different salinities. An ordinal logistic fit model was

used to determine the effect of salinity and other factors on alternating turn behavior. Logistic fit

regression models were also used to determine the effect of salinity on first turn alternation, first

two consecutive turn alternations and any two turn alterations.

Expectations

Random turning behavior would give tadpoles a 12.5% chance of reaching any of the

eight possible endpoints in the multiple t-maze (Figure 4). It was predicted that *H. versicolor*

tadpoles would move randomly as they completed the maze at 0.0 M NaCl and then would make

an increasing number of alternating turns at each of the three higher NaCl concentrations from

0.1 M - 0.20 M. Alternating turns when faced with these chemical stimuli would be consistent

with escape behaviors demonstrated by amphibians (Carbines et al., 1992; Moriyama et al.,

2015) and other animals (Hughes, 1967; Paradis, 2020). Many amphibian species exhibit innate

turning behaviors to avoid predators (Rogers and Andrews, 2002). Directional locomotion

characterized by alternating turns is a common escape mechanism in amphibians (Brown and

Taylor 1995; Domenici et al. 2011).

Maze Time, Latency Time, and Total Time

Maze time, latency time, and total time distributions were tested for normality and

subsequently transformed using a sinh-arcsinh transformation. Fit least squares regression

models were used to determine the effect of salinity and other factors on maze time, latency time, and total time.

Pilot Studies and Prior Salt Exposure

All tadpoles were used in prior multiple t-maze trials. Prior to 6/30/2021, more than 50 tadpoles were used in separate multiple t-maze trials to test the maze design and to develop the experimental methodology. From 6/30-7/1 2021, 101 multiple t-maze trials were run at 0.0 M and 0.1 M NaCl concentrations. From 7/6-7/7 2021 101 multiple t-maze trials were run at 0.0 M, 0.1 M and 0.15 M NaCl concentrations. Finally on 7/14 and 7/17-7/18 2021 102 multiple t-maze trials were run at 0.0 M and 0.1 M NaCl concentrations. These trials lasted no longer than five minutes before tadpoles were removed from the maze.

Throughout the multiple t-maze development and earlier experiments, tadpoles that were used in a multiple t-maze were set aside in separately labeled holding buckets. There were labeled buckets for tadpoles with prior salt exposure and separate buckets for trial-run tadpoles that did not have prior salt exposure. All of these tadpoles were re-run in the final multiple t-maze experiment, running from 7/24-7/31 2021. It was noted during each trial whether the tadpole in the trial did or did not have prior salt exposure. The quickest a tadpole was reintroduced to the multiple t-maze with any concentration of NaCl was six days. The longest period of time between reintroduction to the multiple t-maze with any concentration of NaCl was approximately 32 days. Because exposure was limited to five minutes and at least six days passed between reintroductions, there was not an expectation that prior salt exposure would alter tadpole behavior in the multiple t-maze trials.

Results

Lateralized Turning Behavior in a T-Maze

In 50 trials at 0.0 M salinity, there were 22 (44%) tadpoles that turned left in the t-maze and 28 (56%) that turned right in the t-maze. A binomial test analysis showed that at the population level there was no significant difference between left and right turn preference ($p =$ 0.839) but an exact Agresti-Coull test of the observed vs expected proportion ($N = 50$) yielded low power ($power = 0.163$) for this result (Agresti and Coull, 1998). So while no statistically significant lateralized turning bias was revealed in this initial experiment, the small sample size warrants caution in generalizing about the existence of an overall lateralized bias of the tadpole population. Potential lateralized turning biases were accounted for by randomly selecting among the forced right and left turn maze types for each trial in our subsequent multiple t-maze experiment. These two maze types mirror one another: one forces tadpoles to make an initial left turn while the other forces tadpoles into an initial right turn.

Body Size

Body size ($N = 461$) was not normally distributed ($W = 0.945$, $p < 0.001$) but closely resembled a normal distribution ($M = 1.222$, $SD = 0.136$). The data is slightly skewed to the left ($skewness = -0.055$, $variance = 0.019$, $kurtosis = -0.346$) and there were no outliers in the distribution.

Seven tadpoles (of 468) were excluded from analysis based on body size exclusion criteria. The tadpoles excluded had short tail lengths relative to their body length. Short tail lengths resulted either from damage or developmental abnormalities. This could affect swimming ability and other behavior in the multiple t-maze. The criteria for body size exclusion

was determined by running a bivariate fit plot of tail length by body length and excluding those

data points that fell outside of a bivariate normal density ellipse ($p = 0.990$). All seven of the data

points outside of the density ellipse possessed abnormally short tail lengths relative to body

length. This allowed body length to be used as the definitive variable for body size.

All statistical analyses were completed with the seven data points excluded for the

described body size criteria and without those data excluded. The results were consistent

regardless of whether several data points were excluded based on body size exclusion criteria for

nearly all analyses. Only maze time was not consistent if several data points were not excluded

based on body size exclusion criteria. With no data points excluded, there was not a significant

effect of prior salt exposure on maze time while with those data excluded there was a significant

effect.

Multiple T-Maze Maze Completion

In 461 trials, there were 368 tadpoles that completed the maze and 93 tadpoles that did

not complete the maze. A nominal logistic fit regression model showed that salinity had a

significant effect on maze completion (Table 1). As salinity increased, maze completion rates

increased (Figure 8). There were also significant effects identified for body size and prior salt

exposure. As body size increased, maze completion rates increased. Tadpoles that had prior salt

exposure were significantly less likely to complete the maze (Figure 9). Maze type, maze

orientation, salinity*prior salt exposure, and salinity*body size did not have a significant effect

on maze completion.

Table 1. Nominal Logistic Fit Regression Model of Maze Completion

		$N = 461$	
Variable	***df***	***L-R X^2***	***p***
Salinity	1	11.833	0.001*
Body Size	1	12.717	0.001*
Maze Type	1	1.521	0.218
Maze Orientation	1	2.468	0.116
Prior Salt Exposure	1	4.118	0.042*
Salinity*Prior Salt Exposure	1	0.904	0.342
Salinity*Body Size	1	0.004	0.953

Note. Significant at the $p < 0.05$ level. $R^2(U) = 0.077$

Figure 8. Maze Completion Rates at Tested Salinities
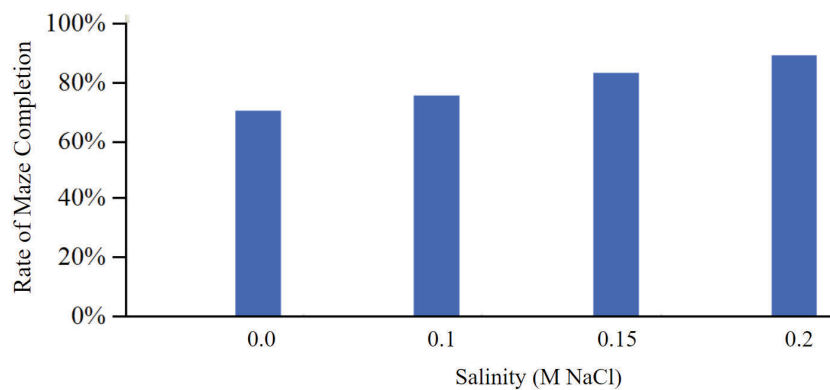
Note. As salinity increased, maze completion rates increased. The maze completion rates ranged from 70% at 0.0 M (far left) NaCl to 89% at 2.0 M NaCl (far right).

Figure 9. Maze Completion Rates by Prior Salt Exposure
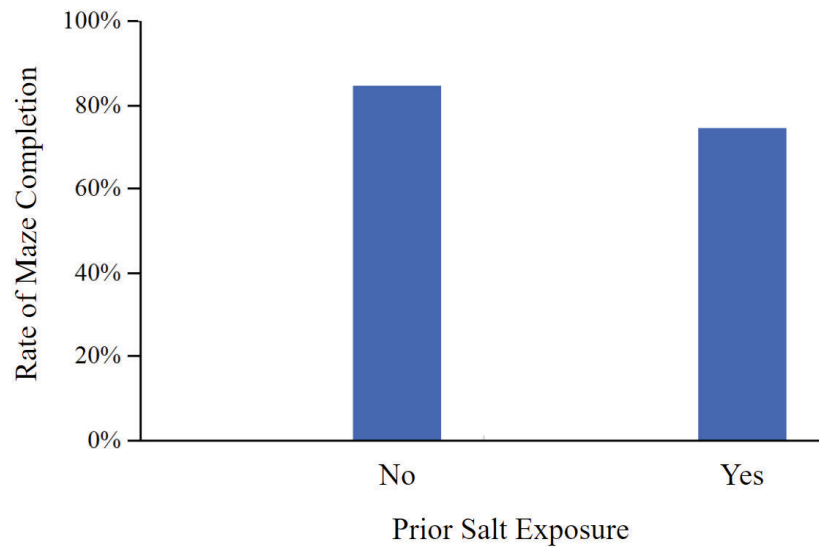
Note. Tadpoles that had prior salt exposure were significantly less likely to complete the maze. The maze completion rate for tadpoles without prior salt exposure was 84% (left) while the maze completion rate for tadpoles with prior salt exposure was 74% (right).

U-Turns in a Multiple T-Maze

In 461 total trials, there were 41 tadpoles that performed u-turns in the multiple t-maze. A standard least squares fit regression model of u-turns showed that there was no significant effect of salinity, body size, maze type , maze orientation, prior salt exposure, salinity*prior salt exposure, or salinity*body length (Table 2). There was however a trend of u-turn behavior increasing as salinity increased (Figure 10).

Figure 10. Mean U-Turns as Salinity Increases
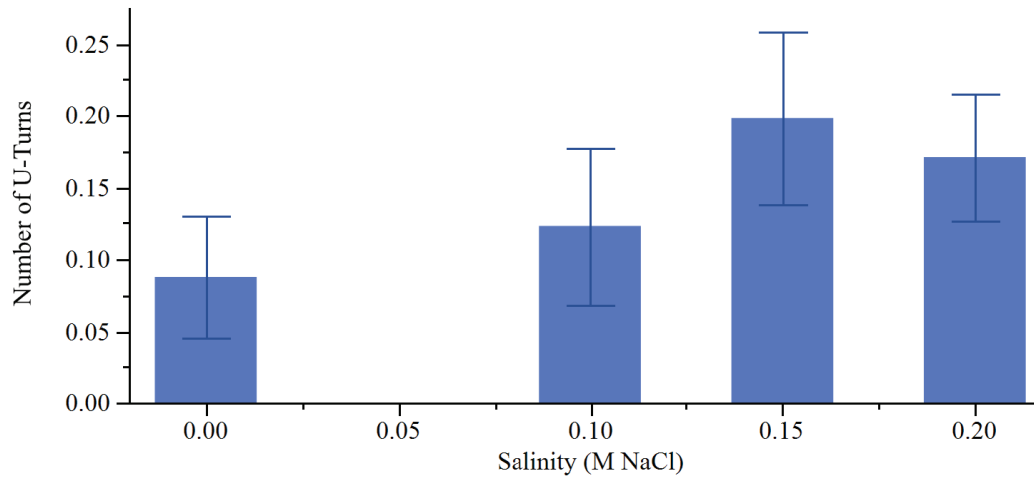
Note. There was a trend of u-turn behavior increasing as salinity increased. The mean number

of u-turns was 0.085 (*SD* = 0.447) at 0.0 M NaCl, 0.120 (*SD* = 0.575) at 0.1 M NaCl, 0.120

(*SD* = 0.646) at 0.15 M NaCl and 0.171 (*SD* = 0.479) at 0.2 M NaCl.

Table 2. Standard Least Squares Fit Regression Model of U-Turns

Variable	df	Sum of Squares	F Ratio	p
		$N = 461$		
Salinity	1	0.563	1.882	0.171
Body Size	1	0.036	0.122	0.727
Maze Type	1	0.084	0.280	0.597
Maze Orientation	1	0.467	1.560	0.212
Prior Salt Exposure	1	0.040	0.133	0.715
Salinity*Prior Salt Exposure	1	0.024	0.079	0.779
Salinity*Body Size	1	0.729	2.436	0.119

Note. * indicates $p < 0.05$. ** indicates $p < 0.01$. $R^2 = 0.015$.

Alternating Turn Behavior in a Multiple T-Maze

There were 337 trials out of 468 total trials included in this data analysis. In these trials, tadpoles completed the maze within five minutes by reaching an endpoint and they did not make any u-turns in that time. A chi-squared analysis of these trials revealed that the observed distributions of the frequencies of alternating turns at each trial condition (0.0-0.2 M NaCl) significantly differed from the expected random distribution of frequencies of 1:3:3:1 (Table 3). The observed frequencies of alternating turn behavior showed that for all salinities tested (0.0 M, 0.1 M, 0.15 M, and 0.2 M) there was a high prevalence of alternating turn behavior in a multiple t-maze. The alternating turn outcome with the highest probability at every salinity except for 0.0M salinity was the maximum alternating turns that could be completed, three (Figure 11).

Table 3. Chi-Squared Analysis of Alternating Turn Distributions

Salinity (M NaCl)	df	*Likelihood Ratio*		*Pearson*	
		X^2	*p*	X^2	*p*
0.0	3	28.227	< .0001**	34.684	< .0001**
0.1	3	40.409	< .0001**	53.420	< .0001**
0.15	3	46.607	< .0001**	58.379	< .0001**
0.2	3	39.191	< .0001**	53.381	< .0001**

Note. * indicates $p < 0.05$. ** indicates $p < 0.01$.

Figure 11. Observed vs Expected Frequency Distributions of Alternating Turns
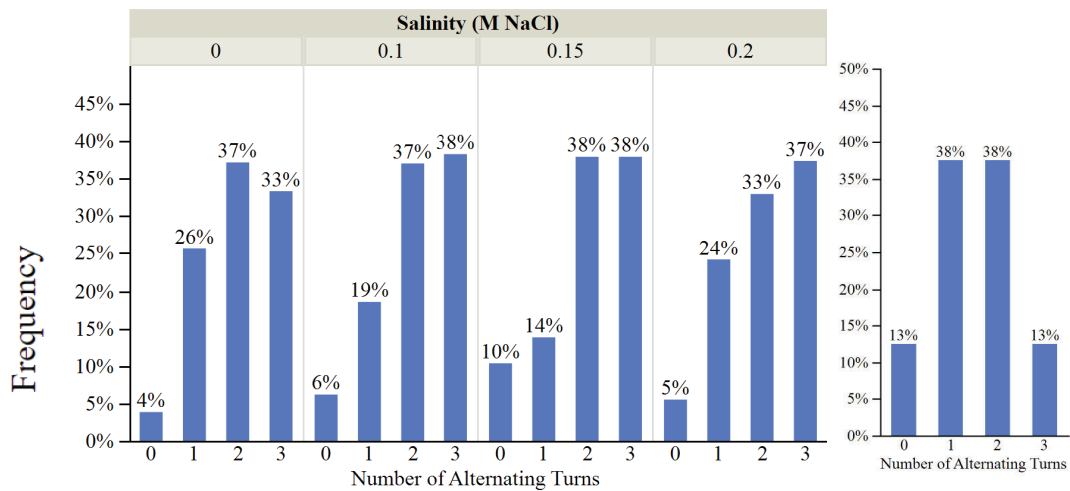
Note. The observed distributions of the frequencies of alternating turns at each salinity ranging for 0.0 M to 2.0 M (left) significantly differed from the expected random distribution of frequencies (right). There was an increase in alternating turn behavior at every salinity.

Salinity and Alternating Turn Behavior

A logistic fit regression model of observed alternating turn distributions showed that there was not a significant difference ($R^2 = 0.001$, $X^2_1 = 0.104$, $p = 0.748$) in the observed alternating turn distributions at different salinities. This is contrary to the prediction that alternating turn behavior would increase as salinity increases.

An ordinal logistic fit model of alternating turns showed that salinity, body size, maze type, maze orientation, salinity*prior salt exposure, and salinity*body size did not have a significant effect on alternating turn behavior (Table 4). Prior salt exposure did have a significant effect on alternating turn behavior. Tadpoles used in prior experiments ranging from 5-30 days before the start of this experiment ($M = 1.904$, $SD = 0.939$) were less likely to exhibit alternating turn behavior when placed in the multiple t-maze than tadpoles without prior salt exposure ($M = 2.140$, $SD = 0.879$) (Figure 12).

Table 4. Ordinal Logistic Fit Regression Model of Alternating Turns

Variable	df	L-R X^2	p
		$N = 337$	
Salinity	1	0.151	0.697
Body Size	1	3.521	0.061
Maze Type	1	2.108	0.147
Maze Orientation	1	0.116	0.734
Prior Salt Exposure	1	4.902	0.027*
Salinity*Prior Salt Exposure	1	1.009	0.315
Salinity*Body Size	1	0.364	0.546

Note. * indicates $p < 0.05$. ** indicates $p < 0.01$. $R^2(U) = 0.015$

Figure 12. Mean Alternating Turns by Prior Salt Exposure
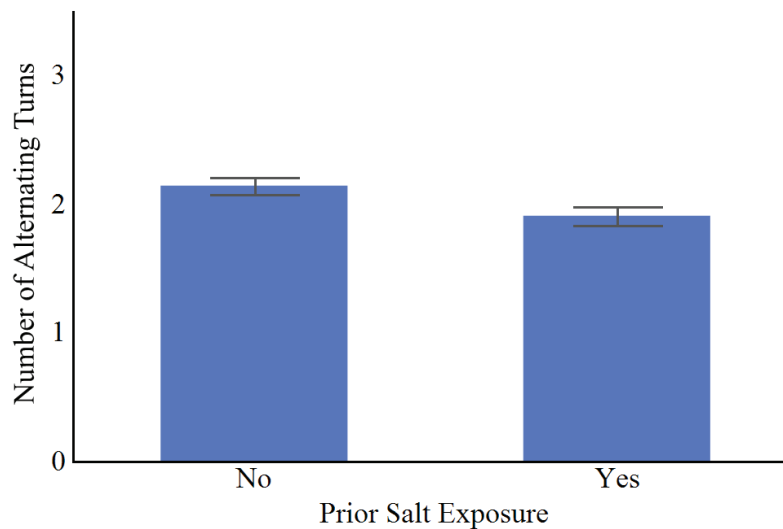
Note. Tadpoles used in prior experiments (*M* = 1.904, *SD* = 0.939) were less likely to exhibit alternating turn behavior when placed in the multiple t-maze than tadpoles without prior salt exposure (*M* = 2.140, *SD* = 0.879)

Other Alternating Turn Behaviors

A nominal logistic fit regression model showed that there was a significant effect of maze type and maze orientation on first turn alternation (Table 5). There was no significant effect of salinity, body size, prior salt exposure, salinity*prior salt exposure or salinity*body size on first turn alternation. A nominal logistic fit regression model showed that there was a significant effect of body size on any two turn alternations (Table 6). There was no significant effect of salinity, maze type, maze orientation, prior salt exposure, salinity*prior salt exposure or salinity*body size on any two turn alternations in a row. A nominal logistic fit regression model showed that there was a significant effect of body size on the first two turn alternations in a row

(Table 7). There was no significant effect of salinity, maze type, maze orientation, prior salt exposure, salinity*prior salt exposure or salinity*body size on the first two turn alternations in a row.

Table 5. Nominal Logistic Fit Regression Model of First Turn Alternation

Variable	df	L-R X²	p
		N = 337	
Salinity	1	0.826	0.363
Body Size	1	0.298	0.585
Maze Type	1	4.261	0.039*
Maze Orientation	1	6.513	0.011*
Prior Salt Exposure	1	0.864	0.352
Salinity*Prior Salt Exposure	1	1.613	0.202
Salinity*Body Size	1	0.450	0.502

Note. * indicates $p < 0.05$. ** indicates $p < 0.01$.

Table 6. Nominal Logistic Fit Regression Model of Any Two Turn Alternations

Variable	df	L-R X^2	p
		$N = 337$	
Salinity	1	0.208	0.648
Body Size	1	8.567	0.003**
Maze Type	1	0.871	0.351
Maze Orientation	1	1.239	0.266
Prior Salt Exposure	1	1.411	0.235
Salinity*Prior Salt Exposure	1	1.353	0.245
Salinity*Body Size	1	0.614	0.433

Note. * indicates $p < 0.05$. ** indicates $p < 0.01$.

Table 7. Nominal Logistic Fit Regression Model of First Two Turn Alternations in a Row

Variable	df	L-R X^2	p
		$N = 337$	
Salinity	1	1.487	0.223
Body Size	1	8.417	0.004**
Maze Type	1	3.348	0.067
Maze Orientation	1	0.220	0.639
Prior Salt Exposure	1	0.269	0.604
Salinity*Prior Salt Exposure	1	1.381	0.240
Salinity*Body Size	1	0.575	0.448

Note. * indicates $p < .05$. ** indicates $p < .01$.

Latency Time

Latency time is the time from when tadpoles are placed in the maze and are ungated to when they make the initial forced turn in the multiple t-maze. A fit least squares regression model, with arcsine transformed latency time, showed that salinity, body size, and maze type had a significant effect on latency time (Table 8). As salinity increased, latency time decreased (Figure 13). As body size increased, latency time increased (Figure 14). Maze orientation, prior salt exposure, salinity*prior salt exposure, and salinity*body size did not have a significant effect on latency time.

Table 8. Fit Least Squares Regression Model of Latency Time

Variable	df	Sum of Squares	F Ratio	p
		$N = 337$		
Salinity	1	5.931	5.901	0.016**
Body Size	1	15.182	15.106	0.001**
Maze Type	1	5.664	5.635	0.018**
Maze Orientation	1	2.976	2.961	0.086
Prior Salt Exposure	1	1.685	1.676	0.196
Salinity*Prior Salt Exposure	1	1.285	1.279	0.259
Salinity*Body Size	1	0.002	0.002	0.963

Note. * indicates $p < 0.05$. ** indicates $p < 0.01$. $R^2 = 0.085$.

Figure 13. Latency Time, Maze Time and Total Time by Salinity
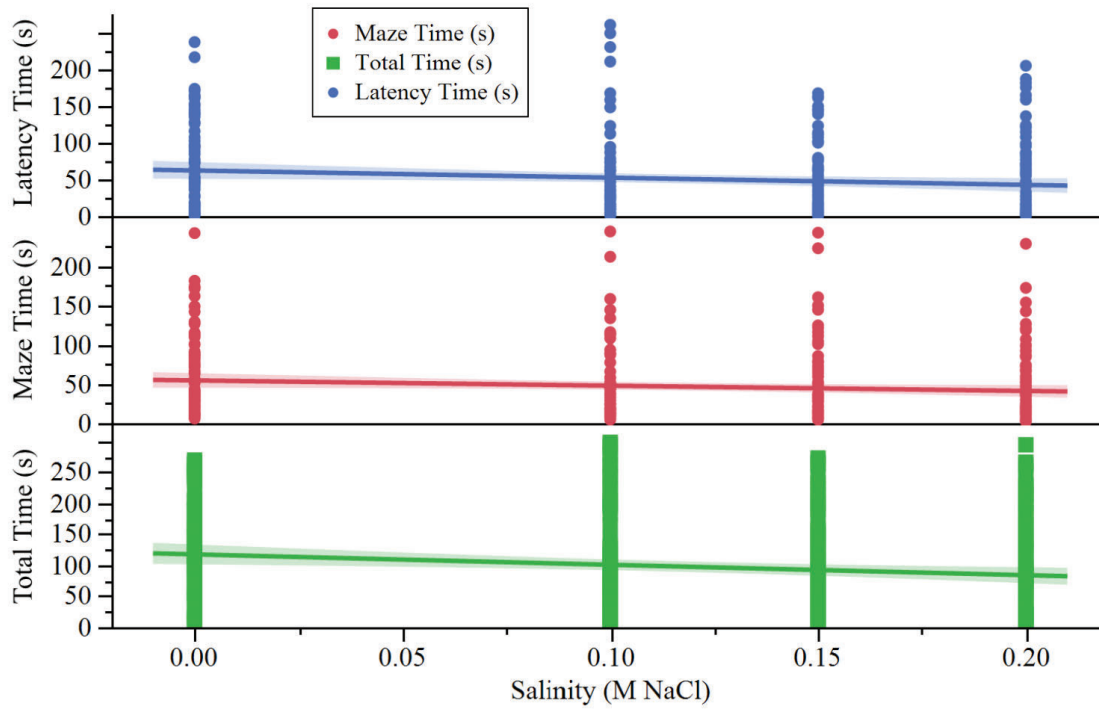
Note. As salinity increased, latency time, maze time, and total time all decreased.

Figure 14. Latency Time, Maze Time and Total Time by Body Size
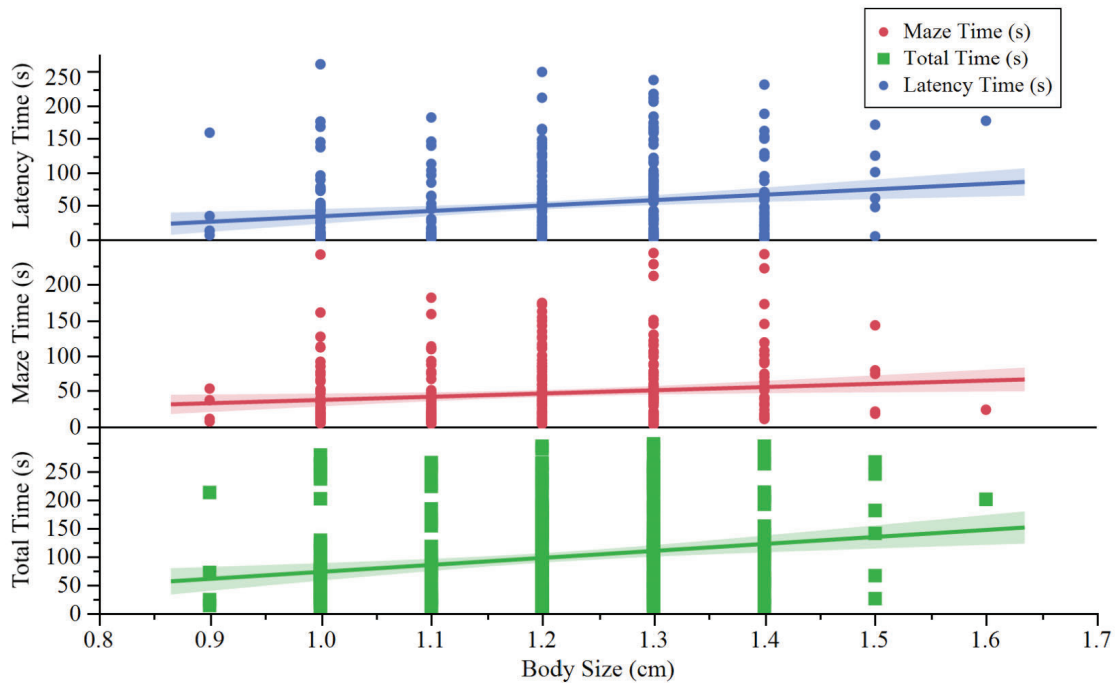
Note. As body size increased, latency time, maze time, and total time all increased.

Latency Time and Lateralization

The latency time for the forced right turn, original maze, was shorter than the latency time for the forced left turn, mirrored maze (Figure 15). This is consistent with the non-significant trend of right turn lateralized bias (28R/22L) seen in the initial t-trials experiment. While no significance was originally observed when an exact Agresti-Coull test of the proportion at $N = 50$ was completed it yielded low power (*power* = 0.163) and could not be used to generalize about lateralized turning biases of the population.

Figure 15. Lateralized Turning Frequency in a T-Maze and Latency Time by Maze Type.
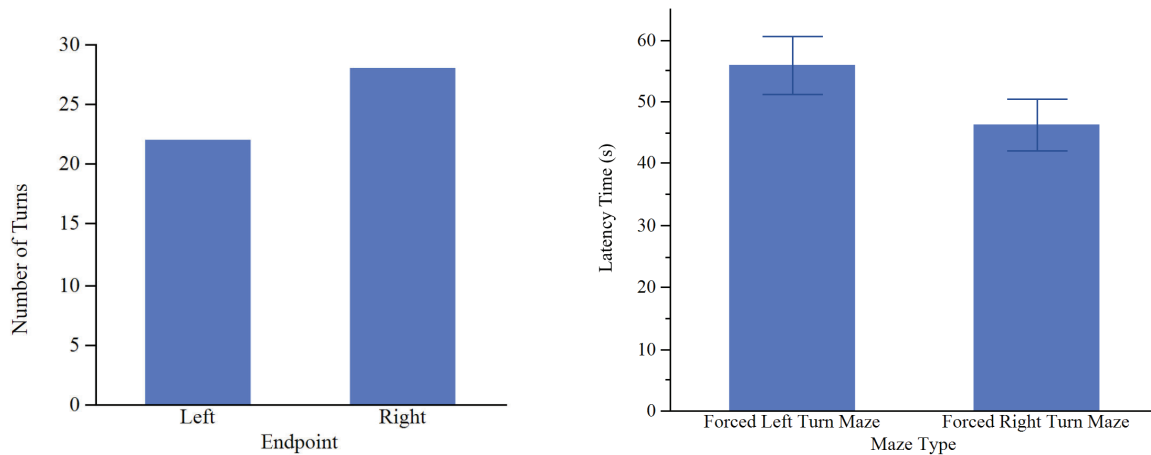
Note. At 0.0 M NaCl, there were 22 (44%) tadpoles that turned left in the t-maze and 28 (56%) that turned right in the t-maze (left). The latency time for the forced right turn maze ($M =$ 55.901, $SD = 59.303$), was shorter than the latency time for the forced left turn maze ($M =$ 46.251, $SD = 55.975$) (right).

Maze Time

A fit least squares regression model, of arcsine transformed maze time, showed that salinity and body size had a significant effect on maze time (Table 9). As salinity increased, maze time decreased (Figure 13). As body size increased, maze time increased (Figure 14). Prior salt exposure, maze type, and maze orientation did not have a significant effect on maze time.

Table 9. Fit Least Squares Regression Model of Maze Time

Variable	df	Sum of Squares	F Ratio	p
		N = 337		
Salinity	1	9650.820	4.583	0.033*
Body Size	1	13892.548	6.597	0.011*
Maze Type	1	2340.550	1.111	0.293
Maze Orientation	1	67.937	0.032	0.858
Prior Salt Exposure	1	8630.596	4.098	0.044*
Salinity*Prior Salt Exposure	1	217.927	0.104	0.748
Salinity*Body Size	1	6067.282	2.885	0.090

Note. * indicates $p < 0.05$. ** indicates $p < 0.01$. $R^2 = 0.055$.

Total Time

A fit least squares regression model, with arcsine transformed total time, showed that salinity, body size, and maze type had a significant effect on total time (Table 10). As salinity increased, total time decreased (Figure 13). As body size increased, total time decreased (Figure 14). As seen for the latency time results, the total time for the forced right turn maze ($M = 90.549$, $SD = 77.093$, was shorter than the total time for the forced left turn maze ($M = 106.164$, $SD = 83.834$. Maze orientation, prior salt exposure, salinity*prior salt exposure, and salinity*body size did not have a significant effect on total time. These results were consistent regardless of whether several data points were excluded based on body size exclusion criteria.

Table 10. Fit Least Squares Regression Model of Total Time

Variable	df	Sum of Squares	F Ratio	p
		N = 337		
Salinity	1	9.202	9.277	0.003**
Body Size	1	17.152	17.292	< 0.001**
Maze Type	1	5.346	5.390	0.021*
Maze Orientation	1	0.415	0.418	0.518
Prior Salt Exposure	1	0.000	0.000	0.999
Salinity*Prior Salt Exposure	1	0.743	0.749	0.388
Salinity*Body Size	1	0.262	0.264	0.607

Note. * indicates $p < 0.05$. ** indicates $p < 0.01$. $R^2 = 0.086$.

Discussion

Lateralization

In single T-maze trials, there was no significant lateralized bias in tadpole turning despite a small trend of rightward turns. A power analysis determined that this result had low power at N = 50. Other studies that examined lateralized biases in amphibians identified turning biases beginning at Gosner stage 25 which is in line with the developmental range of our tadpole population; Gosner stage 26-38 (Gosner, 1960; Malashichev and Robins, 2018). Previous studies examined lateralized behaviors in different ways. These ways included looking at jumping direction in response to predator cues, righting behavior when flipped over, and wiping their snouts (Malashichev, 2006; Malashichev and Nikitina, 2002). Specifically for tadpoles, researchers have examined their startle response to determine lateralized biases (Malashichev and Robins, 2018). A combination of other bias tests with t-maze trials could improve detection of lateralization.

Initial forced turn direction had a significant effect on latency in our trials. In the forced right turn maze latency time was less than in the forced left turn maze. There was also a significant effect on first turn alternation. Tadpoles in the forced right turn maze were more likely to make an alternating turn at the first t-junction. They were also more likely to make two or more alternating turns in the maze. This could be explained by a rightward lateralized bias in the population of tadpoles studied. The sample size of the t-maze experiment ($N = 50$) could have limited our ability to pick up on subtle effects; the effects of initial forced turn direction (N = 337) suggest that a lateralized motor bias exists but was not detected by our t-maze trials due to low statistical power.

Alternating Turn Behavior

Alternating turn behavior in the multiple t-maze was observed at each salinity. This behavior in a complex maze environment is consistent with prior research that describes alternating turns as an innate behavior (Carbines et al., 1992, Hughes, 1967; Moriyama et al., 2015). Alternating turns have been associated with exploration and foraging when animals are introduced to complex environments (Estes and Schoeffler, 1955). Yet elevated salinity, even as high as 0.2 M NaCl, did not have a significant effect on alternating turn behavior (Table 11). Alternating turn behavior did not increase as hypothesized. Rather, at each trial condition there was observed alternating turn behavior regardless of salt concentration.

Table 11. Summary of Chi-Squared Analysis

Performance Variable	N	Effect
Lateralization	50	*ns* (low power) (L↓, R↑ trend)
Alternating Turn Distributions Observed vs Expected	337	↑
Alternating Turn Distributions by Salinity	337	*ns*

Note. ns indicates that there is not a statistically significant effect. ↑ indicates significance at $p < .05$ and an increase in the performance variable caused by an increase in the effect variable. ↓ indicates significance at $p < .05$ and a decrease in the performance variable caused by an increase in the effect variable. L and R refer to forced left and forced right turn maze types respectively.

Salinity

Adult *H. versicolor* have demonstrated aversion for salinity at an EC_{50} threshold of 0.155 M (Jamieson, 2012). The salt concentrations used in this experiment exceeded the threshold of *H. versicolor* tadpoles. In short-term acute exposure to elevated salt concentrations caused by road salt runoff pollution, it would be expected that similar avoidance behaviors would be exhibited by *H. versicolor* tadpoles.

While salinity did not increase alternating turn behavior, salinity did impact other tadpole behaviors (Table 12). At elevated salinities, there was more maze completion. This result was observed in conjunction with lower latency time, maze time and total time at elevated salt concentrations. Tadpoles swam faster in the and were more likely to reach an endpoint in the maze at higher salt concentrations. This suggests that they were less likely to pause at the decision-making t-junctions when salinity increased. Tadpoles were more likely to make u-turns at higher salt concentrations, while still completing the maze more frequently and quickly. Faster swimming tadpoles may have been more likely to attempt to change direction and make a u-turn.

The alternating turn behavior and faster swimming are evidence of avoidance and escape behaviors (Carbines et al., 1992; Moriyama et al., 2015). Turn alternation allowed tadpoles to quickly move in a straight direction after encountering an adverse stimulus.

Table 12. Summary of Regression Model Effects

Performance Variable	N	Effect Variables				
		Salinity	*Body Size*	*Maze Type*	*Maze Orientation*	*Prior Salt Exposure*
Maze Completion	461	↑	↑	*ns*	*ns*	↓
U-Turns	461	*ns* (↑ trend)	*ns*	*ns*	*ns*	*ns*
Alternating Turns	337	*ns*	*ns*	*ns*	*ns*	↓
1st Turn Alternation	337	*ns*	*ns*	L↓, R↑	F↓, B↑	*ns*
1st Two Turn Alternation	337	*ns*	↑	*ns*	*ns*	*ns*
Any Two Turn Alterations in a Row	337	*ns*	*ns*	L↓, R↑	F↑, B↓	*ns*
Latency Time	337	↓	↑	L↑, R↓	*ns*	*ns*
Maze Time	337	↓	↑	*ns*	*ns*	↑
Total Time	337	↓	↑	L↑, R↓	*ns*	*ns*

Note. ns indicates that there is not a statistically significant effect. ↑ indicates significance at $p < 0.05$ and an increase in the performance variable caused by an increase in the effect variable. ↓ indicates significance at $p < 0.05$ and a decrease in the performance variable caused by an increase in the effect variable. L and R refer to forced left and forced right turn maze types respectively. F and B refer to the forwards and backwards maze orientations respectively.

Prior Salt Exposure

Prior salt exposure significantly reduced the frequency of alternating turns and reduced the rate of maze completion. Tadpoles identified as having prior salt exposure were each previously used in a multiple t-maze trial at 0.1 M or 0.15 M NaCl 6-25 days before this

experiment began. It is possible that there was a small degree of learning among the tadpoles after completing the maze once. This is unlikely because there was no reinforcement or reward for this behavior. If learning did occur, it would be expected that tadpoles would complete the maze quicker to be removed from the unfavorable conditions of the salty maze. It is also possible that there were physiological or developmental effects from the earlier exposure. Cumulative exposure to salinity has been shown to reduce activity levels (Denoël et al., 2010; Kearney et al., 2016; Milotic et al., 2017; Sanzo and Hecnar, 2006). These possibilities are unlikely considering that this was a brief (five minute maximum), non-repeated exposure. There also was a six day (which stretched all the way to 32 days for some tadpoles) wait period before reintroduction. Another possibility for these results was that there may have been an unforeseen variable related to the tadpole holding container storage that biased this group of tadpoles. Further studies are needed to establish how this brief prior salt exposure changed tadpole behavior in the multiple t-maze.

Body Size

Body size had a significant effect on maze completion rate, latency time, maze time, and total time. Maze completion increased as body size increased and all three time measurements increased as body size increased. It is assumed that the population of 800 tadpoles initially collected for this study were all from a single clutch of eggs. Clutch sizes of *H. versicolor* range from 1,000 to 2,600 (Dodd 2013). That would mean that the tadpoles were all at the same age but growing and developing at different rates. Larger tadpoles were likely growing and developing at a faster rate than other smaller body size tadpoles of the population. The faster rate of growth and stress of metamorphosis may have reduced their ability to swim. An alternative

explanation is that the channels of the maze, which were intentionally designed to be narrow to prevent u-turns, could have restricted the larger tadpoles and reduced their swimming ability. Less u-turns completed by the larger tadpoles would also explain the higher rate of maze completion. Further studies are needed to clarify how body size changed tadpole behavior in the multiple t-maze.

Experimental Design

Maze orientation had an effect on first turn alternation. There was less first turn alternation when the maze was in the forwards orientation where the tadpole first forced turn is in the direction of the researcher. The position of the researcher at the end of the bench near the maze could have been a visual cue. This may have biased the initial alternating turn. Maze orientation also had an effect on whether a tadpole made any two alternating turns. There were more alternating turns when the maze was in a backwards direction. This could be due to an unforeseen confounding aspect of the lab environment (e.g. lighting, ambient noise). To reduce the potential biases of the maze orientation that had an effect on first turn alternation and two or more alternating turns, future studies with a similar multiple t-maze protocol could use randomized quarter turns.

Chemical cues could have been left behind in the maze by the researcher or by tadpoles from preceding trials. Although the multiple t-mazes were thoroughly rinsed with tap water along with all tools between trials, certain chemical cues may have remained in the maze. While it is unclear how that may have altered tadpole behavior, it may have biased the results. Cues left by tadpoles at an endpoint could have influenced the successive trials. Cues imparted by human

researchers may have been perceived as a predator cue leading to either escape or avoidance behaviors by the tadpoles.

Multiple T-Maze

Multiple t-mazes are an effective tool for observing alternating turn behavior, but it is unclear whether this maze design is effective for determining how those behaviors are affected by a chemical stimulus such as salinity. *H. versicolor* tadpoles exhibited alternating turn behaviors regardless of salinity in the multiple t-maze. If there are salinity driven differences in this behavior, multiple t-maze trials would not aid in elucidating if they may increase. Other data gathered from the maze such as completion and speed times could be collected from simpler tools. For example, a single straight channel design could be used to gauge tadpole motor responses at varying levels of salinity.

This multiple t-maze experimental protocol can be adapted to study the behavior of other species of amphibian tadpoles and aquatic organisms. Further studies could examine a wider range of *H. versicolor* tadpole developmental stages to determine if there are differences in behavior. The multiple t-maze design could also be altered to add an additional t-junction to give the possibility of an individual making a fourth alternating turn in the maze. Beyond salinity, other multiple t-maze experiments could examine the effect of other chemical stimuli such as predator cues or pesticides on alternating turn behavior.

Conclusion

Freshwater amphibian habitat is increasingly becoming salinized from road salt runoff and other pollutants (Corsi et al., 2010). At particular risk is vernal pool habitat that many amphibian species use for reproductive behaviors and larval stage development (Semlitsch, and Skelly, 2008). It is important to understand the behavioral and physiological effects that elevated salt concentrations will have on amphibians. While salinity did not affect alternating turn behavior of *H. versicolor* tadpoles, tadpoles at elevated salinities did continue to make alternating turns and move quickly to reach an endpoint. Acute salt exposure triggered avoidance and escape behaviors. This could have implications for how *H. versicolor* tadpoles in their natural habitat forage, explore and evade predators. These findings could also suggest that there may be similar effects on the closely related *H. chrysoscelis* species that is endangered in New Jersey.

Prior exposure to salinity in earlier pilot studies led to a reduction in alternating turn behavior and decreased maze completion. These findings could offer insight into the impact of longer term salt exposure on turning behavior of *H. versicolor* tadpoles. More work is needed to determine what effect prior salt exposure had on amphibian behavior in the multiple t-maze.

There could be differences in behaviors at different developmental stages. The population of tadpoles studied were within a narrow range of Gosner stage 26-38. Alternating turn behavior and other behavior in the multiple t-maze could vary by developmental stage. Additionally, only one species of amphibian was used for these trials and previous research has demonstrated that among amphibian species there is a wide range of salt tolerance (Collins and Russell, 2009). This limits the generalizability of these results for other species. Future research can explore these behaviors in other species using a similar methodology.

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