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MATE CHOICE, ILLUSIONS, AND BEHAVIORAL DIVERGENCE IN
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MATE CHOICE, ILLUSIONS, AND BEHAVIORAL DIVERGENCE IN
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Abstract

Chapter 1: Illusions are commonplace and distort perception in ways that make objects appear different from reality. Such phenomena may also play a role in mate evaluation because body size and ornament size are signals shaped by sexual selection. Evolution of use of illusions through modifications of color patterns, environmental conditions, or behaviors may be common, yet are poorly understood. The Ebbinghaus illusion refers to the distorted perception of the size of an object depending on the size of surrounding objects, and is traditionally shown using Titchener circles. Because female sailfin mollies *(Poecilia latipinna)* choose mates from shoaling groups of males, I predicted that they would be susceptible to this illusion during mate selection. Specifically males surrounded by smaller Titchener circles (or conspecifics) should be preferred over those surrounded by larger circles (or conspecifics). I tested this prediction by presenting females with males at the center of an Ebbinghaus illusion, surrounded by Titchener circles, designed to make them appear either larger or smaller. I further tested this prediction using fish animations with focal male fish flanked by either larger or smaller males in shoaling groups. Females consistently chose the male flanked by smaller Titchener circles and this illusory effect endured when I replaced the Titchener circles with fish. Traditionally attractive larger males were not chosen, which is also consistent with predictions of the Ebbinghaus illusion. These results show that a male's immediate environment can have a strong effect on generating a deceptive body-size perception to potential mates, and demonstrate that males have the potential to manipulate matings using the Ebbinghaus illusion.

Chapter 2: Identifying how signals can distort the receiver's perception of reality is key to understanding signal evolution. Perceptual biases present in the receiver can create an inaccurate or incomplete perception of an environment, leading to sub-optimal decision-making. In some circumstances, signaling animals are able to deceive receivers by exploiting perceptual illusions, such as the Ebbinghaus illusion.

Female sailfin mollies *(Poecilia latipinna)* have been shown to be susceptible to the Ebbinghaus illusion in a dichotomous choice experiment with contrasting illusions shown at the same time (Chapter 1). However, whether or not sailfin mollies remember the illusory effects while making mate choice decisions remains in question. Here, I tested whether this effect would persist over time using two experiments. In the first experiment I used live flanking males to induce the illusory effects. In the second experiment I used inanimate objects to mimic the Titchener circles found in the classic Ebbinghaus illusion. Specifically, I tested female preference for a male by sequentially presenting the same focal male with three different illusory contexts: 1) focal male flanked by smaller males or smaller inanimate objects, 2) focal male flanked by larger males or larger inanimate objects, or 3) focal male flanked by similar-sized males or inanimate objects. I predicted that, if memory plays a key role in signal interpretation, female *Poecilia latipinna* would be susceptible to the effects of the Ebbinghaus illusion sequentially during mate choice. I found that female *Poecilia latipinna* choice was not influenced in the absence of inducers — both for flanking male treatments and the inanimate stimuli. This pattern suggests that the impact of the illusion was not remembered and did not impact subsequent preference. Thus, illusory effects may only impact mate choice while the receiver is assessing the signal in real time, using working memory, and may not affect longer-term memory.

Chapter 3: Toxic sulfide springs create particularly harsh environmental conditions, driving divergence in physiological, life history, morphological, and behavioral traits. Although few invertebrates or vertebrates can live in hydrogen sulfide springs, several lineages of livebearing fishes have adapted to sulfidic environments. One key behavioral adaptation that can be measured across populations is the performance of aquatic surface respiration (ASR), where organisms in hypoxic conditions exploit the higher dissolved oxygen content at the surface of the water. Generally, organisms that live in sulfidic habitats are highly vulnerable to extinction owing to the

small spatial extent of the habitat, which only supports very small populations, and makes them vulnerable to extirpation by localized natural processes, human activities, and accumulation of deleterious mutations. One such species is the endemic *Limia sulphurophila*, found in a single, small public sulfidic spring on Hispaniola. A closely related species, *L. perugiae*, is historically found downstream and provides a comparative reference for testing ASR in a non-sulfidic species. In this study I (1) characterized the environmental parameters of this system's sulfide-freshwater gradient and (2) evaluated differences in behavioral traits (ASR) between *L. perugiae* and *L. sulphurophila*. I assess behavioral divergence between populations based on time performing ASR to examine whether species could survive outside of their locally adapted environment. I quantify differences in a behavior (ASR) typically found in species adapted to sulfidic condition. This evidence is consistent with the hypothesis that behavioral and physiological adaptions may have arisen multiple times in Poeciliids due to strong selection pressure in an extreme environment.

Chapter 1

Mate choice in a fish is susceptible to the Ebbinghaus illusion: Be a Big fish in a small shoal

1.1 Introduction

Visual illusions are common and can affect an individual's perception by making objects appear larger or smaller, darker or lighter, or farther or nearer than they actually are. Generally, illusions can be defined as any signal that distorts the perception of the receiver (Kelley and Kelley 2014). Illusions are intriguing in the context of evolved behavioral choices because they create a disconnection between what an observer sees and how this is ultimately interpreted. Consequently, reality and perception might be quite different, leaving the observer susceptible to deception. Although the literature covering the mechanisms and properties underlying illusions in humans is vast, to date there has been less of a focus on the perception of illusions and there evolutionary impacts in non-human animals (Coren and Girgus 1978, Kelley and Kelley 2014, Feng et al. 2017). The evolution of use of visual illusions through color patterns, environmental modifications, or behavioral manipulations may be common (Guilford and Dawkins 1991, Kelley and Kelley 2014). Since illusions may influence signal evolution in an array of behavioral domains, the role of visual illusions may be particularly important in intraspecific sexual selection where body size and ornament size are key sexual signals. One visual illusion that is relevant

in this context is the Ebbinghaus illusion (Ebbinghaus 1902), where the perceived size of an object depends on the size of surrounding objects (Figure 1.1a). Furthermore, this illusion can be perceived by non-human animals such as baboons (*Papio papio*), chickens (*Gallus gallus*), dolphins (*Tursiops truncatus*), and fish (*Xenotoca eiseni*) (Parron and Fagot 2007, Salva et al. 2013, Murayama et al. 2012, Sovrano et al. 2015); but see (Nakamura et al. 2014). Because of this illusion, the perceived size of an object could be easily manipulated in various social or environmental contexts, and may be relevant and important in comparative mate evaluation, especially when males display in groups to females (Bateson and Healy 2005).

Over a range of taxa, several examples provide indirect evidence that illusions, and the Ebbinghaus illusion in particular, may significantly affect an individual's perception of the environment and thus influence sexual selection. Fiddler crabs (*Uca mjoebergi*) defend territories from neighboring males, while displaying their claw size to females. The Ebbinghaus illusion might be a determining factor in territorial defense in fiddler crabs with males preferring territories next to smaller males and females preferentially choosing those males (Callander et al. 2012). Similarly, male mate choice in guppies may be affected by a similar phenomenon, with males preferring females flanked by drab males (Gasparini et al. 2013). Although this is not a size-based illusion since there is no size comparison, the predicted result is the same: females choosing a male they otherwise might not. In birds the Ebbinghaus illusion might influence mate choice in the Great Bowerbird (*Chlamydera nuchalis*). Males construct their bower in a way that creates a forced perspective; so that the bower appears larger than it is to the female observer (Kelley and Endler 2012). Finally, triggerfish (*Rhinecanthus aculeatus*) were impacted by the Purves and Lotto lightness cube illusion, where perceived color and brightness are affected by perceived shadows that may play a role in foraging success (Simpson et al. 2016).

The effects of known illusions are not universal. As such, these illusions are one of many possible hypotheses from which to predict behavioral choices. For example, humans were not susceptible to size contrast illusions, such as the Ebbinghaus illusion, when rating the attractiveness (body mass index) of potential mates. This result was due to the flexibility of the receiver psychology of humans in mate choice and showed that body mass is an honest signal in humans (Bateson et al. 2014). This study, however, was a representation of only a partial Ebbinghaus illusion because the focal subject was not fully surrounded by flanking objects. This experimental design can be problematic because a female observes not only the displaying male(s) but also the entire environment in which the male displays, contributing to the receiver's psychological landscape and possibly directly or indirectly influencing mate choice (Guilford and Dawkins 1991). Therefore, if illusions do play a role in signal evolution, it is important to understand the receiver psychology and how signals may either directly distort or take advantage of the cognitive biases of the receiver (Ryan and Cummings 2013, Rosenthal 2007).

In the present study, I directly tested for the presence of the Ebbinghaus illusion using *Poecilia latipinna* (sailfin molly), a livebearing fish widely distributed along the Atlantic coast of southern North America. *Poecilia latipinna* has a promiscuous breeding system and males provide no parental care. This system is driven by female choice and, because of this, males are highly ornamented with elaborate dorsal fins and symmetrical vertical bars on each lateral side. One important feature of this system is the great natural variation in one key trait, male body size: the largest males are almost four times larger than the smallest males (Travis and Woodward 1989). Furthermore, sailfin mollies have complex social interactions and typically form non-permanent mixed-sex groups, where individuals have the opportunity to choose with whom they will compete for mates, although forced copulations are also common (Schlupp et al. 1994, Bisazza 1993). Sailfin molly females, like females in

many species, prefer the largest males. Hence it is adaptive for males to be large in order to be chosen by a female (Rosenthal and Evans 1998). Males can be perceived as being large in two ways: they can actually be large, or they can deceptively appear large. To appear large, males may take advantage of visual illusions.

Here, I quantified the change in female preference for a male by simultaneously presenting the same male in different social contexts and environmental treatments will all treatments mimicking the Ebbinghaus illusion to make the focal male appear either smaller or larger. I predicted that sailfin mollies would be susceptible to the Ebbinghaus illusion, meaning that males would be perceived as larger and more attractive or smaller and less attractive based on the forced context. If the illusions were not perceived, females would simply prefer the largest males, regardless of the presence of the illusions because larger body size is a more salient signal (Ryan et al. 2007). I also predicted that females would have a generalized response to illusions and that surrounding a focal male with non-fish objects to create a literal Ebbinghaus illusion would create the perception of either an increase or decrease in body size. Although this exact scenario is not likely to occur in nature, it is useful in evaluating the impact of the surrounding environment in mate evaluation. Furthermore, I also investigated whether a robustly honest signal like body size can be dishonest and even illusory based on context.

1.2   Methods

1.2.1   Fish care and management

I collected Sailfin mollies at in Brownsville, Texas, USA in April of 2015. I transported the fish back to 113-liter tanks at the University of Oklahoma, fed twice daily, and kept under a 12:12 dark/light cycle. I measured the standard length (snout to caudal peduncle) of all males and, based on the natural variation that occurred,

separated the males into three discreet sizes classes: small (35-36 mm), intermediate (42-50 mm), and large (55-60 mm). I separated the fish by sex and size for one month prior to the trials. All fish used for the experiment were at least 35 mm in standard length to ensure sexual maturity.

1.2.2   Test for illusion using Titchener circles

To test the Ebbinghaus illusion, I used a standardized dichotomous choice test where the female (N=30) was exposed simultaneously to treatments of a focal male surrounded by smaller Titchner circles and a focal male surrounded by larger Titchner circles (Figure 1.2a). In order to create the illusion, I surrounded a live wild-caught male with circular clay models of either smaller or larger size to create a literal Ebbinghaus illusion using Titchener circles (Figures 1.1, 1.2, 1.3). I used dark brown modeling clay to form a 3-D version of the illusion so that it would be clear that the focal male was on the same plane as the inanimate clay models. Based on the three classes of males I found in this population, I shaped the clay into the small size class that consisted of 6 half spheres each with a 25-mm diameter and the large size class that consisted of 6 half spheres each with a 75-mm diameter. I secured the objects to a white backboard on either end of the experimental tank. The focal male was contained in a clear plexiglas container so that he would remain in the center of the illusion. Both displays would be visible from the center of the tank for the acclimation period.

The experimental tank (61×39×30 cm) was divided into three equal sections, containing a neutral zone in the center and preference zones on both lateral ends (Figure 1.2). At the beginning of each trial, the female was placed in a clear plexiglas container in the center of the neutral zone. This acclimation period lasted for 10-min so that the female could observe both focal males. After the acclimation period, I

measured the amount of time the female spent in either preference zone for a 5-min test period. I switched the sides of the stimuli, allowed another 10-minute acclimation period with the female in the center of the tank, and tested female preference again to control for any side bias. After excluding females that displayed strong side-bias, the sample size was reduced to 22 individuals. To control for the effect of the clay models, I also tested each female (N=28) for an existing preference for either small or large clay models. The sample size was reduced due to mortality. After removing individuals with a side-bias the sample size was reduced to 20. I ran trials without the focal males and measured the amount of time the female spent in either preference zone (Figure 1.2c).

1.2.3   Test for illusion with flanking males

This protocol generally followed the methodology described above. However, instead of using live focal males surrounded by Titchener circles, I used a standardized dichotomous choice test where the female (N=32) was exposed simultaneously to animations of a focal male surrounded by males of either smaller or larger sizes (Figure 1.2c). I created video animations to control for any variation in male behavior or differences in color patterns. I did so by taking a digital photograph (Canon EOS Digital Rebel XTi with EF 50mm 2.5 macro lens, Tokyo, Japan) of an intermediate-sized male swimming in order to capture all attributes of the fish. I then used Adobe Photoshop to isolate images and remove the background. To create the animated swimming movement, I used an animation in Keynote and lengthened the animation to the final 5-minute video using iMovie to display on an iPad2. To create the illusion, I manipulated the size of the focal male to belong to either the smaller or larger size class. I created a separate set of animations for each of the three wild-caught sailfin molly males. Each animation was then randomly assigned to playback on an iPad2

during the mate-choice trials and females were exposed to animation from only one male.

Wild-caught females were exposed simultaneously to treatments using a dichotomous choice design. Each female was exposed to one set of videos of the same male, adjusted to various size treatments to create the Ebbinghaus illusions. The same experimental tank (61×39×30 cm) was divided into three equal sections, containing a neutral zone in the center and preference zones on both lateral ends (Figure 1.2). Apple iPad 2s were displayed at both ends of the tank, which displayed one of three possible animations creating the illusion. The females were shown videos of an intermediate-sized male of 45 mm SL flanked by either three smaller males of 35 mm SL or three larger males of 55 mm SL. Each treatment was shown simultaneously on either end of the test tank. Again, all images were of the same male, only the size of the male was manipulated. The focal male was the unique-sized male in each video, with all moving together in a shoaling group.

At the beginning of each trial, the female was constrained to the center of the neutral zone of the experimental tank in a clear plexiglas cylinder for a 10-min acclimation period to observe the animations on either end of the tank (Figure 1.2). Both animations were designed so that they would be visible from the center of the tank. After the acclimation period, I carefully removed the cylinder so that the female was free to swim around the experimental tank. Once she began to move, I began the 5-min test period during which I recorded the amount of time the female spent in either preference zone. To control for any side-bias, after the first test period, I switched the sides on which the iPad 2s were displayed on and repeated the procedure. If a female spent 85% or more time on one side of the tank across both trials, then it was considered a side bias, and that individual was removed from any analysis (McCoy et al. 2011). After the removal of side-biases, the sample size was reduced to 21 females, and the amount of time the female spent in each preference

zone was compared between treatments. Time in preference zones has been shown to accurately reflect mate choice when females were allowed to actually interact with males, and I have followed established mate choice testing procedures for this group of fishes (Tobler et al. 2009, Tobler M. et al. 2009, Schlupp and Ryan 1997); but see: (Gabor 1999).

1.2.4   Statistical analysis

For all trials, I tested for normality using the Shapiro-Wilks normality test with all pairs representing a normal distribution. Therefore, I conducted a paired *t*-test with time (in seconds) as the dependent variable between treatments. I eliminated trials where there was a side-bias was present. All analyses and graphics were performed using R version 1.0.136 (R Core Team 2017). To display the data, I used the mean with standard deviation (SD) as well as an alternate view of the data distribution using a box and whisker plot using the median (Figure 1.3).

1.3   Results

1.3.1   Tests with Titchener circles

There was the strongest female preference for the male flanked by smaller non-fish objects over the male flanked by larger non-fish objects. The females significantly spent more time in the preference zone of the focal sailfin molly male surrounded by smaller clay models than in the preference zone of the male surrounded by larger clay models (t_{21}=4.97, p=0.00006, Figure 1.3). This result shows there was some innate bias in which females are susceptible to this illusion in a generalized way. Although this is still within the realm of mate choice, the bias is strong enough that non-fish objects in the environment would be enough to elicit this illusory response. There was no difference in female preference in the trials with small and large clay models

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(t_{19}=-1.38, p=0.18; Figure 1.3).

1.3.2   Tests using video stimuli with flanking males

There was also a female preference for a male surrounded by smaller flanking males than for a male surrounded by larger flanking males. The females spent more time in the preference zone for the smaller flanking treatment (t_{20}=2.12, p=0.047, Figure 1.3). Therefore, the same male was perceived as more attractive in the illusory scenario that made him appear larger, than in the counter illusory scenario. There was no effect of the standard length of the choosing female (F=3.03, p=0.09).

1.4   Discussion

These results are consistent with the hypothesis that sailfin molly females are susceptible to the Ebbinghaus illusion during mate choice. Females consistently chose the male flanked by smaller males over that same male flanked by larger males. This illusory effect occurred even when the flanking males were removed and replaced with inanimate objects (Titchener circles). This result is significant for two main reasons. First, the traditionally attractive, larger males that were available were not chosen in what appears to be a direct result of this illusion. Many previous studies have shown that females would be most visually stimulated by the largest males, since larger males are more detectible, memorable, and desirable (Ryan and Cummings 2013). However, the present results show that a male's immediate environment can have a strong effect on generating a deceptive body-size perception to potential female mates. Second, these results show that male sailfin molly males have the potential to manipulate matings through the illusory effects of the Ebbinghaus mechanism. This effect is important when males signal not only in groups of males, but even when surrounded by inanimate objects that could be present in the environment.

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To directly test whether or not the Ebbinghaus illusion was responsible for the females' behavior, I surrounded a focal male with Titchener circles using 3D clay objects (Figure 1.1). This is where I saw the strongest effect of the illusion. The females clearly preferred the focal male surrounded by smaller Titchener circles to the focal male surrounded by larger Titchener circles. This result gives insight into the underlying mechanism of how the Ebbinghaus illusion is perceived in multiple contexts, since its effects can be seen from inanimate objects (Titchener circles), not only from fish displaying in groups. During the control trials, the females had no preference for either the smaller or larger objects, narrowing the potential explanations of the results to illusory effect of the experimental trial. There were one to two females in the experimental trial and the control trial that had the opposite preference relative to other females. These were different females in each of the trial, which suggests some receiver variation with respect to susceptibly to the Ebbinghaus illusion, and that the response may not be constant across time for all individuals. Furthermore, since there was such a long acclimation period (10-minutes) to view the illusion before the experimental trials, the effect of the illusion must be relatively powerful if individuals are susceptible for that length time. For humans for similar size illusions, the amount of exposure to an illusion diminishes the effect of that illusion; the more time you have to view, or solve, the illusion, the more likely you are to accurately perceive reality (Brouwer et al. 2014). Here, I saw that the illusory effects withstood a long examination period, suggesting my results are a robust response to the illusion.

Although the landscape scenario used in this study is not naturally occurring, it is important to note that, by default, the female observes not only the displaying male(s) but also the entire environment in which the male displays. This view contributes to the receiver's psychological landscape and possibly either directly or indirectly influences mate choice (Guilford and Dawkins 1991). In this way, the surrounding environment (either landscape or social) can serve to modify the signal of

the sender to appear either larger or smaller than it actually is. It is important to emphasize that the signaler in this experimental design (the focal male) alone is not manipulating the signal, for the illusory effect of this signal is dependent upon his surrounding context, either landscape or social. More specifically, this illusory behavior serves to amplify the existing signal of body size. In *Poecilia reticulata*, males can actively choose to associate with less attractive males when females are present, manipulating their social environment in their favor (Gasparini et al. 2013). This amplification or manipulation of signals is well studied, but direct use of illusions for amplification of signals is a new area of research (Cummings et al. 2006). In fact, this aspect of the illusion reveals a way in which the "noisy environment" where the signals are often observed can become adaptive in certain contexts (Endler et al. 2005, Rosenthal 2007). For example, Great Bowerbirds (*Chlamydera nuchalis*) were able to use their surrounding environment to their advantage to create a forced perspective to increase mating success, selecting for a behavior to manipulate the environment in a way that reduces that noise (Kelley and Endler 2012). The effects of illusions or forced perspectives to magnify sexually selective traits, therefore, may be adaptive.

In the second experiment, using flanking male animations, there was female preference for the focal male that was surrounded by smaller males, creating the illusion that the focal male was larger than he really was. Since the focal male was indeed the largest in that group, and therefore most desirable, the female subject was confronted with the option to choose that same male surrounded by larger males, simultaneoulsy on the opposite side of the tank. If the illusion had no effect, the female would have chosen the treatment with the larger flanking males, since that was the treatment group with the largest males possible from both groups. This result demonstrates how powerful the effects of this illusion may be on how female sailfin mollies compare and perceive sizes when males display in groups. Although most females in this experiment preferred the focal male flanked by smaller males, there

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were two individuals (Figure 1.3) who had a clearly contradictory reaction, which suggests that not all individuals are susceptible to this illusory effect.

The experiments demonstrated that a robust honest signal like body size can actually become deceptive or manipulative based on the context of the social environment or immediate landscape surrounding the focal individual. Body size is often considered an honest signal with regard to fitness (Maynard Smith and Harper 2003, Searcy and Nowicki 2005, Rowell et al. 2006) but, as shown from the present experiments, can be misinterpreted under certain social and environmental contexts. An illusion is clearly dependent on whether or not the receiver can perceive the illusion. For instance, the effect of the Ebbinghaus illusion was not perceived by all receivers of the signal and was not always consistently perceived by a given receiver across all trials. In this way, the illusory signal could be maintained within the population as a deceptive signal.

Furthermore, female sailfin mollies chose the focal male surrounded by smaller males even though this choice may have been more costly. In this species, smaller males in this species are more likely to perform coercive copulations while larger males typically court females (Schlupp et al. 2001, Ptacek and Travis 1997). Therefore females would then be susceptible to more harassment through associating with the smaller flanking males. Despite this cost, the females still spent more time with the smaller-male flanking treatment than the larger-male flanking treatment. Even though the benefit is clear for the focal males in the smaller-male flanking treatment, the benefit to the female is not, which again brings into question the honesty of the signal that is being perceived by the receiver. Although I attempted to control for this effect using animations that controlled for any differences in courtship behavior or aggression, the behavior associated with the size of the males may have had a residual effect on the female preference. This possibility may also explain the inconsistency of choice of some females across trials. Furthermore, the illusory effect using video

animation was not as strong as when using the live focal male with the surrounding Titchener circles. One explanation of this pattern may be that I used a video animation and sometimes the strength of preference is lower when a video is used as opposed to live fish (Trainor and Basolo 2000). While the direction of preference is the same, the strength is not.

Illusions themselves are by no means specific to mate choice and can affect other behavioral domains where context has been shown to be important, such as predator-prey interactions and foraging ability. For example, in guppies, the light environment plays a large role in both mate choice and predation (Endler 1993). Some color patterns may be conspicuous for both potential mates and predators in certain light environments, while being cryptic to those same receivers in other light environments. This fact makes the choice of a microhabitat an important one, with strong fitness consequences imposed on individuals (Endler 1980). Just like the signal of color intensity can be perceived differently in varying light environments, so too can the signal of body size in varying landscape and social conditions. Hence, the environment, and even microhabitat choice, can play a key role in the perception of signals, in mate choice and in predator-prey interactions. The role of illusions in these contexts deserves additional attention.
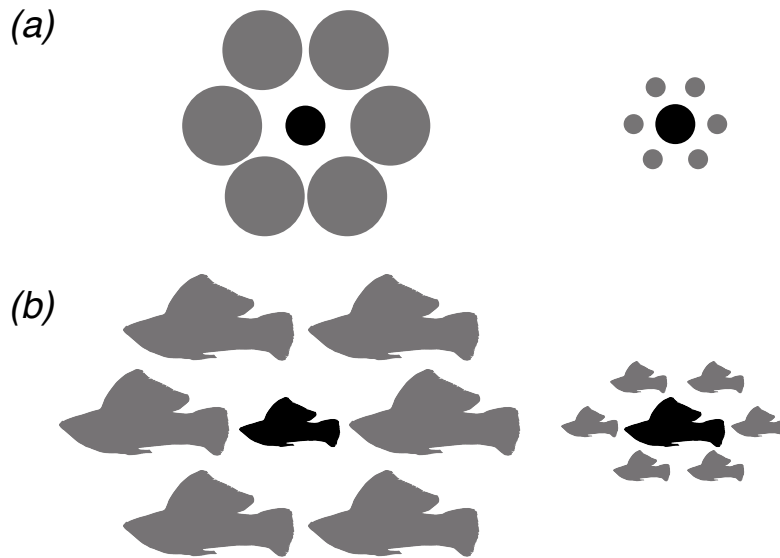
Figure 1.1: (*a*) Classic example of Ebbinghaus illusion using Titchener circles. The black dots in the center are the same size but appear to differ due to surrounding grey dots. (*b*) Example of the Ebbinghaus illusion using silhouettes of male sailfin mollies (*Poecilia latipinna*). The two black fish are the same size.
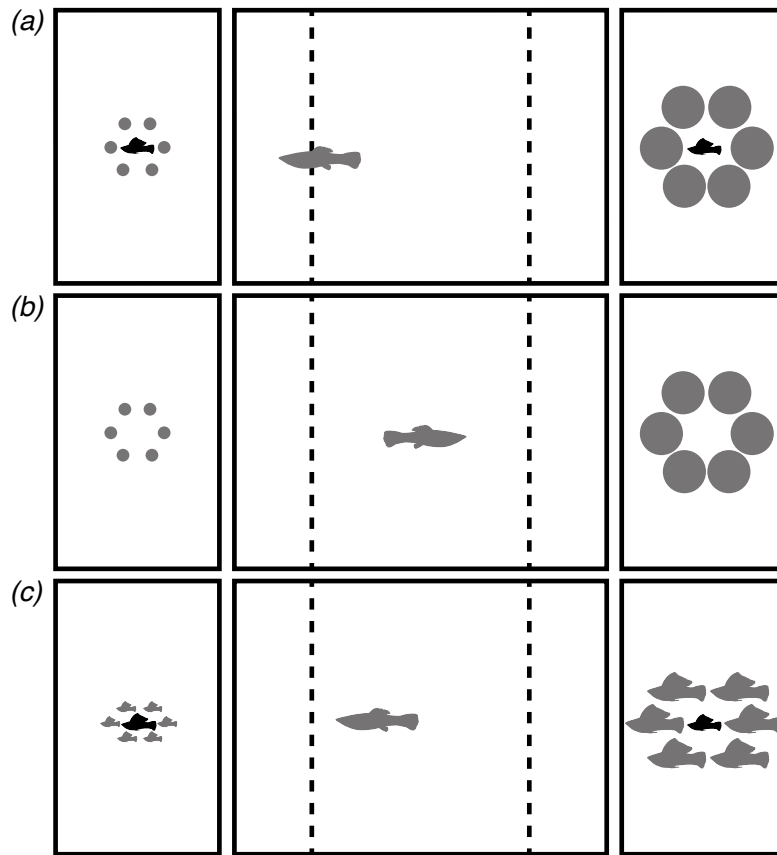
Figure 1.2: Top view of the experimental setup. In each panel, the female is in the center of the test tank, with the choice zones on either end denoted by the dotted lines; the center zone is a neutral zone. The stimuli for each experiment were placed at each end. (*a*) Trials using clay inanimate objects to create the Titchener circles of the illusion. A live focal male was flanked by either smaller or larger objects. (*b*) Control trials with clay objects to create the Titchener circles only, no focal male. (*c*) Experiment using video animations of a focal male flanked by either smaller or larger males. The focal male was the odd-sized male in each video.
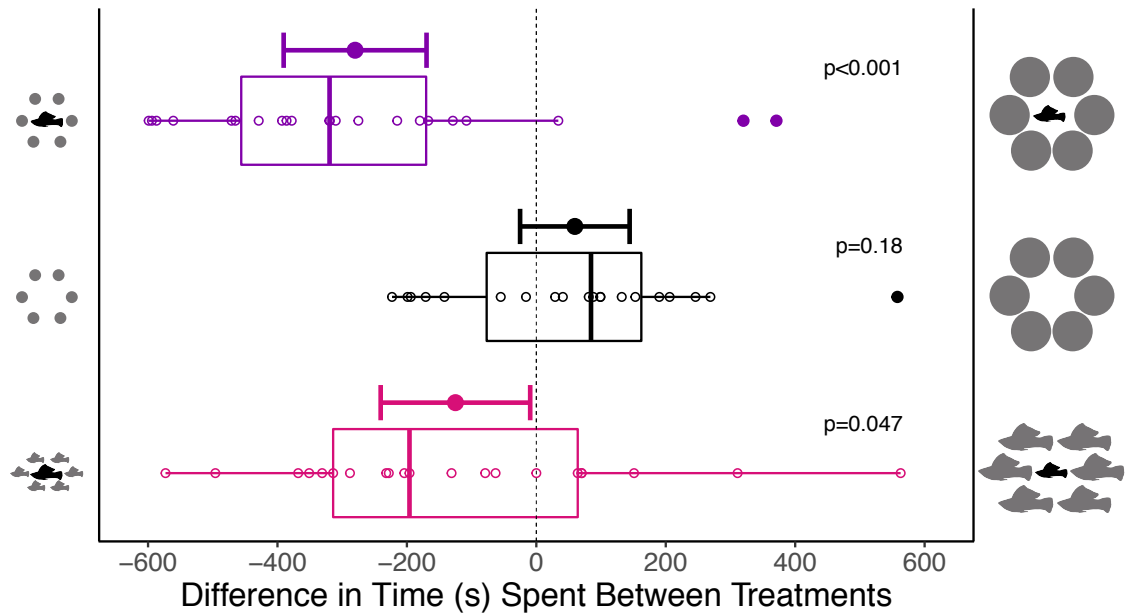
Figure 1.3: Results from all experiments using the difference in time (seconds) spent between treatments to measure female preference. Negative scores indicate a preference for the focal fish flanked by smaller fish and/or inanimate objects (Titchener circles) supporting the Ebbinghaus illusion; positive scores indicate a preference for the focal fish flanked by larger fish and/or inanimate objects (Titchener circles), which would be the a priori prediction of a non-illusory effect. The top boxplot and mean distribution show results for the non-fish (Titchener circle) flanking experiment, the middle boxplot and mean distribution shows results for the control experiment, and the bottom boxplot and mean distribution show results for the flanking fish experiment. Each boxplot depicts median value and 25th and 75th percentiles. The tails indicate data 1.5 times the interquartile range below the lower quartile or above the upper quartile. Data points outside of this range are outliers. The boxplots are present to show the entire dataset, centered on the median, while the mean is pictured above each boxplot with 95% confidence intervals.

Chapter 2

Ebbinghaus illusion does not always fool sailfin mollies (*Poecilia latipinna*)

2.1 Introduction

Sexual signals can be costly to produce, and consequently, are a source of honest information of individual quality in most cases (Zahavi 1975, Andersson 1986, Pomiankowski 1989, Grafen 1990, Johnstone 1998, Maynard Smith and Harper 2003, Searcy and Nowicki 2005). However, multimodal and multidimensional signals enable subtle and complex communication that can impact how a signal is perceived (Rosenthal 2017, Ryan 2018). This complexity, combined with background noise in the environment, can overload the ability of the receiver to correctly perceive critical information for mate choice. This overload of information can create opportunities for errors when receivers are perceiving signals (Rosenthal 2007, Ryan and Cummings 2013). Furthermore, perceptual biases present in the receiver can create an inaccurate or incomplete perception of an environment, leading to sub-optimal decision-making (Rosenthal 2007, Ryan and Cummings 2013). Evolutionary constraints on how animals interpret color, motion, brightness, depth, light, etc., lead to errors in signal interpretation — a cognitive bias (Ryan and Cummings 2013).

Illusions are one source of perceptual error. In some circumstances, signaling animals are able to deceive receivers by exploiting perceptual illusions (Kelley and

Kelley 2014, Lea and Ryan 2015). For example, a size-contrast illusion is a determining factor in territorial defense in fiddler crabs (*Uca mjoebergi*), with males preferring territories adjacent to smaller males, and females preferentially choosing those males (Callander et al. 2012). Therefore, understanding the "receiver psychology", how salient and memorable the signal is in reference to the receiver, at the time of perception and focusing on the higher-end of cognitive processing —not just on sensory reception — can lead to a greater understanding of the evolution of animal communication (Roper 1990, Guilford and Dawkins 1991, Maynard Smith and Harper 2003, Endler 2012, Rosenthal 2017).

Visual signals used in mate choice are commonplace and may be easily augmented by size, geometric, or light illusions (Guilford and Dawkins 1991, Kelley and Kelley 2014). The Ebbinghaus illusion is a size-contrast illusion, where the size of the focal object is dependent upon the size of the surrounding objects. This illusion has been shown to impact behavioral choices in several taxa (Murayama et al. 2012, Salva et al. 2013, Nakamura et al. 2014, Fuss and Schluessel 2017), including in the Sailfin molly (*Poecilia latipinna*) (see Chapter 1). This previous work demonstrated that sailfin mollies (*Poecilia latipinna*) were susceptible to the Ebbinghaus illusion in a dichotomous choice experiment with contrasting illusions shown simultaneously. In the previous experiment, illusions were present during both the acclimation and testing periods, and illusions were perceived by the receiver using both flanking fish and inanimate objects, mimicking the classic Ebbinghus illusion used with Titchener circles (Chapter 1). These illusory effects potentially have broad and adaptive consequences, but how long do these illusory effects last? Specifically, is female choice modified after the illusion is removed? Identifying how signals distort the receiver's perception of reality is key to understanding their evolution. An open question is the role played by memory in whether illusory effects have a lasting impact on mate selection.

To investigate this question, I use Sailfin mollies (*Poecilia latipinna*), which have been shown to be susceptible to the Ebbinghaus illusion within a mating context (Chapter 1). Memory is an important aspect of signal perception and it has been previously shown that Poecilia can make decisions based on prior experiences and memory (Schlupp et al. 1994, Schlupp and Ryan 1997, Bierbach et al. 2011, Bateson et al. 2014). To test whether these illusory effects remain in the absence of the Ebbinghus illusion, I examined selection preference by females for live males in three different shoaling social contexts: 1) focal male flanked by three smaller males, 2) focal male flanked by three larger males, or 3) focal male flanked by three similar-sized males. These same treatments were repeated with flanking males replaced with clay spheres to represent Titchener circles to mimic the class form of the Ebbinghaus illusion. I predicted that, if memory plays a key role in signal interpretation, female *Poecilia latipinna* would be susceptible to the effects of the Ebbinghaus illusion, even in the absence of inducers (i.e., flanking males or Titchener circles). Additionally, I predicted that this illusory effect would remain consistent across the two shoaling groups (i.e., live flanking males and Titchener circles).

2.2   Methods

To specifically test the lasting and direct effects of the Ebbinghaus illusion sequentially on mate choice, I removed the illusion inducers during all testing periods, after an initial exposure period where the male was observed inside the illusion. Female preference was not measured during this exposure time, since I had shown in Chapter 1 that females were susceptible to the illusion. This exposure period was established to test the working memory of the illusory effects on receivers, and to also observe the direct effect of the illusion on the focal male. During the previous simultaneous experiments in Chapter 1, I was unable to separate female preference for the

focal male from preference for the flanking males, or inducer objects. Here, I wanted to test the direct effects of the illusion on preference for the focal male, so I measured female preference for the focal male immediately after all inducers were removed. I evaluated the results during a preference function test, because preference function choice tests are designed to measure the preference for a trait across multiple groups over time (Ritchie 1996, Wagner 1998). Preference functions have also been shown to correlate with mate choice or shoal choice in Poeciliids (Schlupp and Ryan 1997, Plath et al. 2006, Arriaga and Schlupp 2013, Makowicz et al. 2016). Furthermore, since I tested only one illusory effect at a time, I created a detailed response area in order to measure preference over a wider range. These response zones included the interaction zone (the strongest response), followed by a preference zone, and an association zone, each indicating a weaker response for the stimulus than the one before.

2.2.1 Fish collection and maintenance

Fish were collected in Brownsville, Texas, USA in June of 2014 and April of 2015. For each collection trip, fish were transported back to the University of Oklahoma and kept under a 12-hour light/dark cycle and fed twice daily. Fish were separated by sex and standard-length measurements were taken to determine the natural size variation in the population. I divided wild caught males into three discreet size-classes of small (35-36 mm), intermediate (42-50 mm), and large (55-60 mm) for a total of 20 males. Those size classes were used for the following experiment and in previous experiments (see Chapter 1). There were seven available males that could be used as a focal male and were chosen in a randomized order. Since the illusory effect was stronger using live male stimuli in the previous set of experiments, I decided to use live males to test the direct effects of the Ebbinghaus illusion over time. While this introduces more variation among trials, it also creates a more realistic shoaling

environment and better represents populations in the wild.

2.2.2   Sequential illusion with live flanking males

Females (N=39) were exposed to the same focal male in the following three experimental treatments in a randomized order: 1) focal male flanked by three smaller males, 2) focal male flanked by three larger males, or 3) focal male flanked by three similar-sized males. If a female spent 85% or more time on one side of the tank across trials, then it was considered a side bias, and that individual was removed from any analysis (McCoy et al. 2011). After the removal of side-biases, and individuals who did not show a response, the sample size was reduced to 29 females, and the amount of time the female spent in each preference zone was compared among treatments.

The experimental setup (Figure 2.1 a, b, and c) contained an experimental tank (61×39×30 cm) marked with the three zones in which I measured female preference and a separate, smaller tank containing the focal and flanking males. Zone 1 is referred to as the interaction zone and is the zone closest to the male display tank measuring one average female standard length (4 cm). Zone 2 is referred to as the preference zone (15 cm), and Zone 3 is the association zone (30 cm), which extended to the midline of the tank. Using these three zones, I determined if the female's preference for the focal male varied with distance. Measurement by zone was important in quantifying the relationship between distance and the illusion. The right half of the tank was considered the neutral zone, signifying absence of female preference. The stimulus tank is located on the left-hand side. Due to constricted tank space (1-gallon), only three flanking males were used in the stimulus tank, instead of the six inducers used in the classic Ebbinghaus illusion.

For each treatment, the focal male was physically separated from the flanking males in a clear plexiglas cylinder so that the female did not lose sight of the focal male. The female observed the focal male surrounded by the flanking males for 10

minutes during the exposure period while being restricted to a plexiglas cylinder on the opposite side of the tank. The flanking males were then removed so I could directly measure the female's specific preference for the focal male, without the flanking males, and test whether the illusory effects lasted over time. Based on my previous work in Chapter 1, we know that fish were susceptible to the Ebbinghaus illusion for the entire 10-minute testing period. 10-minutes was more than long enough to see effects of the illusion on mate choice (Chapter 1). Measuring the lasting effect of the illusion is important for assessing how robust or adaptive this effect could be, which has consequences for understanding mate choice. After the exposure period, I measured the amount of time the female spent in each of the testing zones for the next five minutes to compare across treatments. After each treatment, the female was constrained to the plexiglas cylinder at the far end of the tank, while I placed the next treatment of flanking males immediately into the observation tank. The females observed the new treatment for 10 minutes and this process was repeated until the female observed the focal male in all social treatments.

2.2.3   Sequential illusion with inanimate stimuli

Here, I follow the methodology described above, but replace live flanking males with the clay inanimate objects to mimic Titchener circles (Figure 2.1 d-f). To mimic the classic Ebbinghaus illusion, I used six inducers. I used 27 females to test this hypothesis. This total was reduced to 23 individuals for analyses after elimination of any side-bias using the same procedure as above. Each female was exposed to three different treatments: focal male surrounded by smaller clay models, that same male surrounded by clay models of the same size, and that same male surrounded by

larger clay models. There was a ten-minute exposure period and a five-minute test period for each treatment. The order of the treatments was randomized for each female.

2.2.4 Statistical analysis

I used an ANOVA to evaluate time differences (response) across each of the three social context treatment groups. A random effect of fish ID was used to account for the repeated measurements across each of the treat groups. In total, I ran six ANOVAs, one for each preference zone for the live and Titchener circle experiments. All analyses were conducted in R (R Core Team 2017) and the lme4 package (Bates et al. 2015) was used to implement the random effect.

2.3 Results

Females spent a significant majority of their time in the interaction zone during both the live (65% of time; N = 29 live males) and Titchener circles (68%; 23 Titchener circles). Individuals that spent the majority of time in the three zones, also spent a significantly greater percent of time within the interaction zone (live: $F_{1,85}$=27.23, p<0.001, R^2=0.242; Titchener: $F_{1,67}$=68.76, p<0.001, R^2=0.507). For sequential trials using live flanking males, I found no difference in treatment for the time spent in the response zones (interaction, $F_{2,56}$=0.127, p=0.880), preference ($F_{2,56}$=1.156, p=0.322), and association ($F_{2,56}$=0.988, p=0.379) (Figure 2.2a). Similarly, I found no difference for time spent in the response zones when using Titchener stimuli (interaction, $F_{2,44}$=0.961, p=0.390), preference ($F_{2,44}$=1.948, p=0.155), and association ($F_{2,44}$=1.457, p=0.244) (Figure 2.2b). Using fish ID and social context as random effects, I found that time spent in the interaction zone was greater during the Titchener circle experiments ($F_{1,50}$=4.358, p=0.042), yet no difference was observed in the

preference ($F_{1,50}$=0.718, p=0.401) and association zones ($F_{1,50}$=0.259, p=0.613).

2.4   Discussion

How illusory effects are perceived during mate choice is key to understanding their role in signal evolution. While I have previously shown that female sailfin mollies (*Poecilia latipinna*) were susceptible to the Ebbinghaus illusion in a dichotomous choice experiment with contrasting illusions shown at the same time (Chapter 1), whether or not sailfin mollies remembered the illusory effects while making mate choice decisions was unknown. Here, I tested whether this effect would last over time, to directly quantify the illusory effect on the focal male. Sailfin molly females were not susceptible to the Ebbinghaus illusion in mate choice over time. This was true for the trials using both live fish and Titchener circles as stimuli to create the Ebbinghaus illusion. This pattern suggests that the impact of the illusion is not remembered and does not impact subsequent preference. Therefore, in order for the illusory effect to be perceived, the entire illusion may need to occur in real time, or the observing female will not be susceptible and choose the otherwise predictable male. This means the illusory effects may only impact mate choice while the receiver is assessing the signal in real time, using working memory, and may not be affecting longer-term memory for decision making (Ryan et al. 2009).

The Ebbinghaus illusion is a size-contrast illusion. The size of the focal object is dependent upon the size of the surrounding objects. In order for the illusion to be perceived, the proximity of the flanking objects to the center object, the number of the surrounding objects, and the size of the flanking objects can all alter the perception of the illusory effects. This could partially explain why the receivers were not susceptible to the illusory effects during the live-male flanking treatments. A similar effect was observed in humans (Bateson et al. 2014), wherein, they were also not fooled by a size-contrast effect in mate choice where the focal subject was not completely surrounded

by flanking inducers. In both scenarios, the size-contrast arrangement was not strictly adhering to the psychophysics of the illusion, and therefore could not be perceived. However, this does show how flexible receivers can be in determining an honest signal related to mate choice, and that receivers are not always fooled by illusions in all contexts or over time.

I was able to measure the preference at three separate response zones that varied with distance from the stimulus within the experimental tank. While I did not see a difference in preference among response zones, previous studies have found that distance from a source can also change receiver responses to a stimuli. Halfwerk et al. (2014) tested whether male Túngara frogs responded to distance-dependent cues of a competitor based on multimodal signals (water ripples and vocal calls), and demonstrated that the receiver response to the signal(s) did vary based on distance to sender. Although viewing distance was not important to preference over time, it could still have an impact on preferences while the illusion is still in effect.

Working memories exist over a short duration of time and these memories are thought to be most important during mate choice (Ryan et al. 2009). The complexity of a trait or signal can also affect the strength or accuracy of the working memory. For example, in Túngara frogs, the complexity of courter male's calls was correlated a female's ability to remember the location of that preferred male in the future (Akre and Ryan 2010). This complexity or saliency may make it easier to both detect and, more importantly, remember that signal over time and drive the evolution of more complex, salient signals (Guilford and Dawkins 1991, Akre and Ryan 2010, Rosenthal 2017). Therefore, the effects of the illusion could be more salient when simultaneously compared in real-time (Chapter 1), instead of sequentially. And, so, the effect of illusions and the constraint on illusions may be especially frequent when males display in groups and multiple males are compared simultaneously by receivers (Bateson and Healy 2005). The simultaneous scenario is also more likely to occur in

nature, with the receivers making decisions in real-time while the information is still available.

One such cognitive constraint that impacts evolution of visual signals and receiver's ability to perceive them is Weber's Law (Fechner 1912). As the absolute size of the male visual stimulus increases, females are less able to distinguish between differences among male ornaments. According to Weber's Law, it is the ratio of the difference in size that matters and not the actual size of the ornament itself. This dependence on ratios could affect the female's ability to perceive the attractiveness of males relative to other potential mates. Furthermore, female cognition limited the evolution of signal elaboration in male Túngara frogs. As the calls became more elaborate, the females were unable to differentiate between preferred and non-preferred calls because the ratio of the difference between calls was reduced (Akre et al. 2011). So, even though the females preferred the more elaborate calls, there is a constraint on what the females can perceive, showing the importance of female sensory recognition in processing signals from males in mate choice. The actual magnitude only matters if it can be perceived. Also, the perceived magnitude can be manipulated in ways other than changes in the actual magnitudes, such as visual illusions. Here, for the Ebbinghaus illusion, once the difference is determined, it no longer matters if it does not happen in real time, constraining the evolutionary effects on this signal.
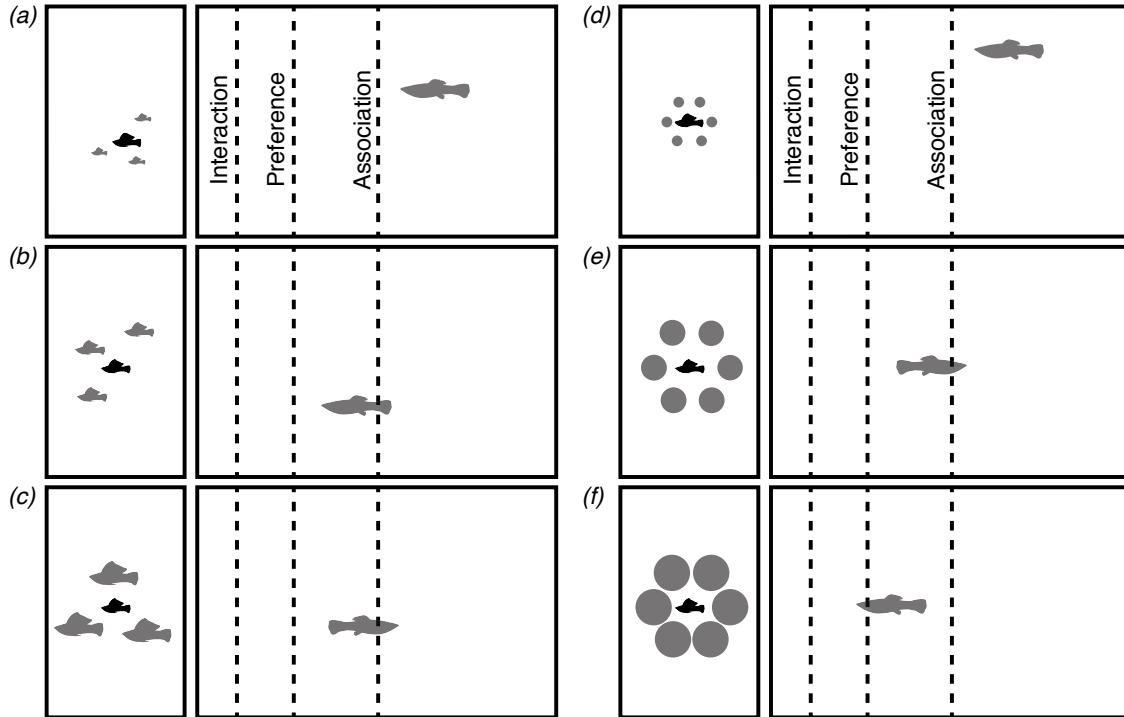
Figure 2.1: Experimental design for live (*a-c*) and synthetic (*d-f*) Ebbinghaus illusion trails. The focal male with live flanking males (*a-c*) or clay Titchener circles as flanking inducers (*d-f*). Experimental tanks are demarcated with three preference zones (Interaction, Preference, and Association) to measure female preference. Tanks *a* and *d* represent the small flanking treatment, *b* and *e* represent the similar-sized flanking treatment, and *c* and *f* represent the large flanking treatment.
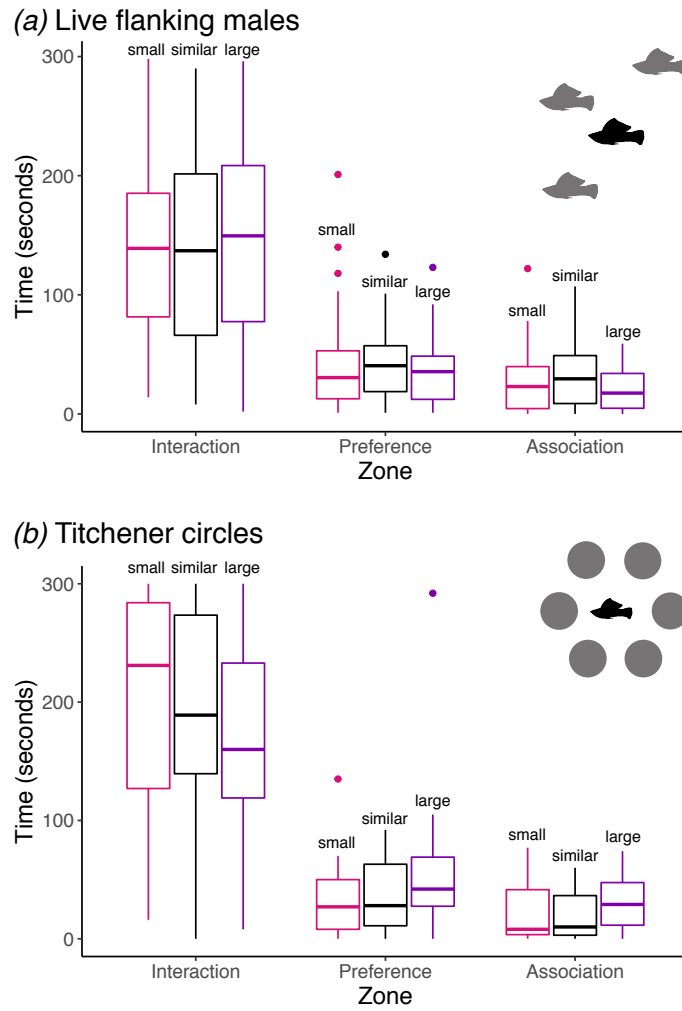
Figure 2.2: Results from all experiments using the difference in time spent among treatments to measure female preference. The boxplots show the entire dataset, centered on the median. Each boxplot depicts median value and 25th and 75th percentiles. The tails indicate data 1.5 times the interquartile range below the lower quartile or above the upper quartile. Data points outside of this range are outliers. Pink indicates the small flanking group, black the similar flanking group, and purple the larger group, replicated across each zone. There was no difference in preference measured across each group of live-flanking male treatments (a). There also was no difference across the treatments for males flanked by Titchener circles (b).

Chapter 3

Behavioral divergence of sulfide tolerance in endemic *Limia* fishes

3.1 Introduction

Extreme environments can be characterized by toxicity, hypoxia, and severe temperatures and pressures. Few metazoans are able to migrate to or invade these extreme habitats, restricting gene flow to the adapted populations already there (Mc-Mullin 2000, Tobler et al. 2018). In that way, extreme environments often give rise to endemic populations, much in the way that islands can (MacArthur and Wilson 1967, Rundle and Nosil 2005, Losos and Ricklefs 2009, Nosil 2012). Generally, organisms that live in extreme habitats are highly vulnerable to extinction owing to the small spatial extent of the habitat, which only supports very small populations, and makes them vulnerable to being destroyed by localized natural processes, exacerbated by climate change and other human activities, or by the accumulation of deleterious mutations (Contreras-Balderas et al. 2002, Willi et al. 2006, Bouzat 2010). Thus, extreme habitats present a major conservation concern in that they have driven the evolution of unique, isolated lineages, which have limited abilities to expand into other, more benign habitats (Nosil et al. 2005, Plath et al. 2013, Rosenblum et al. 2014).

One extreme environment that is of particular interest are toxic sulfide springs, because they create particularly harsh environmental conditions which can drive divergence in physiological, life history, morphological, and behavioral traits (Tobler et al. 2008a, Riesch et al. 2014). Hydrogen sulfide is known as a strong respiratory toxicant that can be acutely toxic or lethal to most animals. This has been shown to be true even in small macromolar amounts for short periods of time (Evans 1967, Smith et al. 1977, Bagarinao 1992, Grieshaber and Völkel 1998, Tobler et al. 2008a). Most species that have been found to inhabit sulphidic environments are invertebrates (Grieshaber and Völkel 1998, McMullin et al. 2000). Few vertebrates have locally adapted to hydrogen sulfide springs, but several lineages of livebearing fishes (Poeciliidae) have done so multiple times. Examples include *Poecilia mexicana* near Tabasco, Mexico, *Cyprinodon bobmilleri* in northern Mexico, and *Poecilia sulphuraria* and *Gambusia eurystoma* in Tabasco, Mexico (Alvarez del Villar 1948, Miller 1975, Lozano-Vilano and Contreras-Balderas 1999, Tobler et al. 2006, 2008a, Riesch et al. 2014). Generally, these livebearing fishes have adapted to sulfidic environments morphologically, physiologically and behaviorally (Kramer and McClure 1982, Plath et al. 2007a, Riesch et al. 2014). One key behavioral adaptation that can be measured across populations is their performance during aquatic surface respiration (ASR) where organisms in hypoxic conditions exploit the higher dissolved oxygen content at the surface of the water. This behavior, in combination with enlarged gill areas, is thought to be a key adaptation to low oxygen conditions (Chapman et al. 1991). It has been well documented that Poeciliids perform ASR in hypoxic, sulfidic conditions by swimming to the water-air interface to respire. ASR is also a plastic behavior, with Poeciliids adjusting how much time they spend performing the energetically costly ASR behavior based on the levels of hypoxia present in the water (Kramer and McClure 1982, Timmerman and Chapman 2004a, 2004b, Plath et al. 2007a).

While several endemic species have been described from toxic, hydrogen sulfide rich springs, including the Sulfur Limia (*Limia sulphurophila*) investigated here, not much is known about the behavior or physiology, or about the sulfide environment it inhabits. Typically, the toxic nature of these sulfidic environments and their extreme hypoxia keep locally adapted populations isolated and limit introgression from non-adapted populations. The species, *L. sulphurophila*, Sulfur Limia, (described by Rivas 1980) is found in a single, small sulfidic spring, La Zurza, on the island of Hispaniola. The La Zurza spring is upstream of Lake Enriquillo, the largest freshwater lake in the Dominican Republic, creating a creek with a gradient between sulfidic and freshwater environments. A closely related species, *L. perugiae*, is historically found downstream of La Zurza in Lake Enriquillo and freshwater creeks near the lake. Lake Enriquillo has risen recently, doubling in surface area since 2004 (Wright et al. 2015), altering the dynamics of the aquatic habitats in the region. Furthermore, La Zurza is used as a public pool for therapeutic sulfur baths, contributing to habitat disturbance and degradation. *L. sulphurophila* is at risk both due to destruction of the habitat to which they are adapted and possibly due to potential hybridization with *L. perugiae* in intermediate habitats created by the rising lake.

In this study, I (1) characterized the environmental parameters of this sulfide-freshwater gradient and (2) evaluated differences in behavioral traits (ASR) between *L. perugiae* and *L. sulphurophila* to assess behavioral divergence between populations and begin to determine if either species has the potential to survive outside of their locally adapted environment. I predict that *L. sulphurophila*, but not *L. perugiae*, would be better adapted to sulfidic conditions by performing ASR, supporting the evidence that this behavioral and/or physiological adaption may have arisen multiple times in Poeciliidae due to strong selection pressure in an extreme environment (Riesch et al. 2014).

3.2 Methods

3.2.1 Water chemistry

Hydrogen sulfide concentration at La Zurza was determined by collecting 2 ml of water at twenty-two sites within the study area (Figure 3.1). I collected two samples at each site and took an average of the concentrations to determine the final concentration shown in Table 3.1. Using a syringe, water samples were placed in individual anoxic vials containing 2 ml of zinc acetate ($ZnC_4H_6O_4$) in a N_2 atmosphere with no O_2 present. I transported the preserved samples back to the University of Oklahoma to estimate the amount of dissolved sulfide present at each site by using a sulfide assay. For each sample, I added 3.925 ml H_2O, 0.5 ml DPDA reagent (2g dimethyl-p-phenylenediamine in 1 L of 20% H_2SO_4), and 25 μL $FeCl_3$. I then measured the extinction of the samples at 660 nm in a spectrophotometer against a sample of distilled H_2O. I created a standard curve to analyze the sulfide assay performed on the samples. The extinction coefficient of the blank sample was subtracted from the extinction coefficient of the field sample in order to determine the relative concentration present on the standard curve. For the standards, I used an anoxic solution of Na_2S and added a sulfide crystal. This was then diluted using 40 g of zinc acetate and 0.5 ml of acetic acid in 1 L of H_2O to create the standard curve ranging from 0.1 mM up to 3 mM of sulfide concentration. For more detailed methods see Trüper and Schlegel (1964) and Tobler et al. (2008b).

I used a Horiba Sensor Probe (Model U-5000) to collect abiotic measures at four shallow locations (immediately below the surface) and four deep locations (2 meters below the surface) at the sites along the sulfide gradients. Water temperature was measured in °C, specific conductance in μS/cm, turbidity in nephelometric units

(NTU), and dissolved oxygen in mg/L.

3.2.2   Sulfide tolerance

The aquatic surface respiration (ASR) experiment was done on-site at La Zurza on March 3, 2014 from 12:00-16:00. I tested both the latency (the amount of time it takes until the fist starts performing ASR) and the total amount of ASR performed by *L. sulphurophila* and *L. perugiae* when placed in sulfidic water obtained from the sulfide spring. While the original methodology included the reciprocal test using non-sulfidic water, extenuating circumstances prevented those trials from being tested. This is an important and necessary follow-up experiment, but nevertheless, I thought it was still valuable to present the data I was able to collect. *L. sulphurophila* were collected downstream at site 2E and the *L. perugiae* were collected 400 meters downstream at Lake Enriquillo (site 1I). I tested 8 *L. sulphurophila* and 12 *L. perugiae*. Each single fish was placed into an open glass container filled with water containing 7.32 μM of hydrogen sulfide water. No mixing of the water occurred to maintain the natural sulfidic condition collected at the spring. There was a five-minute acclimation period prior to the five-minute test period. I observed the fish from 3 meters away and recorded both the latency (first time to surface to perform ASR) and the total amount of ASR during the testing period. I randomly selected either *L. sulphurophila* or *L. perugiae* for each trial and replaced the water every five trials in order to maintain the high amount of hydrogen sulfide in the water. The sex and standard length of each fish was determined after each trial and the fish were returned immediately to their original habitat.

3.2.3   Statistical analysis

I used a linear regression to examine the effects of species, sex, and standard length on total time performing ASR and latency. Because there was no effect of sex

and standard length on ASR (p=0.232; p=0.762) or on latency (p=0.186; p=0.974), I used a Welch's two-sample t-test designed for samples of unequal variance to explicitly test the difference of species on ASR. I used the parametric Welch's t-test instead of the student's t-test to account for the small sample sizes (N<10) and differences in standard deviation of ASR and latency across species. All analyses were conducted in R (R Core Team, 2017).

3.3   Results

3.3.1   Description of La Zurza

La Zurza is a sulfidic springhead found in the Dominican Republic on the island of Hispaniola (18°23′52.3″N; 71°34′11.5″W; Figure 3.1). There are two man-made pools that are fed by separate sources of hydrogen sulfide and freshwater, both of which drain northward into Lake Enriquillo. At the time of our study, Pool 1 contained *L. sulphurophila*, *L. perugiae*, *Gambusia hispaniola*, and *Gobiomorus dormitor*, and Pool 2 contained no fishes. *Limia perugiae* was also found at sampling sites 1I and 2I in Lake Enriquillo, while *Limia sulphurophila* was found at sampling site 2E but at no other downstream sites (Figure 3.1).

3.3.2   Water chemistry

There were no differences in water chemistry between shallow and deep samples. Both Pool 1 and Pool 2 showed a steep gradient in hydrogen sulfide concentration, with the highest concentration at the source of the springs (Table 3.1). The most extreme environment was found at the sulfidic springhead in Pool 1 at 20.31 μM. Moving downstream in stream 1 and stream 2, the sulfide concentration decreases, except where stream 1 meets Lake Enriquillo (site 1I) (Figure 3.1). The concentration of hydrogen sulfide also coincides with where the fish were observed. In Pool

1, all fish were only seen between sites 1A and 1B, in the lowest hydrogen sulfide concentrations. No fish were observed in sites 1C or 1D where the hydrogen sulfide concentration was the highest.

3.3.3   Sulfide tolerance

L. sulphurophila (N=8) and *L. perugiae* (N=12) differed in their response time in total amount performing air surface respiration (ASR), with *L. perugiae* performing significantly more ASR when compared to *L. sulphurophila* ($t_{13.1}$=-2.479, p=0.028, CI=-237.1 to -16.38) (Figure 3.2). *L. sulphurophila* and *L. perugiae* did not differ in their latency, or amount of time until first performance of ASR ($t_{11.0}$=-1.438, p=0.178, CI=-81.44 to 388.4) (Figure 3.3).

3.4   Discussion

Endemic species in extreme environments provide a unique opportunity to better understand adaptation and the processes of speciation. While several Poeciliid species have been described in extreme sulfidic environments, little is known about the extremophile *L. sulphurophila*, endemic to Hispaniola. Here, I tested whether there was behavioral divergence in response to the toxic suflidic environment between *L. sulphurophila* and *L. perugiae*. I showed that *L. sulphurophila* and *L. perugiae* vary in their tolerance to toxic hydrogen sulfide, with *L. perugiae* performing higher levels of ASR than *L. sulphurophila*. This difference may be due to the moderate sulfide concentration present in the experimental water collected from the springhead. I also measured the concentration of the sulfide gradient present in the La Zurza spring and surrounding area, and documented the occurrence of these species along the sulfidic gradient. While 7.32 μM of hydrogen sulfide is a relatively high concentration when

compared to freshwater, it is relatively low compared to other known toxic hydrogen sulfide springs inhabited by Poeciliids (Tobler et al. 2008a). This finding may mean that *L. sulphurophila* are better at maintaining normal physiological functions at higher concentrations than *L. perugiae*. *Poecilia mexicana* (atlantic molly) living in highly toxic sulfidic springs are better at detoxification and excretion when compared to *P. mexicana* living in freshwater springs and transplanted into sulfidic water (Tobler et al. 2018). Here, *L. perugiae* may also be less adapted to higher sulfide concentrations, and have to perform ASR at a higher rate in order to cope.

Behavioral divergence between *L. sulphurophila* and *L. perugiae* was apparent from my observations, in spite of the fact that the concentration of H$_2$S is lower at La Zurza (20.3 μM) relative to other systems, such as Arroyo El Azufre, Mexico (540 μM) and the Cueva del Azufre, Mexico with H$_2$S values as high as 530 μM. Even low concentrations of sulfide may be toxic to fish (Tobler et al. 2008a), and so even low levels of sulfide have the potential to drive the divergence between populations (Riesch et al. 2014). This divergence can be seen through the differences in time spent performing ASR. However, I did not find a difference in the latency when starting to perform ASR. Because sample sizes were small, my power to detect differences was limited, pointing to the need for further sampling. Still, there was a large difference in the amount of time performing ASR, even despite having a small sample size. Furthermore, having to perform ASR at a higher rate, also exemplifies how sulfide could act as a potential cause for reproductive isolation. *L. perugiae* are unable to cope in an environment with a moderate concentration of sulfide without performing a greater amount of ASR, which is very energetically costly (Plath et al. 2007b).

In addition to the behavioral contributions of this work, the chemical analysis of water chemistry provide a necessary characterizations of the spring area, filling a nearly 40-year gap in our knowledge of the habitat of *L. sulphurophila*. Rivas (1980) mentioned the La Zurza spring when describing an endemic species from the spring

area, *L. sulphurophila*, the specimens used for this description were collected decades earlier. At that time, a chemical characterization of the spring area was not possible, and here I provided this description to supplement his foundational work. Since the original description, there have been significant changes to the spring. Rivas (1980) uses the term Balneario in the description of the spring, hinting at human usage of the site as a bath, but I believe the concrete pools presently used for swimming were built much more recently, and present new environmental challenges from human disturbances.

I found a steep sulfidic-freshwater gradient between the La Zurza springs and Lake Enriquillo habitats. Furthermore, the behavioral differences seen in the fish from both locations matched the sulfidic-freshwater gradient. Specifically, where *L. sulphurophila* was shown to be more tolerant of the sulfidic conditions, *L. perugiae* was comparatively less tolerant. This difference in behavior matches the patterns present in other sulfidic systems (Tobler et al. 2008a).

Due to the restricted range of *L. sulphurophila* and its incredibly small population size, this species may be inherently endangered and in need of proactive conservation measures. Previous work has shown that other extremophiles in Poeciliidae have convergently evolved to produce fewer, but larger, offspring compared to non-extremophiles (Riesch et al. 2014), and are lecithotrophic (Cohen et al. 2015), potentially leading to a slower population growth, which would allow *L. perugiae* to outcompete *L. sulphurophila* in the absence of H_2S. If Lake Enriquillo keeps rising and the freshwater and sulfidic habitats converge, freshwater species of Limia may likely out-compete the extremophile, either leading to hybridization and/or extinction of Sulfur Limia altogether. From observations, it appears that there may be movement between the populations along the sulfide gradient; however, these are only snapshots in time, as there are not long-term datasets or DNA analyses to further clarify this question.

Hybridization can also be a new source of genetic variation that can quickly allow species to adapt to rapidly changing or degrading environments. Human alterations can have swift and drastic impacts on the environment, and instead of losing a species in this process, this hybridization may be a fast source of evolutionary rescue (Hamilton and Miller 2016, Oziolor et al. 2019). With the added vulnerability of these Limia species by human interaction at La Zurza, hybridization may not always mean a loss of a species, but rather can lead to strains that become resistant to human-mediated or natural toxins, or even more resilient to the impacts of climate change. Continued monitoring of this highly specialized habitat, as well as in the streams and surrounding areas, is needed, especially in times of rapid landscape modification and climate.
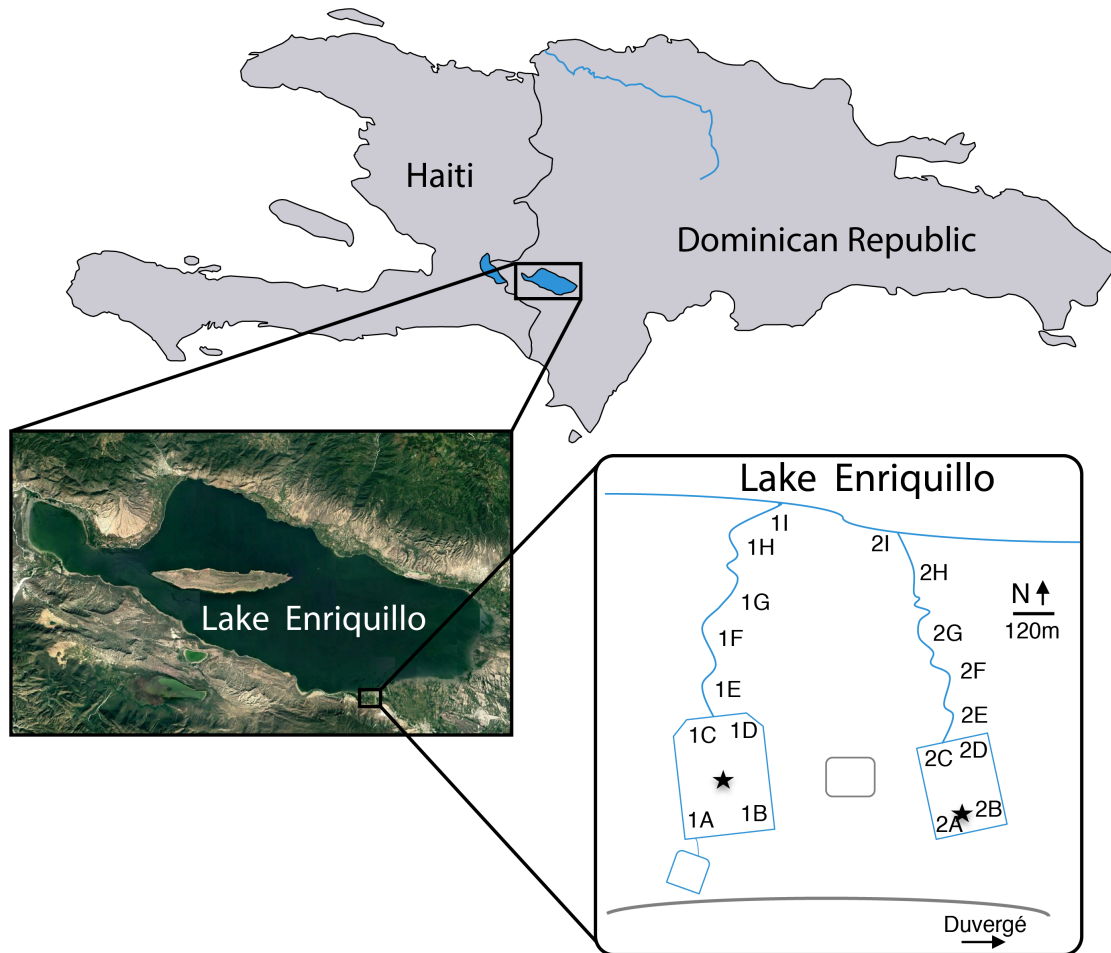
Figure 3.1: Location of La Zurza on Hispaniola (top and left). Overview sketch of La Zurza and Lake Enriquillo with labeled sampling sites (right). The grey lines represent roads and buildings and the blue lines represent waterways. Sulphidic springs are denoted with a star.
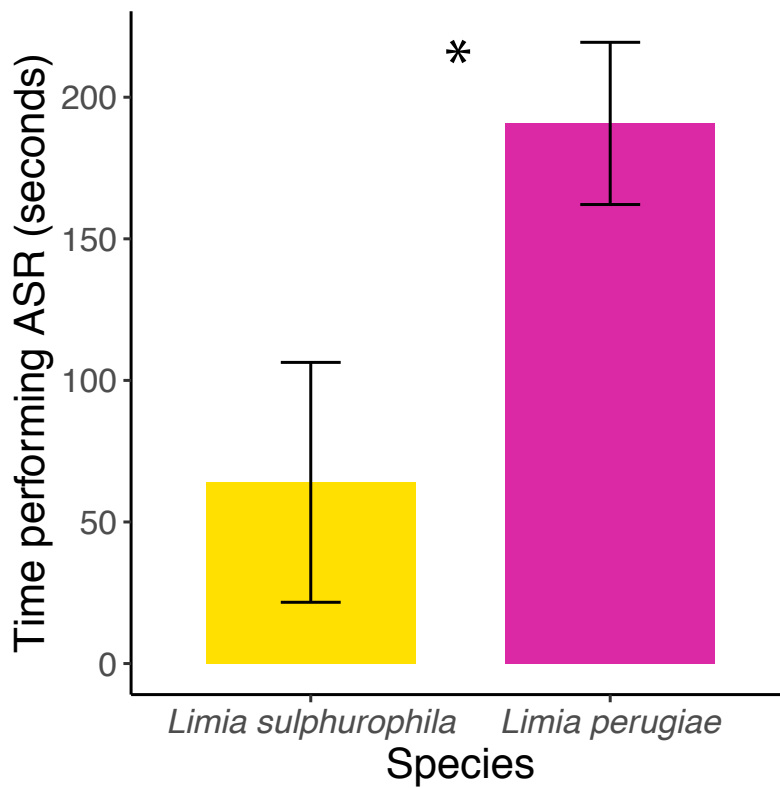
Figure 3.2: Mean time (seconds) spent performing air surface respiration (ASR) for *Limia sulphurophila* (N=8) and *Limia perugiae* (N=12). *Limia perugiae* spent significantly more time performing ASR when compared to *Limia sulphurophila* in the sulfidic water. Standard error bars shown and * denotes p<0.05.

Figure 3.3: Mean latency (seconds) to observed air surface respiration (ASR) for *Limia sulphurophila* (N=8) and *Limia perugiae* (N=12). No difference (p=0.178) was observed between species response time to performing ASR in sulfidic water. Standard error bars shown.

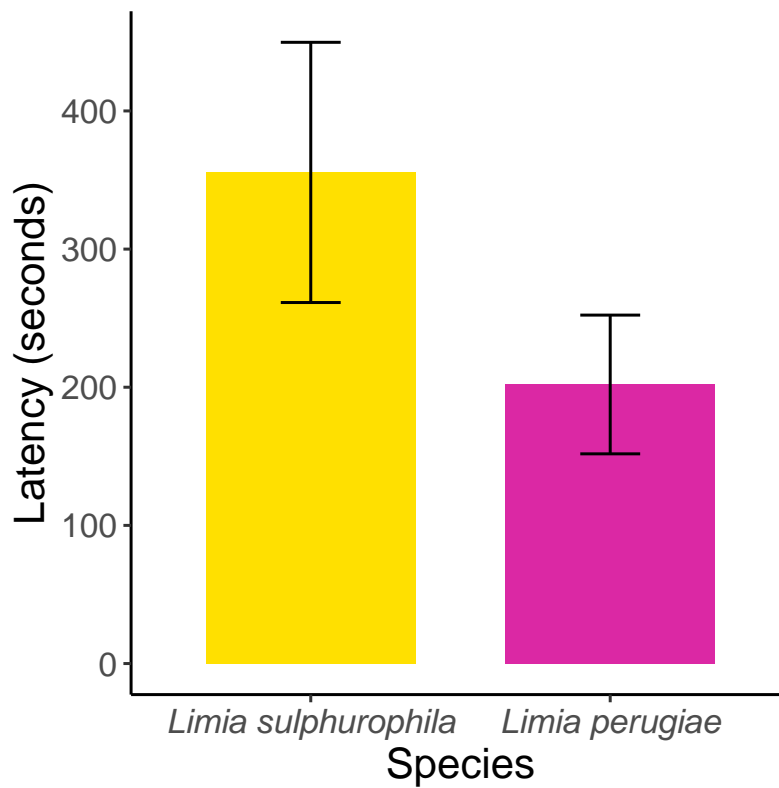**Table 3.1:** Summary of water chemistry for La Zurza sampling sites. See Figure 1 for site layout. For sites within the two pools, samples were collected at a depth of two meters and at the surface.

Site	H$_2$S Concentration (μM)	Water Temperature (°C)	pH	Conductivity (mS/cm)	Turbidity (NTU)	Dissolved Oxygen (mg/L)
1A (surface)	2.01	22.21	7.30	1.55	3.2	12.81
1A (two-meter)	—	22.17	7.23	1.55	4	8.01
1B (surface)	2.05	22.1	7.32	1.61	0.9	4.48
1B (two-meter)	—	22.17	7.17	1.64	1.2	4.35
1C (surface)	20.31	21.96	7.25	1.67	1.1	3.55
1C (two-meter)	—	21.97	7.09	1.71	0.6	3.13
1D (surface)	3.42	22	7.20	1.69	0.6	3.39
1D (two-meter)	—	22.00	6.94	1.71	0.6	3.00
1E	2.37	22.01	7.31	1.71	4.1	8.22
1F	0.58	22.2	7.32	1.69	10	7.76
1G	0.47	22.47	10.17	1.71	41.9	24.04
1H	0.38	23.78	8.48	1.21	18.9	9.70
1I	4.29	26.00	7.43	12.60	22.1	5.76

Site	H$_2$S Concentration (μM)	Water Temperature (°C)	pH	Conductivity (mS/cm)	Turbidity (NTU)	Dissolved Oxygen (mg/L)
2A	5.49	21.99	6.96	1.86	0.7	2.30
2B	5.3	21.95	6.99	1.86	0.2	2.15
2C	5.51	22.28	6.7	1.87	0.6	10.62
2D	5.29	21.96	6.94	1.85	0	2.56
2E	2.26	24.13	7.52	1.73	5.5	9.62
2F	1.05	23.46	7.53	1.88	9.0	22.79
2G	0.66	23.91	6.53	1.40	67.5	9.52
2H	0.77	23.94	6.22	0.61	105	10.78
2I	0.51	26.35	6.35	7.92	73.2	10.83

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