

BEHAVIORAL AND NEUROPHYSIOLOGICAL RESPONSES TO
MECHANOSENSORY SIGNALS ASSOCIATED WITH THE HULA BEHAVIOR IN
MALE AXOLOTLS (*AMBYSTOMA MEXICANUM*)

By

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ABSTRACT

Studying communication signals from the perspective of both senders and receivers allows us to create a greater understanding of how signals are exchanged between animals. For my doctoral dissertation, I leveraged the courtship ritual of the axolotl, *Ambystoma mexicanum*, a fully aquatic salamander, to investigate mechanisms of signal generation and detection. I focused specifically on a courtship behavior called the “hula”, which involves a rhythmic swaying motion of the pelvic region combined with an undulating movement of the tail. Although the hula has been historically been considered a male behavior, female axolotls play a critical role in the hula behavior as the receiver, and may potentially participate in the male hula by nudging the cloaca or changing proximity to the hula-ing male. Additionally, both males and females perform the hula behavior during courtship; here, I focused on male to female communication mediated by the hula. The hydrodynamic stimuli generated by the hula behavior may serve as communication signals; female axolotls can presumably detect these stimuli via their lateral line system, which is capable of sensing both mechanical and electrical signals in aquatic environments. For my dissertation research, I characterized the motion patterns that males displayed while hula-ing and measured how females responded to a range of hydrodynamic stimuli, both through a behavioral and neurophysiological lens.

Critically, I also aimed to determine if a principle known as the sender-receiver matching hypothesis occurs in the mechanosensory lateral line system; this hypothesis posits that the physical properties of signals are shaped by the sensory responses of the receiver, and vice versa. This principle has been most notably demonstrated in

auditory communication; for example, the frequency and rise time of calling songs in tree crickets and songbirds (respectively) have both been shown to match the sensitivity of their receiver's auditory system. However, it is currently unclear if sender-receiver matches occur within vibratory communication. Importantly, my research serves as a building block to ascertain whether sender-receiver matching occurs in the mechanosensory lateral line system.

In Chapter 1, I characterized courtship interactions between male and female axolotls. I accomplished this by placing pairs of male and female axolotls into an aquarium together and then observing their behaviors over a 24 hr period. Chapter 2 describes the typical motion patterns that male axolotls exhibit during the hula behavior, as well as female behavioral responses to specific combinations of hula parameters. Chapter 3 describes neurophysiological responses of the anterodorsal lateral line nerve to the hydrodynamic stimuli that are generated during the hula behavior.

I was able to demonstrate a moderate degree of sender-receiver matching in the female lateral line system; I found that male axolotls were most likely to display a sweep angle of 30° while hula-ing, and that the anterodorsal lateral line nerve of females had a significant excitatory response to 30° . However, given that I only tested sweep angles and speeds within the range of what male axolotls actually display during courtship, it is unknown if females exhibit significant excitatory responses to more extreme sweep angles and speeds. Thus, my findings serve to progress research on the lateral line system by providing insight into the level of matching between hydrodynamic signals generated by male axolotls as well as the behavioral and neurophysiological responses exhibited by female axolotls.

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*For Rick,
whose contributions to my research are immeasurable.*

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CHAPTER 1: Characterization of Axolotl Courtship Behaviors

ABSTRACT

Animals exhibit a wide diversity of behaviors during their courtship rituals, which creates a wealth of opportunities to understand important topics in behavioral biology such as communication strategies, sexual selection, and the evolution of behavior. The axolotl, *Ambystoma mexicanum*, a fully aquatic salamander, has a dynamic repertoire of courtship behaviors as well as the ability to breed year-round, which makes them especially suitable for courtship studies. The goal of our behavioral survey was to describe the durations of time that male and female axolotls spend performing various courtship behaviors to establish a baseline for comparison in future studies; to accomplish this, we quantified courtship behaviors of both sexes, as well as female locomotion patterns specifically, throughout their entire courtship ritual.

The axolotl “hula” behavior (an undulating motion of the tail and hips) was previously thought to be a male courtship behavior, but we found that males and females generally performed the hula to the same degree. Additionally, we observed that males performed the tactile “bump” behavior (making physical contact between the male’s cloacal gland and female’s snout) during spawning attempts more frequently than females did. Furthermore, female axolotls modulated their locomotion patterns based on their proximity to a hula-ing male. These results revealed the significance of both male and female hula-ing, the necessity of tactile cues in successful courtship and spawning, and a potential locomotion strategy for localizing a hula-ing male. Overall, our results allowed us to gain a greater understanding of the courtship dynamics between

male and female axolotls and also provided important context for the upcoming chapters of this dissertation.

INTRODUCTION

Animal courtship rituals provide researchers with a unique substrate for studying communication strategies and understanding complex behavioral interactions. Courting animals exchange countless cues and signals with conspecifics, which includes not only competitors, but potential mates as well. Males and females display a wide range of courtship behaviors that engage multiple senses (e.g., visual, tactical, auditory, olfactory) both alone, and in combination. Therefore, there are many opportunities for researching specific communication events that include both signalers and receivers. Salamanders specifically have exceptionally diverse courtship behaviors which affords behavioral researchers with opportunities to evaluate inputs of multiple senses alone and in combination (Arnold, 1977; Arnold, 1987; Halliday, 1990; Houck & Arnold, 2003; Salthe, 1967; Verrell, 1999), especially within the family Ambystomatidae (Anderson, 1961; Arnold, 1976; Shoop, 1960).

The axolotl (*Ambystoma mexicanum*), a fully aquatic ambystomatid salamander, is an excellent model system to evaluate complex courtship behaviors. Axolotls can breed year-round (Armstrong & Malacinski, 1989) and do not adhere to a breeding seasonality, allowing for courtship evaluation studies to be conducted year round. Both males and females exhibit a diverse repertoire of courtship behaviors (Arnold, 1972), but a full characterization of courtship behaviors and their frequencies has not yet been documented. The purpose of this chapter is to characterize both male and female courtship behaviors and to establish a contextual foundation for the other chapters in

this dissertation. To accomplish this aim, we staged 30 encounters between male and female axolotls and quantified their courtship behaviors as well as female locomotion patterns throughout the courtship ritual.

In the section that follows, we describe the axolotl's courtship ritual, which we divided into 2 stages for our survey: the "preliminary stage" and the "mating stage". This labeling scheme is a modified version of Salthe's (1967) general description of salamander courtship, which includes 5 distinct stages of courtship that are labeled stages A - E. Salthe also labels "stage A" as "a preliminary stage"; here, we also used the phrase "preliminary stage" but consolidated Salthe's remaining stages into what we call the "mating stage". Thus, we will refer to male and female interactions across both stages overall as the "courtship ritual" or simply "courtship".

Axolotl courtship ritual

The function of the axolotl's courtship ritual is to coordinate the process of transferring sperm from male to female. Female axolotls fertilize their eggs internally but rely on external sperm transfer, which is facilitated by the use of a spermatophore; each one, produced by the male, consists of a gelatinous base with a short stalk capped by a round pellet of sperm (sperm cap) that the female will pick up with her cloaca. Females are active receivers at the sperm collection phase, and successful copulation necessitates female involvement during courtship and while receiving sperm (i.e., picking up the sperm cap). Axolotl courtship tends to progress in a cyclical fashion, both within and between stages. Thus, a courting pair may alternate between preliminary stage behaviors several times before transitioning to the mating stage, and the pair may then return to the preliminary stage to begin the ritual over again (Salthe, 1967).

The preliminary stage begins with a male approaching a female and is punctuated by the male performing a “push” behavior; the male presses his snout against the female, typically against her lateral torso but sometimes against her head. Pushing can either involve a slight tapping motion or it may escalate to shoving; males often move females across the arena by forcefully walking or swimming into them. The preliminary stage also includes a behavior known as the “hula” (Verrell, 1982), which is exhibited by both sexes. Hula-ing involves a rhythmic swaying of the hips and posterior legs combined with an undulating motion of the tail; we have observed both sexes either holding their tails horizontally or upwards at an angle. Axolotls may hula at a distance from each other (which we have defined here as occurring at least one male tail-length away) or at a closer range (which we defined as within one male tail-length).

After the preliminary stage ends, the pair typically (but not always) proceeds to the mating stage. The mating stage constitutes an escalation of the preliminary stage in that it involves all of the behaviors from the preliminary stage, but also includes additional behaviors that are necessary for successful spawning such as the “follow” behavior (Shoop, 1960). The follow behavior facilitates spawning by coordinating the process of external sperm transfer between male and female. The female initiates a follow bout by walking closely behind the male as he slowly walks forward. The female’s snout remains close to the male’s cloacal gland as he walks and elevates his tail slightly to accommodate the female’s head. During the mating stage, male axolotls will typically deposit at least one spermatophore onto the substrate; then, the male will move forward about one body length and stop, positioning the female’s cloaca directly over the

spermatophore. Finally, the female will remove the sperm cap with her cloaca, leaving the gelatinous base behind.

Several additional courtship behaviors may occur while the female is following behind the male. The mating pair's forward progress is often interrupted by a behavior called "bumping" (Shoop, 1960), which can be initiated either by the male or female. A male axolotl initiates a bump when he steps backwards and touches either his cloacal gland or the proximal half of his tail to the female's snout; female bumping is similar, except that the female initiates physical contact. Males also perform frequent bouts of hula-ing during following periods and can be observed raising and lowering their tails onto the female's snout, which comprises a "tail-tapping" behavior (Arnold, 1976). Additionally, we have defined a "nudge tip" behavior such that either the male or female initiates contact between the female's snout and distal half of the male's tail. Once the mating stage has ended, the pair will separate from one another and may begin part or all of the courtship ritual over from the beginning.

MATERIALS AND METHODS

Subjects

Adult axolotls (*Ambystoma mexicanum*) were obtained from the Ambystoma Genetic Stock Center at the University of Kentucky. Animals were housed in ~114 L aquaria in 40-100% Holtfreter's solution (Armstrong et al., 1989) supplemented with Replenish™ solution at temperatures between 18 and 22°C; animals were separated by sex and each aquarium contained 1-3 animals. We programmed the lights in our facility to match the natural sunrise and sunset of Mexico City, Mexico (the native habitat of

axolotls) with monthly updates to the axolotls' photoperiod. Housing and experiments were conducted with the approval of the Institutional Animal Care and Use Committee of Michigan State University (approval number: 10/15-154-00).

Behavioral trials

We recorded behavioral interactions from 30 pairs of male and female axolotls (N = 10 males, 19 females); each male was paired with 3 different females. We randomly chose 10 males from our colony for the first 10 trials and then repeated the order of males twice to complete the set of 30 trials. Female axolotls were selected at random for the entire study, with two caveats: 1) no female was used twice in a row and 2) females that laid eggs were not used again later in the survey. No male was paired with the same female twice, but some females were paired with multiple males; 10 females were used once, 7 females were used twice, and 2 females were used three times.

For each trial, we placed a male and a female together into a ~114 L aquarium (approximately 90 cm long x 45 cm wide x 30 cm wide) filled with Holtfreter's solution at the same concentration as their home aquaria. Because axolotls are nocturnal, we placed the pair together shortly before the lights in our animal facility turned off for the evening. We allowed the courting pair to interact overnight and then returned them to their home aquaria the following morning. We used two Sony Nightshot camcorders (model: CMOS) to record top-down and side views of each trial for roughly 12 hrs.

Because the infrared (IR) light on the camcorders did not sufficiently illuminate the recording area, we placed a lightbox equipped with a series of waterproof infrared LED lights underneath the aquarium. We also placed two IR lamps behind the aquarium

and attached a layer of light-colored paper to the outer surfaces of the aquarium to diffuse the IR light. We do not believe that the behavior of our study animals was impacted by our use of infrared lighting. The absorbance spectra of isolated rod cells from a closely related species, the tiger salamander (*Ambystoma tigrinum*), indicate that their absorbance drops to approximately 0 between 640 and 700 nm (Cornwall et al., 1984). Given that infrared radiation has wavelengths between 700 nm and 1 mm, it is unlikely that our subjects were able to detect the infrared lighting that we used in our study.

Definition of courtship behaviors

We conducted a preliminary behavioral survey of 12 complete trials (out of 30 total) to 1) understand which courtship behaviors were relevant to our study and 2) determine an appropriate amount of video footage to score for each trial. The subset of 12 trials consisted of data from 4 male and 10 female axolotls, and we examined 9 hrs of each trial. Two of these males were in trials that led to a spawning event, which were the only two trials in the entire survey that resulted in spawning; the other 2 males were selected at random and we examined each male's trio of trials. We used Shoop's (1960) description of mole salamander (*Ambystoma talpoideum*) courtship to guide our behavioral survey because it is similar to axolotl courtship.

We first quantified a small number of courtship behaviors described by Shoop (1960), e.g., push, follow, male bump, and female bump. Shoop's "follow" is the same as our follow behavior. However, Shoop (1960) refers to what we call "push" and "female bump" behaviors simply as "bumping"; in our survey, we distinguished them to avoid duplicates in our ethogram. While surveying the subset of trials, we noted

additional behaviors that we included in our final coding (female walk, swim, and pause, female locomote/pause near male, orient, male and female close/distant hula, nudge tip, deposit or pick up spermatophore, nip, bite, and attack) and described which behaviors can and cannot co-occur (Fig. 1.1). Additionally, we found that courting pairs are most active during the first 2.5 hrs after the lights turned off; thus, we used this time window for all subsequent analyses. After completing our initial survey, we then quantified the behaviors that are defined in our ethogram (Table 1.1) for all 30 trials.

Categorization of courtship behaviors

We organized our axolotl behaviors of interest into several categories: female locomotion, preliminary stage, mating stage, and aggressive behaviors. The co-occurrence patterns of these categories (and the behaviors within them) are summarized in Figure 1 below. The “female locomotion” category refers to patterns of walking, swimming, and pausing, which is the absence of locomotion (Coombs et al., 2014) (Table 1.1); we recorded these behaviors throughout the trial except during the mating stage. The females in our study were motionless for highly variable amounts of time (0.25 sec up to 2.5 hr), and although the term “pause” is typically reserved for bouts up to 50 sec (Kramer & McLaughlin, 2001) we will use “pause” to signify that the female was motionless. “Locomote/pause near male” (Park et al., 2008) is meant to differentiate female locomotion behaviors that occur near and farther away from the male when the pair is not engaged in the preliminary or mating stage (Fig. 1.1); all of the behaviors within these stages are already defined such that they designate the proximity of the pair to one another. Although “orient” is not technically a locomotion behavior, we included it in the female locomotion category because it occurs during

periods of “locomote/pause near male”; a female axolotl orients toward a male when she is “near” the male and swivels her head to face him (Park et al., 2008).

The “preliminary stage” category includes push behaviors as well as male and female hula behaviors; all hula bouts were measured when the courting pair was not engaged in following, except for “female close hula” which may occur at any point during the trial. We defined the end of a preliminary stage such that the pair fails to exhibit pushing and/or hula-ing for at least 5 min or they start to engage in a follow bout (Fig. 1.1). The “mating stage” category consists of all preliminary stage behaviors, plus “follow” and behaviors associated with following; for example, we recorded the number of times a male deposited a spermatophore onto the substrate as well when a female picked one up. We did not independently measure bouts of male hula-ing or female locomotion during follow bouts because they both consistently co-occur with following. We defined the mating stage as starting when a pair begins to follow, and ending when the pair stops following, pushing, and/or hula-ing for 5 min or more (Fig. 1.1).

The last behavioral category, aggression, includes three rare but notable behaviors that we have defined for this survey. Females may exhibit aggressive behaviors at any point during the courtship ritual. “Nip” occurs when the female bites the male’s tail and then immediately releases it. “Bite” is similar to “nip” except that the female keeps her jaws engaged on the male’s tail for a few seconds. “Attack” is an escalated version of “bite”; a female bites the male’s tail for an extended period of time while thrashing her head from side to side.

We also recorded several non-behavioral events that we did not include in the ethogram (Table 1.1). We observed how many spermatophores were floating in the

aquarium during the trial, as they can detach from the glass surface easily and we could not always see when a male axolotl was actively depositing a spermatophore. We also counted the number of spermatophores that were present in the aquarium once the trial had ended the following morning; this value encompasses the total number of spermatophore depositions (floating and still attached to the aquarium floor). Additionally, we noted if the female laid eggs, which typically occurred within 24 hrs of the trial ending.

Behavioral coding

We used Behavioral Observation Research Interactive Software (BORIS; version 7.9.RC1) to quantify axolotl courtship behaviors (Friard & Gamba, 2016). BORIS aids in behavioral coding by allowing users to assign individual behaviors to particular computer keys; the user then watches a video and presses the corresponding keys when they observe a behavior of interest occurring. Once the user has finished coding a trial, BORIS will output the number of occurrences and the duration (where applicable) of each behavior into a data sheet.

Importantly, BORIS allows users to set an “exclusion matrix”, which designates which behaviors can co-occur with one another and which behaviors are mutually excluded. For example, male bumps can be coded while a follow period is occurring; the user would press the key for “follow” to begin the behavior, then press the key for “male bump” any time one occurred, and finally press the key for “follow” again once the follow period had ended. In contrast, a female axolotl cannot walk and swim at the same time, so these behaviors are mutually excluded. If a swim behavior is followed by a walk behavior BORIS will automatically toggle off swimming once the walking bout begins.

Statistical analyses

We calculated summary statistics (mean, standard deviation, minimum, maximum) on all courtship behaviors (Table 1.2) using the “psych” package (version 2.4.3; Revelle, 2009) in R (R Core Team, 2020), with zeros removed for total durations and average bout durations (i.e., total duration divided by count) but retained for count variables. Summary statistics are written as mean($\pm$ SD) in the text that follows. We also assessed data on female locomotion during male hula-ing, which we obtained by performing conditional behavior analyses in BORIS; we then calculated transitions between walking, swimming, and pausing (Table 1.3), as well as the proportions of time that females spent performing these behaviors (Table 1.4). Because females were repeated multiply in the dataset an unequal number of times, we also evaluated behaviors using general linear mixed-effects models in R (using packages “lme4” (version 1.1.35.3; Bates et al., 2015) and “lmerTest” (version 3.1.3; Kuznetsova et al., 2017)) with female ID as the random effect and restricted maximum likelihood (REML) set to “true”. We evaluated sex-based differences in hula durations (total and average bout duration), and occurrence of “bump” behaviors; in addition, we assessed differences in female locomotion transitions based on the presence or absence of a hula-ing male as well as the female’s proximity to a hula-ing male (Table 1.5).

RESULTS

Female locomotion

Of the 150 min of each trial, female axolotls spent an average ($\pm$ SD) of 50($\pm$ 20) min locomoting (i.e., walking and swimming). Females spent the most amount of time

pausing ($90(\pm 27)$ min), followed by walking ($43(\pm 21)$ min), and spent the least amount of time swimming ($7(\pm 10)$ min) (Fig. 1.2). One female in our study did not locomote (“paused”) for the entirety of the trial, but performed non-locomotive behaviors and therefore was not excluded from analyses. . Females paused on average for $3(\pm 13.6)$ min per bout. Female axolotls that spent any amount of time near the male spent a total of $24(\pm 10)$ min near him when he was not in the preliminary or mating stage. Females spent on average $0.5(\pm 1.4)$ min near the male per bout. Females oriented towards the male an average of $6(\pm 7)$ times during a trial.

Female locomotion during male hula-ing

The presence of a hula-ing male impacted female locomotive activity during the courtship ritual; however, the proximity of the male had little bearing on female locomotion. For reference, the males that hula'd for any amount of time spent $5(\pm 4)$ min close hula-ing, $4(\pm 4)$ min distant hula-ing, and $8(\pm 8)$ min hula-ing in total when the pair was not engaged in a bout of following. Female transitioned between walking, swimming, and pausing significantly more often ($7.90(\pm 8.62)$ times/min) when a male was hula-ing versus when there was no hula-ing occurring ($2.76(\pm 1.27)$ times/min; $t = -3.474$, $p = 0.00128$; Fig. 1.3). Females transitioned between walking, swimming, and pausing an average of $9.99(\pm 18.61)$ times/min when a male was hula-ing close by and transitioned $10.09(\pm 18.32)$ times/min when the male was hula-ing at a distance ($t = 0.025$, $p = 0.9803$). Thus, the presence of a hula-ing male caused females to increase their transitions between locomotive states, but females exhibited overall the same activity levels regardless of whether the male was hula-ing close to them or at a distance.

Although the proximity of a male (close vs far) did not affect how often a female transitioned between locomotive states, the proximity of a hula-ing male did impact the proportions of time that female axolotls spent locomoting and pausing. When a male axolotl was hula-ing close to a female, she spent an average of 44% of the time walking, 0.01% of the time swimming, and 55% of the time pausing. In contrast, when a male was hula-ing at a distance, females spent an average of 59% of the time walking, 2% of the time swimming, and 39% of the time pausing. Thus, females allocated time differently to locomotive and pausing behaviors when a male was hula-ing at a distance, compared to when a male was hula-ing at close proximity to the female.

Preliminary and mating stages

The 27 pairs of axolotls that courted spent an average of 31($\pm$ 29) min in the preliminary stage (pushing and hula-ing), and the 2 pairs that mated spent an average of 75($\pm$ 44) min in the mating stage (preliminary stage behaviors plus following) per 150-min trial. Among males that exhibited pushing behaviors, males pushed females for an average of 8($\pm$ 7) min total; each bout of pushing lasted on average for 0.1($\pm$ 0) min. The pairs in our study invested a substantial amount of time in the preliminary stage and an even greater period of time in the mating stage, suggesting that the axolotl courtship ritual involves a high level of effort and coordination between the sexes.

In general, males and females did not differ substantially in the overall amount of time spent hula-ing, but the duration of each bout of hula-ing was longer for males than females. Among individuals that exhibited the hula (10 males, 14 females), males hula'd in total (close + distant) for 8($\pm$ 8) min and females hula'd for 5($\pm$ 13) min ($t = 1.43$, $p = 0.16$). However, male axolotls hula'd for longer bouts of time for each hula event

($0.1(\pm 0.1)$ min) than females did ($0.1(\pm 0)$ min; $t = 2.38$, $p = 0.02$). At distant ranges, males hula'd for $4(\pm 4)$ min and female hula'd for $3(\pm 9)$ min ($t = 0.55$, $p = 0.59$). Male axolotls hula'd distantly for longer bouts of time ($0.2(\pm 0.1)$ min) than females did ($0.1(\pm 0.1)$ min; $t = 3.80$, $p = 0.0008$).

When in close proximity, males hula'd more than females. Males hula'd for an average of $5(\pm 4)$ min, whereas females hula'd for an average of $2(\pm 5)$ min ($t = 2.45$, $p = 0.02$; Fig. 1.4). On average, each bout of close hula-ing that males performed lasted $0.1(\pm 0.1)$ min; for females the average was similar at $0.1(\pm 0)$ min ($t = 0.87$, $p = 0.39$; Fig. 1.5).

The 27 courting pairs in our survey spent approximately 15% of their time in the mating stage, and frequently exhibited behaviors related to spawning during those periods, including following, bumping, and spermatophore depositions. The males and females that participated in the mating stage engaged in following for an average of $23(\pm 14)$ min per trial; each bout of following lasted an average of $0.8(\pm 0.3)$ min. We found that males bumped females ($90(\pm 145)$) significantly more often than females bumped males ($51(\pm 71)$, $t = 2.45$, $p = 0.02$; Fig. 1.6). Courting pairs that exhibited “nudge tip” did so an average of $7(\pm 13)$ times. Male axolotls deposited spermatophores in 40% of trials (i.e., 12 out of 30 trials) determined by both observation on the video and through inspection of the aquaria after each trial. Males deposited a maximum of 3 spermatophores per trial where deposition was visible in the video footage, but we counted up to 5 in the aquarium when each trial was completed; certain spermatophores were not visible in our footage because they are clear, mostly colorless, and small. Due to the recording angle of the video camera we were unable to

observe females picking up a spermatophore; however, 2 females laid eggs after their trials had ended.

Aggression

Female aggression towards the male during the courtship ritual was minimal and restricted to nipping behavior. Nipping occurred in 27% of our trials (8 out of 30), however the number of nips was limited; of the trials where nipping happened, we observed an average of 1.25 nips. We never observed females biting or attacking the male.

DISCUSSION

Female locomotion behaviors

Over the course of their entire courtship ritual, female axolotls spent the greatest amount of time pausing, followed by walking and then swimming (Fig. 1.2). When the male axolotl was performing the hula behavior, females transitioned between walking, swimming, and pausing significantly more often (~8 times/min) than when the male was not hula-ing (~3 times/min; Fig. 1.3). In contrast, the proximity of a hula-ing male (i.e., close vs distant) did not have a significant impact on how many times the female transitioned between locomotive states, but did impact the allocation of time in each locomotive behavior.

Females modified the proportions of time that they spent walking, swimming, and resting in response to the proximity of a hula'ing male. When a male was hula-ing in close proximity, females spent ~40% of the time walking and ~60% of the time pausing, whereas the female spent ~60% of the time walking and ~40% of the time pausing is

the male was hula-ing at a distance. Therefore, females spent a larger proportion of time locomoting when the male was hula-ing at a distance, and paused rested more when the male was hula-ing at close proximity.

Thus, it appears that females change their rates of transitioning between locomotive states depending on the presence or absence of a hula-ing male, but they modify the proportions of time spent on walking, swimming, and resting relative to the proximity of the hula-ing male. The male's hula behavior likely generates sensory cues that can be propagated over short and long distances, including electrical cues (via muscle contractions (Soleymani et al., 2017)) and mechanosensory cues (i.e. water perturbations (Satou et al., 1991)), visual stimuli (Verrell, 1982), and the dispersal of odorants (Maex et al., 2016). The combination of these cues would allow for the female to detect the hula-ing male's presence over multiple distances and adjust her locomotion levels accordingly.

A possible explanation for the modulation of female locomotion patterns during male hula-ing is that females may increase their level of searching (i.e. walking more than pausing) if they detect vibrations in the water. From a distance, a male's hula movements may indicate the presence of another animal in the water, potentially a conspecific or a prey item, which may increase the female's searching efforts. However, once the hula-ing male gets close to the female she may walk less and pause more often to gather sensory information from the male's tail motions more effectively. Analogously, Kramer & McLaughlin (2001) posit that stillness reduces blur in the visual field, allows for detection of faint sounds, and decreases vibrations that may interfere with object localization across many invertebrate and vertebrate species; thus, pauses

in locomotion can help animals detect objects in their sensory fields more easily by reducing self-generated noise (Montgomery et al., 2014).

The mate-search strategy of courting animals are known to be influenced by the presence of stimuli that are produced by conspecifics. Park et al. (2004) demonstrated that male axolotls, regardless of their prior experience level with females, increased their general activity levels when exposed to whole-body odorants from gravid (egg-laden) or recently spawned females; similarly, the searching effort of male guppies, *Poecilia reticulata*, increased when they were exposed to female odorants (Guevara-Fiore et al., 2010). Male axolotls have also been shown to decrease their general activity levels when presented with gravid female odorants; however, the experience levels of these males are unknown (Eisthen, unpublished). Park et al. (2004) also demonstrated that female axolotls did not change their activity levels when exposed to male whole-body odorants. We speculate that the females in our survey received other sensory signals from the hula-ing male in addition to any male odorants present in the aquarium, and that the presence of these multimodal signals may have increased the female's activity levels, whereas isolated male odorants alone may not have been sufficient to increase the female's search efforts in the experiments of Park et al. (2004).

In contrast, the female may be prompted to pause more often once she is in closer proximity to the hula-ing male. An animal's ability to sense their surroundings can be modulated by their own locomotion behaviors. For example, when the motor (efferent) nerve fibers of the mechanosensory lateral line system in the African clawed frog, *Xenopus laevis*, are electrically stimulated the corresponding sensing (afferent) fibers are inhibited from firing (Russell, 1968); under normal circumstances, the activity

of these afferent fibers would be suppressed by the animal's own body motions (Lunsford & Liao, 2021; Plazas & Elgoyhen, 2021; Wullimann & Grothe, 2014). Given that *X. laevis* and axolotls share a similar lateral line system, female axolotls may experience a decrease in the sensitivity of their mechanosensory organs (i.e., neuromasts) during periods of walking or swimming. Thus, it may be advantageous for the female to pause more often once the hula-ing male is close to her to more effectively gather sensory information from the male without the interference of her own motions.

Preliminary and mating stages

Our pairs of male and female axolotls spent about 31 min out of each 150-min trial (approximately 21% of their time) in the preliminary stage, which was dominated by push behaviors as well as male and female hula behaviors. Tactile feedback via pushing appears to play an important role in the mating success of axolotls. The total duration of pushing had statistically significant, positive correlations with both spermatophore depositions (i.e., those that we observed during behavioral coding) and the total number of spermatophores we counted in the aquarium after each trial; in other words, males that pushed more often also tended to produce more spermatophores. In *Ambystoma laterale*, pushing behaviors may aid in the male's recognition of the species and/or sex of other animals in the area. When placed in an arena with both sexes, *A. laterale* males will nudge males and females initially but then shift to nudging females only; it is thought that males may be able to detect a female-specific skin secretion or the presence of eggs (Storez, 1969). Thus, it appears pushing may be associated with

more frequent spawning attempts that are also aimed towards the correct species and sex.

Although males hula'd more than females at close ranges (Fig. 1.4) and hula'd for longer bouts of time at distant ranges (Fig. 1.5), males and females hula'd to approximately the same degree in many other instances. Despite our findings, hula-ing and similar behaviors in axolotls and other salamanders have typically been regarded as a male behavior (Halliday, 1990; Maex et al., 2016; Verrell, 1983). It appears that researchers tend to assign more importance to male hula-ing in their descriptions of the behavior; in males, tail undulations are generally discussed in greater detail and given explicit labels like “hula” or “tail fan”. Although researchers have described hula-ing in female axolotls (Park et al., 2004) and hula-like behaviors in other female ambystomatids like *Ambystoma macrodactylum columbianum* (Verrell & Pelton, 1996) as well as female plethodontids like *Desmognathus wrighti* (Verrell, 1999), female tail undulations are often given simpler descriptions and typically are not included in courtship ethograms. Observers often have preconceived notions about the behavioral roles of male and female animals (Ahnesjö et al., 2020), and the gender biases of observers may explain the discrepancy in the treatment of male and female hula-ing in the literature.

Pierotti et al. (1997) suggest that the reason male behaviors are emphasized in ethology journals is because both male and female researchers have largely been trained to focus on male-centered narratives. Given that many of the researchers who laid the groundwork for salamander courtship behaviors did so before gender biases in ethology were called into question starting in the mid-1990s, it is unsurprising that a

focus on male hula-ing has taken precedence over female hula-ing. In contrast, the observer's gender appears to make little to no difference in the outcomes of behavioral coding of non-courtship behaviors, aggression and foraging, in salamanders specifically (Marsh & Hanlon, 2004). Although the function of hula-ing in female salamanders is unclear, it appears to play a significant role in the courtship dynamics of axolotls given that males and females hula to approximately the same degree.

The axolotls in our study spent approximately 75 min in the mating stage (approximately 50% of the trial), and roughly 36% of that period was dedicated to bouts of following. All pairs that transitioned to the mating stage at least once exhibited the follow behavior multiple times over the course of a trial. Multiple spawning attempts are also a common feature in the courtship rituals of other ambystomatid species (Petranka, 1982; Shoop, 1960; Verrell & Pelton, 1996). The bump behavior (which occurs during following) appears to be an important factor in the outcome of each spawning attempt. The function of courtship bumping has been described in the Alpine newt, *Ichthyosaura alpestris* (Denoël & Doellen, 2010), which exhibits a similar behavior during the spermatophore transfer process. In the Alpine newt's courtship ritual, a female "tactile stimulus" (which we call a "female bump" in axolotls) serves to 1) communicate her level of responsiveness to the male and 2) lower the male's chance of breaking contact with the female. Further, male Alpine newts perform a "push-back" behavior (which we call "male bump"), which pushes the female back toward the spermatophore in the event that she failed to locate it with her cloaca.

We posit that male bumping in axolotls may also serve to correct the female's lateral movements as she positions herself over the spermatophore; in our survey, we

observed males bumping their cloacal glands into the sides of the female's snout as well as the center. Although male axolotls bumped females more than vice versa (Fig. 1.6), both types of bumping seem to be important to overall spawning success; one of the two trials that resulted in spawning had the greatest number of combined bumps (772) out of any trial. The other trial with a spawning event had 175 combined bumps, which is moderate compared to other trials; however, this trial had the highest overall duration of male hula-ing. This observation suggests that the interaction between physical contact and male hula-ing may be an important component of successful mating.

Aggression

Occurrences of serious aggressive behaviors (biting, attacking) in our survey were not observed. Nipping, while occurring in 27% of trials, was not a consistent feature of the axolotl courtship ritual; of the trials that featured nipping, females nipped at males on average ~1 time per trial. We were unable to discern if females were directly aggressive toward males or if they were instead exhibiting a predatory response to the male's tail or limbs.

CONCLUSION

The courtship ritual of the axolotl is complex and dynamic, as the signaler and receiver respond not only to a range of touch and motion-based cues, but also modify their behaviors depending on the proximity to one another. Axolotl courtship involves a substantial time investment where the courtship behaviors are repeated and allows for changes and dynamic response based on specific current conditions. In general,

courting pairs spent more time in the mating stage than in the preliminary stage. We found that female axolotls transitioned between locomotion states more frequently when males performed hula-ing behaviors. This suggests that females are active receivers of male motion-based behaviors and modify their behavior in response. Additionally, females alter the time spent in each locomotive state depending on whether males are in close or far proximity, suggesting that they can respond to rapidly to changing signals. This survey allowed us to establish a baseline of courtship behaviors, define common and contrived male behaviors, and understand the context for how females naturally respond, serving as a foundation for future work on female behavioral and neurophysiological responses.

TABLES

		Behavior	Description
<i>Female locomotion</i>		Walk	Female walks forward or backward at least 3 steps, with all four legs moving.
		Swim	Female swims forward using her tail, with legs pressed against the body.
		Pause	Female is not walking or swimming.
		Locomote/pause near male	Female is walking, swimming, or pausing and her head is within one male tail length of the male.
		Orient	Female has her head within one male tail length of the male and she turns her head to face the male.
<i>Mating stage</i>	<i>Preliminary stage</i>	Push	Male pushes against the female's torso or head with his snout.
		Hula	Animal sways hips and posterior legs while undulating tail.
		Male close hula	Male performs hula near the female, within one tail length.
		Male distant hula	Male performs hula at least one tail length away from the female.
		Female close hula	Female performs hula near the male, within one male tail length.
		Female distant hula	Female performs hula at least one male tail length away from the male.
		Follow	Female walks closely behind the male as he steps forward. Starts when female snout is within one female head-length of the male's cloacal gland and ends when the male cloacal gland is at least one tail length away from the female's snout.
		Male bump	Male walks backwards and touches his cloacal gland or proximal half of the tail to the female's snout.
		Female bump	Female walks forward and touches the male's cloacal gland or proximal half of the tail with her snout.
		Nudge tip	Contact is made between the female's snout and distal half of the male's tail. May be initiated by male or female.
		Deposit	Male deposits a spermatophore onto the substrate.
		Pick up	Female picks up a spermatophore with her cloacal gland.
<i>Aggression</i>		Nip	Female bites the male's tail and immediately releases it.
		Bite	Female bites the male's tail and briefly keeps jaws engaged.
		Attack	Female bites the male's tail and thrashes her head side to side.

Table 1.1: Ethogram of male and female courtship behaviors. We classified courtship behaviors into four categories: female locomotion, preliminary stage, mating stage, and aggression. All of the preliminary stage behaviors may occur during the mating stage, whereas “follow” (and all behaviors associated with following) may only occur during the mating stage.

		Behavior	Count	Total Duration	Average Duration
Female locomotion		Walk	NA	43(21) 11-76	0.2(0.1) 0.1-0.3
		Swim	NA	7(10) 0-44	0.2(0.1) 0.1-0.3
		Locomote (walk + swim)	NA	50(20) 11-83	0.2(0.1) 0.1-0.3
		Pause	NA	90(27) 49-150	3(13.6) 0.2-75
		Locomote/pause near male	NA	24(10) 6-61	0.5(1.4) 0-7.6
		Orient	6(7) 0-32	NA	NA
Mating stage	Preliminary stage	Push	NA	8(7) 0-27	0.1(0) 0-0.1
		Male close hula	NA	5(4) 0-14	0.1(0.1) 0-0.2
		Male distant hula	NA	4(4) 0-15	0.2(0.1) 0.1-0.5
		Male all hula	NA	8(8) 0-29	0.1(0.1) 0-0.3
		Female close hula	NA	2(5) 0-19	0.1(0) 0-0.2
		Female distant hula	NA	3(9) 0-39	0.1(0.1) 0-0.2
		Female all hula	NA	5(13) 0-58	0.1(0) 0-0.2
		Total preliminary stage	NA	31(29) 0-99	NA
		Follow	NA	23(14) 0-54	0.8(0.3) 0.2-1.3
		Male Bump	90(145) 0-583	NA	NA
		Female Bump	51(71) 0-297	NA	NA
		Nudge Tip	7(13) 0-59	NA	NA
		Deposit Spermatophore	1(1) 0-3	NA	NA
		Pick Up Spermatophore	0(0) 0-0	NA	NA
		Observe Spermatophore	0(0) 0-2	NA	NA
		Total Spermatophores	1(2) 0-5	NA	NA
		Eggs	0(0) 0-1	NA	NA
		Total mating stage	NA	75(44) 0-146	NA
Aggression		Nip	0(1) 0-2	NA	NA
		Bite	0(0) 0-0	NA	NA
		Attack	0(0) 0-0	NA	NA

Table 1.2: Summary statistics of male and female courtship behaviors. Data are displayed as mean(SD) minimum-maximum; all durations are listed in minutes. Zeroes have been removed from duration data but left in count data. Count and total duration data were rounded to the nearest whole number, and average duration was rounded to the nearest tenth, meaning that some durations show a minimum value of zero.

Analysis period	Transitions / min
Entire trial	3.06(1.22) 0.01-5.31
During close male hula-ing	9.99(18.61) 0-93
During distant male hula-ing	10.09(18.32) 0-75
During all male hula bouts	7.90(8.62) 0-37.43
No male hula-ing	2.76(1.27) 0.01-5.15

Table 1.3: Summary statistics of female transitions between locomotive states.

Data represent the number of times per minute that females transitioned among walking, swimming, and pausing states and are displayed as mean(SD) minimum-maximum. All values were rounded to the nearest tenth.

	Close Hula	Distant Hula	All Hula
Walk	0.44(0.25) 0.10-1.00	0.59(0.26) 0-0.91	0.47(0.25) 0-1
Swim	0.01(0.05) 0-0.21	0.02(0.08) 0-0.33	0.03(0.08) 0-0.33
Pause	0.55(0.25) 0-0.90	0.39 (0.23) 0.09-0.82	0.50(0.22) 0-0.82

Table 1.4: Summary statistics of proportions of time females spend locomoting during male hula-ing.

Data represent the proportions of time that females spent walking, swimming, and pausing while the male was hula-ing at close proximity or at a distance and are displayed as mean(SD) minimum-maximum. All values were rounded to the nearest tenth.

Dependent variable	Independent variable	t statistic	p - value
Close hula total duration	Sex	2.45	0.02 *
Close hula average duration	Sex	0.87	0.39
Distant hula total duration	Sex	0.55	0.59
Distant hula average duration	Sex	3.80	0.0008 ***
All hula total duration	Sex	1.43	0.16
All hula average duration	Sex	2.38	0.02 *
Bump count	Sex	2.45	0.02 *
Female locomotor transitions	Proximity to hula-ing male	0.025	0.98
Female locomotor transitions	Presence/absence of male hula	-3.474	0.00128***

Table 1.5: Results of general linear mixed-effects models. Analysis of sex differences in hula durations, bump counts, as well as female locomotion patterns during bouts of male hula-ing revealed significant differences in close hula-ing, distant hula-ing, bumping, and female transitions between locomotive states. Female ID number was used as the random effect in all analyses. All t statistics and p-values were rounded to 2 decimals, except when the p-value was less than 0.01.

FIGURES

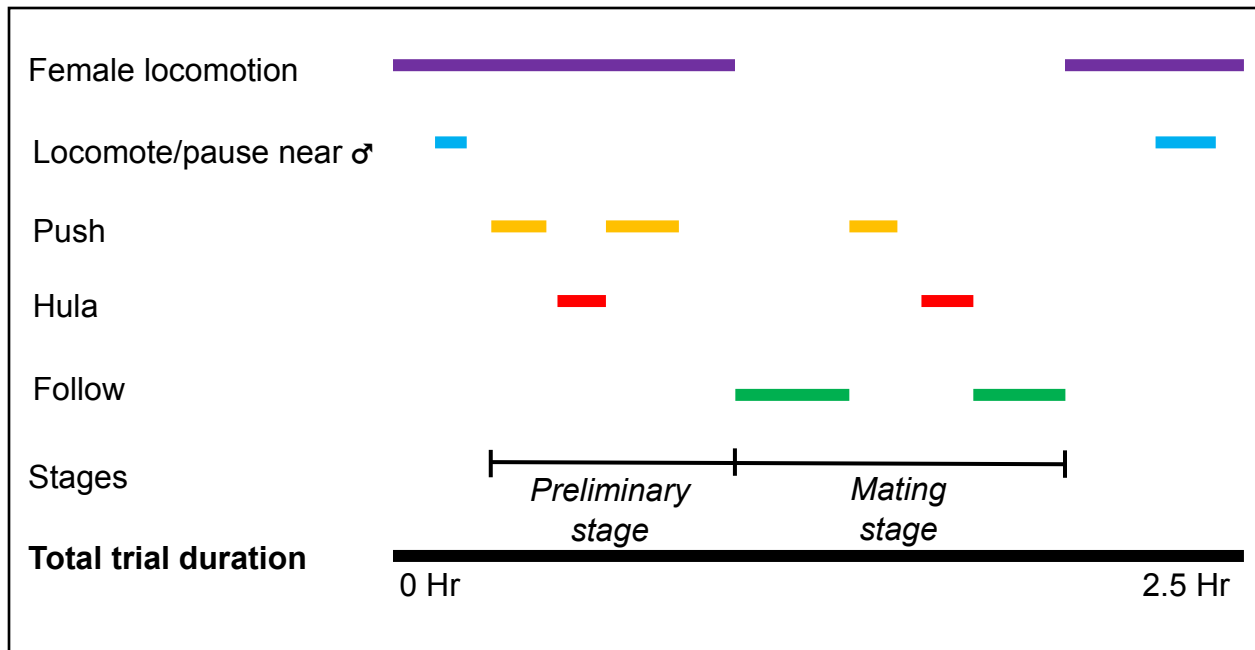


Figure 1.1: Example time budget of courtship stages. Illustration displaying which behavioral categories may co-occur and which are mutually excluded: includes female behaviors (female locomotion, locomote/pause near male), male behaviors (push, hula), and follow, in which both sexes are engaged in the behavior. All behaviors displayed in this illustration are listed in the ethogram (Table 1).

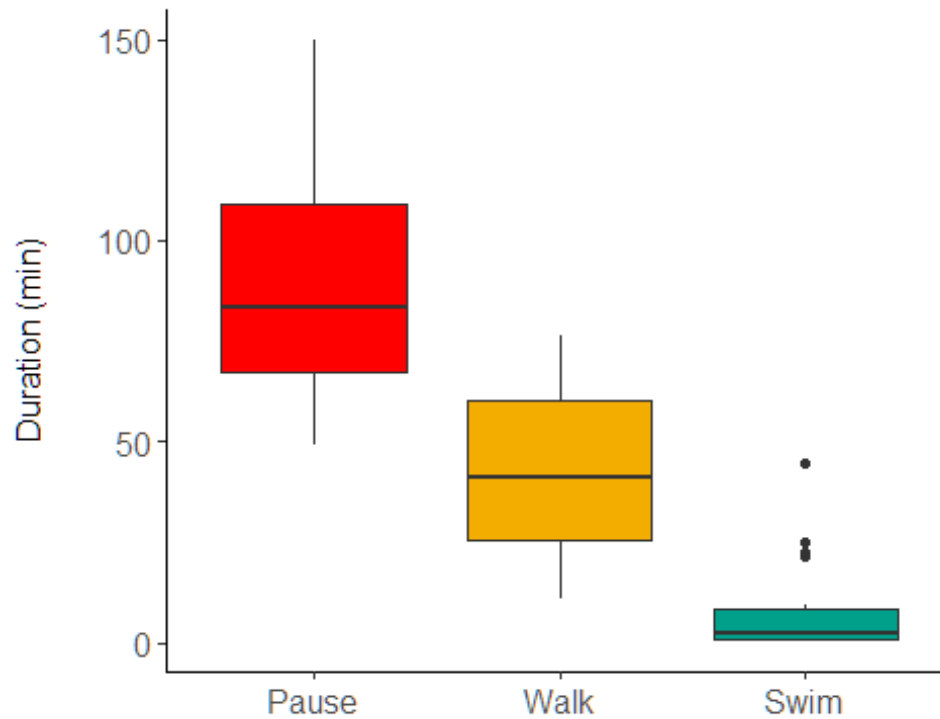


Figure 1.2: Female locomotion throughout the courtship ritual. Overall, females spent the greatest amount of time pausing, followed by walking and then swimming. Each box and whisker plot represents five statistical values: the lower end of the bottom vertical line is the minimum, the bottom edge of the box is the lower quartile, the line in the center of the box is the median, the upper edge of the box is the upper quartile, and the upper end of the top vertical line is the maximum. Dots represent outlier values and zeroes have been removed.

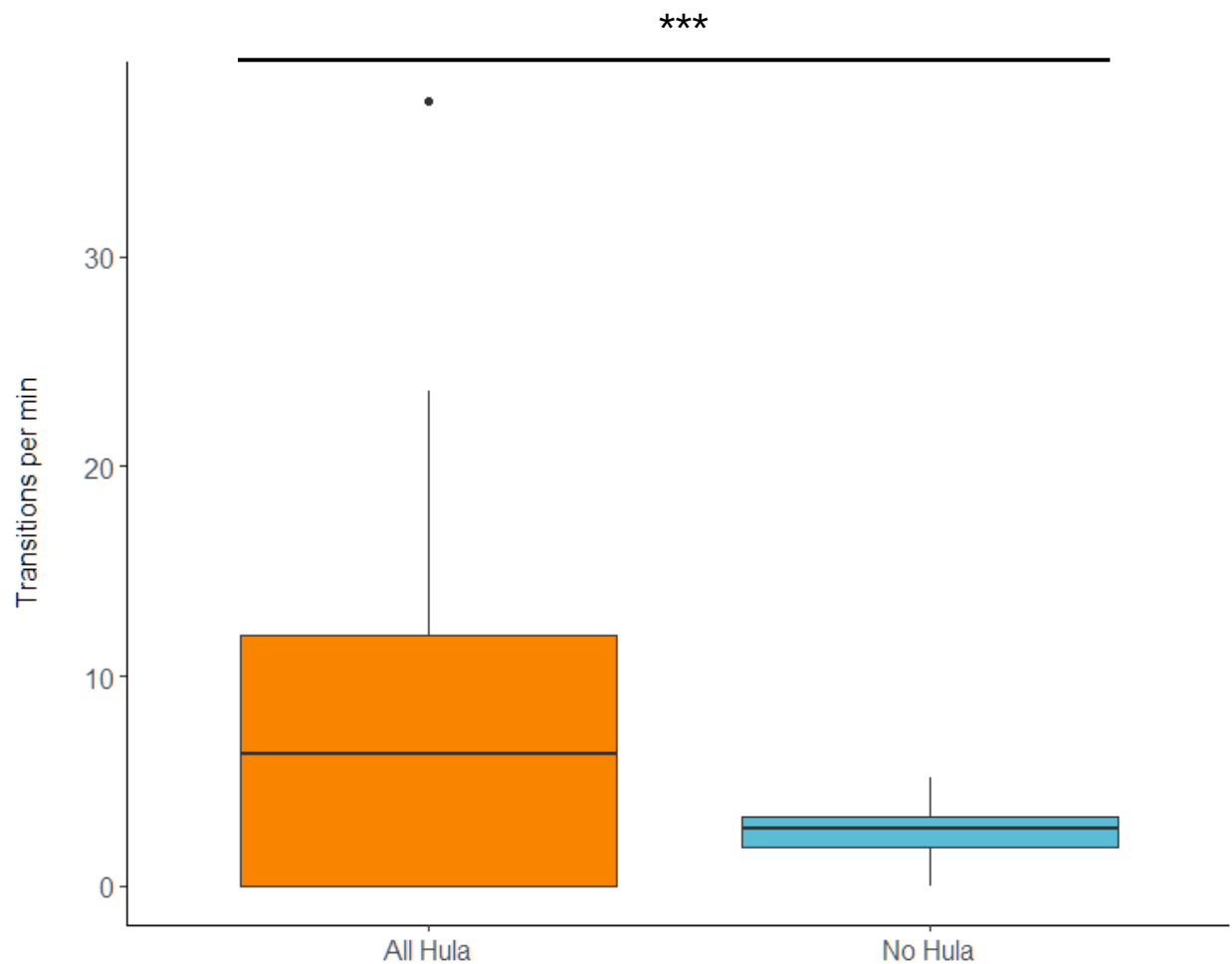


Figure 1.3: Female transitions between locomotive states with the presence or absence of a hula-ing male. Female axolotls transitioned between walking, swimming, and resting more often when the male was hula-ing versus when he was not hula-ing ($p = 0.00128$). Each box and whisker plot represents the minimum, the lower quartile, the median, the upper quartile, and the maximum value; dots represent outlier values and zeroes have been removed.

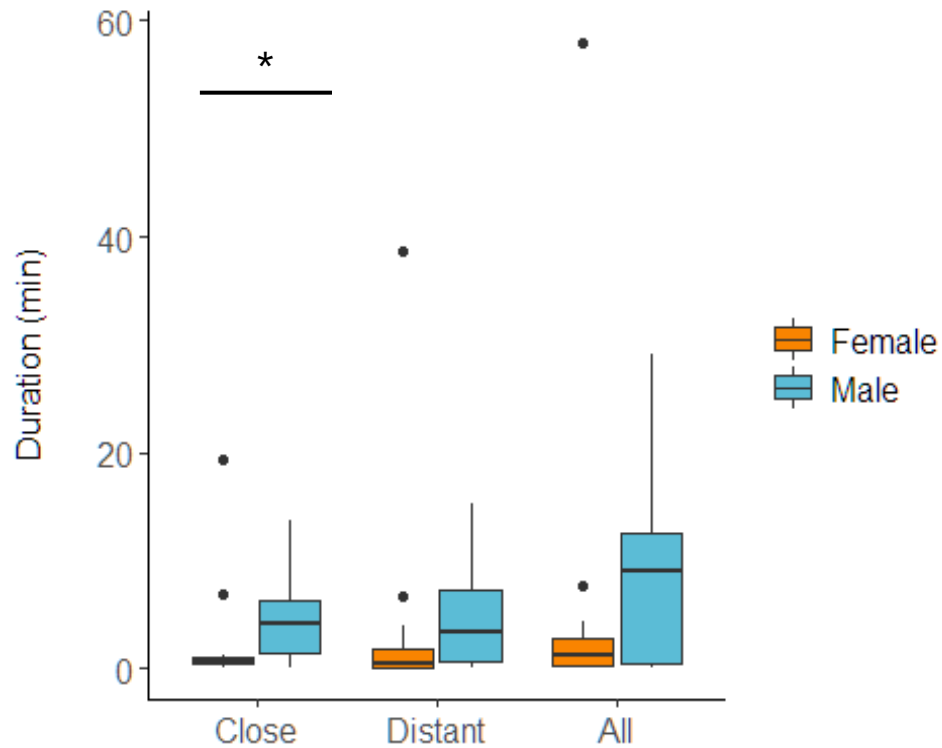


Figure 1.4: Total duration of male versus female hula behavior. Male axolotls performed “close” hula-ing longer than did females ($p = 0.02$). Each box and whisker plot represents the minimum, the lower quartile, the median, the upper quartile, and the maximum value; dots represent outlier values and zeroes have been removed.

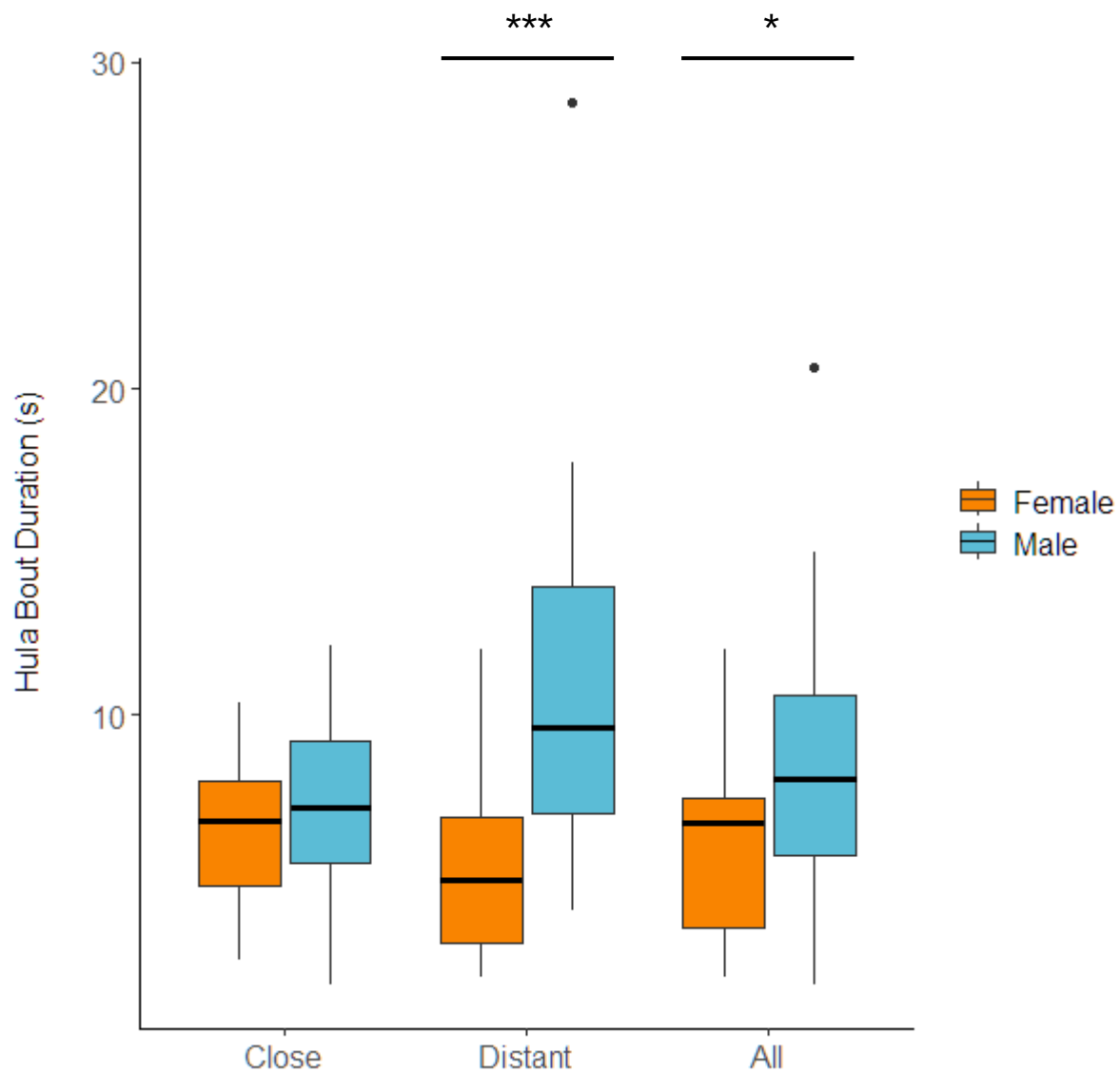


Figure 1.5: Average bout duration of male versus female hula behavior. Male axolotls exhibited significantly longer bouts of distant hula-ing than females did ($p = 0.0008$); the same was true when we pooled all bouts of hula-ing ($p = 0.02$). Each box and whisker plot represents the minimum, the lower quartile, the median, the upper quartile, and the maximum value; dots represent outlier values and zeroes have been removed.

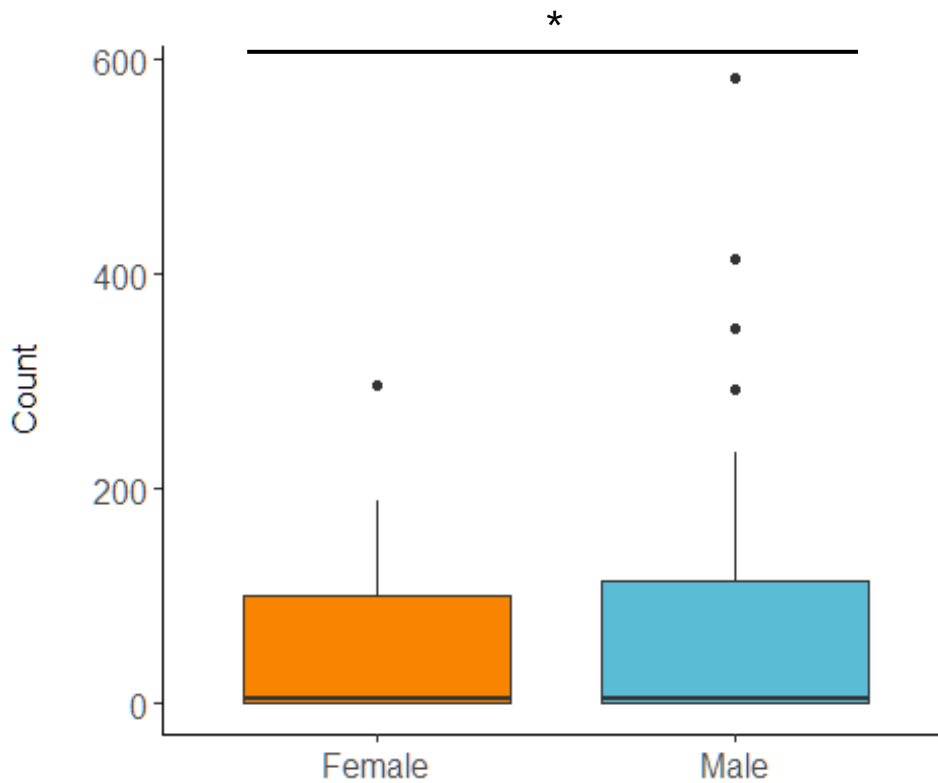


Figure 1.6: Male versus female bumping behaviors. Male axolotls performed the bump behavior more frequently than did females ($p = 0.02$). Each box and whisker plot represents the minimum, the lower quartile, the median, the upper quartile, and the maximum value for each data set; dots represent outliers.

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CHAPTER 2: Behavioral Responses of Female Axolotls to the Male Hula Behavior

ABSTRACT

Hydrodynamic communication signals are important for aquatic vertebrates to coordinate complex schooling behaviors, mediate aggressive encounters, and synchronize mating. Some fully aquatic salamanders, including the axolotl, *Ambystoma mexicanum*, create hydrodynamic stimuli by performing a rhythmic wriggling or fanning motion of the tail. Unlike amphibians that metamorphose into terrestrial adults, axolotls maintain functionality of the lateral line systems throughout their lives. The lateral line system detects electrical and mechanical stimuli in aquatic environments, and tail movements can be detected readily by the lateral line. During courtship, both male and female axolotls exhibit a behavior known as the “hula”, which involves a rhythmic undulation of the tail combined with a swaying motion of the pelvic region. We investigated the potential communicative role of the hydrodynamic stimuli generated during the male hula behavior and response of the female to these signals.

We first characterized the range of speeds and angles that males can perform with their tails (sweep angle, speed, and elevation angle), and investigated females' behavioral responses to particular motion parameters at both close proximity and at a distance. We then designed a robotic device (the “Robotail”) to mimic the hula, which we programmed to produce speeds and angles that reflect 27 potential combinations of these parameters that reflect both normal behaviors and abnormal male behaviors that males were unlikely to perform or were never observed naturally.

We found that when males were in close proximity to females, males performed predominantly moderate combinations that represented a wider sweep angle ($\sim 20^\circ$ to 93°), slightly slower speed (~ 0.25 Hz to 1.4 Hz), and higher elevation angle ($\sim 15^\circ$ to 75°) compared to when they were hula-ing at a distance from a female. When we examined the behavioral effects of single hula parameters, we found that female axolotls responded to the Robotail such that wider sweep angles (90°) and faster speeds (1.5 Hz) caused females to exhibit shorter bouts of walking, and wide to moderate sweep angles (30° to 90°) caused females to exhibit shorter bouts of swimming. Concordantly, females transitioned between locomotive states (walking, swimming, pausing) more often when we stimulated them with sweep angle/speed combinations of $90^\circ/1.0$ Hz, $30^\circ/1.5$ Hz, or $90^\circ/1.5$ Hz. Additionally, faster speeds (1.5 Hz) caused females to spend more time near the tail overall. Importantly, we found that while males generally performed moderate behaviors, females responded to more extreme behaviors from the Robotail. These results suggest that females would respond strongly to real males that exhibit very fast or wide hula patterns; perhaps males are physically limited in their ability to perform these extreme behaviors.

Our experiments serve to describe how males generate hydrodynamic stimuli during courtship and how females behaviorally respond to a range of hula motion parameters. This research represents an important first step in understanding whether the lateral line system is physiologically tuned to hydrodynamic signals generated during courtship.

INTRODUCTION

The roles of auditory (Baker et al., 2020; Woolley & Moore, 2011) and visual signals (Osorio & Vorobyev, 2008) in animal communication are well established; however, a comparatively small amount of research exists on the communicative function of vibrational signals. Although vibrational communication has been documented in insects like katydids (De Luca & Morris, 1998) and spiders (Joel et al., 2017) as well as terrestrial vertebrates such as elephants (O’Connell-Rodwell, 2024) and mole rats (Hrouzková et al., 2013), an even smaller body of literature exists on vibrational communication in aquatic systems. Hydrodynamic stimuli (i.e., water disturbances) are known to play an important communicative role in the lives of aquatic animals. In particular, some animals produce body movements to direct hydrodynamic stimuli towards conspecifics during social interactions, such as aggression (Butler & Maruska, 2015) and mating (Butler & Maruska, 2016). For example, the body movements of some teleost fishes generate vibrational stimuli that facilitate schooling behaviors (Coombs & Montgomery, 2014) as well as coordinate mating efforts between males and females (Satou et al., 1994). Aquatic invertebrates such as male water mites, *Neumania papillator*, are also known to wave their front appendages near females during courtship to attract them (Proctor, 1991).

Many male salamanders perform a courtship behavior that involves a repetitive, undulating motion of the tail; variations of this include the “tail fan” in which just the tip of the tail is moved, as seen in the great crested newt, *Triturus cristatus* (Green, 1989) as well as “pelvic wagging” exhibited by the mole salamander, *Ambystoma talpoideum*, in which the male sways the hips and tail simultaneously (Shoop, 1960). These types of

salamander behaviors may serve to stimulate the lateral line system of conspecifics; the lateral line is a sensory system that can detect both electrical (Baker et al., 2013) and mechanical stimuli (Coombs et al., 1989) in aquatic environments. Park et al. (2008) demonstrated that male Korean salamanders (*Hynobius leechii*) detect vibrational stimuli via their lateral line systems by testing salamanders' behavioral responses to a model that mimics the tail motions of rival males in courtship settings. Males oriented toward and approached the model less often when vibrations were blocked with an acrylic barrier or when the lateral line was chemically blocked with cobalt chloride. Here, we aim to further elucidate the role of hydrodynamic stimuli in salamander courtship by studying the behavioral responses of the female axolotl, *Ambystoma mexicanum*, to a variety of stimuli produced by males.

We chose to use the fully aquatic axolotl in our experiments for several reasons: axolotls breed year-round in laboratory settings (Voss et al., 2015) and males reliably perform a behavior known as the “hula” throughout their courtship ritual, which involves a swaying motion of the hips combined with an undulating motion of the tail (Eisthen, 1989). Although hula-ing in axolotls is similar to the pelvic wagging behavior in *A. talpoideum* (Shoop, 1960), this type of behavior in axolotls has customarily been called the “hula” (Park et al., 2004). Additionally, given their neotenic life cycle, axolotls maintain their lateral line systems throughout their entire lives, whereas salamanders that undergo metamorphosis tend to lose their lateral line functionality upon the transition to terrestrial life (Fritzsche & Wahnschaffe, 1983). Here, we designed two experiments to investigate the potential mechanosensory role of the hula behavior during axolotl courtship. Specifically, we aimed to characterize the typical range of

motions and speeds that males can perform with their tails during the hula and assess female behavioral responses to various combinations of hula motion parameters.

We first defined three natural motion parameters of the male's tail during the hula behavior, which we called the "sweep angle" (the degree of side-to-side motion), the "speed" of undulation (measured in Hz), and the "elevation angle"; males periodically raise and lower their tails during courtship, so we described this axis of motion by measuring the angle between the substrate and the male's tail. Video recordings of male and female courtship interactions helped to establish the range of natural movements and behaviors (Chapter 1). Once we established common motion patterns we assessed female behavioral responses to a range of hula motion parameters using the "Robotail", a robotic device we created. We designed the Robotail to mimic the undulating tail motion of the male hula behavior; importantly, this device allowed us to test the effects of specific parameters on female behavior in a controlled and repeatable fashion.

We tested three values from each of the three motion parameters for our experiment, which represented the minimum, median, and maximum values that males produced with their tails during courtship; we then evaluated all 27 potential combinations of these parameters. Some of these motion combinations were naturalistic (i.e., male axolotls perform them) whereas others were contrived (i.e., males were unlikely to perform them). Our research will allow us to understand how the individual parameters of the hula influence female behavior. Importantly, these experiments serve as a framework to support further studies to understand if the lateral

line system is physiologically tuned to detect the hydrodynamic stimuli generated by conspecifics in a courtship context.

MATERIALS AND METHODS

Subjects

Adult axolotls (*Ambystoma mexicanum*) were obtained from the Ambystoma Genetic Stock Center at the University of Kentucky. Animals were housed in ~114 L aquaria in 40-100% Holtfreter's (HF) solution (Armstrong et al., 1989) supplemented with Replenish™ solution (Seachem Laboratories, Madison, GA) at temperatures between 18 and 22°C; animals were separated by sex into aquaria containing 1-3 animals each. We programmed the lights in our facility to match the natural sunrise and sunset of Mexico City, Mexico (the native habitat of axolotls) with monthly updates to the axolotls' photoperiod. Housing and experiments were conducted with the approval of the Institutional Animal Care and Use Committee of Michigan State University (approval numbers: 10/15/-154-00, PROTO201800106).

Hula motion parameters

To assess the range of tail motions that male axolotls exhibited while hula-ing, we recorded behavioral interactions between 30 pairings of male and female axolotls (N = 10 males, 19 females); each male was paired with 3 different females. For each trial, we placed a pair together into a ~114 L aquarium (90 cm long x 45 cm wide x 30 cm wide) filled with HF solution at the same concentration as their home aquaria shortly before the lights in our animal facility turned off for the evening; we then allowed the pair

to interact overnight because axolotls are nocturnally active. We used 2 Sony Nightshot camcorders (model: CMOS) to record top-down and side views of each trial.

We analyzed hula motion parameters for 6 different males; 2 spawned with females and the other 4 were selected at random. For each male, we only analyzed trials that contained at least one bout of “close” hula-ing that was at least 2 min long and/or a bout of “distant” hula-ing that was at least 30 sec long; thus, we included 3 trials for 1 male, 2 trials for 3 males, and a single trial for the remaining 2 males, resulting in 11 trials total. We defined close hula-ing as occurring within one male tail-length of the female and distant hula-ing as occurring at least one male tail-length away from the female. For each trial, we analyzed 2 hrs of footage starting when the lights in our animal facility turned off for the evening.

We first used BORIS (Behavioral Observation Research Interactive Software; version 7.9.RC1; Friard & Gamba, 2016) to catalog the frequency and duration of all hula bouts, which we trimmed to 2 min for close bouts and 30 sec for distant bouts. We used a modified version Altmann's (1974) “instantaneous sampling” method to analyze the males’ tail movements within each bout. For close hula bouts, we divided each bout into 4 30-sec windows and took measurements for the first 10 sec of each window; for distant bouts, we analyzed 2 15-sec windows by measuring the first 5 sec of each one. We used Kinovea software version 0.8.27 (www.kinovea.org) to measure the angle of the male’s tail as it moved from side to side (sweep angle), the frequency (in Hz) of tail movement, and the elevation angle of the tail.

We measured tail sweep angles and frequencies in tandem from the overhead perspective by watching video footage frame-by-frame and measuring the lateral angle

whenever the proximal third of the tail changed direction. We recorded the maximum and minimum sweep angle for each measurement period (i.e. 10 sec for close bouts, 5 sec for distant bouts), discarding any measurements less than 5° because the resolution of our videos footage made it difficult to accurately discern smaller lateral motions. The frequency of tail motion was calculated by dividing the total number of angle measurements by the duration of the measurement period. Elevation angles were measured from the side view by measuring the ventral edge of the male's tail relative to the aquarium floor. We obtained a baseline measurement for the elevation angle 30 sec before any bout of hula-ing began and then recorded the maximum and minimum elevation angles during each measurement period.

Construction of a hula-mimicking robot

To assess the behavioral effects of specific motion parameters on females in a controlled and repeatable manner, we constructed a robotic tail, the “Robotail”, that mimics male hula behaviors. The Robotail consisted of a silicone “tail”, 3D printed components to mount the silicone tail on the floor of an aquarium, and a suite of electronics to program and drive the tail's motions. We ensured that all components within the aquarium were made of non-metallic materials to 1) prevent metal ions from leaching into the HF solution, which can be harmful to salamanders (Bazar et al., 2009) and 2) prevent the generation of electrical cues and noise in the test arena, as the lateral line system of axolotls can detect electrical stimuli (Münz, Claas, & Fritzs, 1984).

To create the silicone tail, we measured the tail dimensions of 5 adult male axolotls by anaesthetizing each male in a solution of 0.1% pH-corrected MS-222; we

measured the smallest and largest male in our colony (based on weight) and randomly chose an additional 3 males. The total length of the male's tail (posterior edge of cloacal gland to tail tip) was divided into 5 equal segments; at each landmark, we measured the width and height of the dorsal and ventral fins, the width at the middle of the tail, and the total height (Fig. 2.1). We used the median value of each measurement to generate a "representative" tail, which we modeled, along with a 2-part mold, in SolidWorks® software (version: SolidWorks 2016; Dassault Systèmes, Vélizy-Villacoublay, France). We made the silicone tail from Smooth-On Ecoflex™ 00-30 (Smooth-On, Inc., Macungie, Pennsylvania) using a 3D print of our custom mold from Shapeways (Livonia, Michigan); during the casting process, we embedded a custom 3D printed clip in the proximal end of the silicone tail to attach the tail to the mounting components (Fig. 2.2A).

The mounting components consisted of a 3D printed triangular base plate and a pivoting cylinder with a slot to hold the tail in place (Fig. 2.2); the triangular base was mounted to the aquarium floor using waterproof silicone. We also sourced a silicone salamander toy resembling an ambystomatid salamander and removed the tail; we mounted the remainder of the toy in front of the base plate to resemble the shape of a male axolotl (Fig. 2.3). Finally, we attached EVA foam tiles to the aquarium floor to make the floor flush with the mounting plate and rubber salamander. A length of braided nylon fishing line was used to connect the pivoting cylinder to the gimbal, which was outside of the aquarium, without the use of metal (Fig. 2.2). When the Robotail was submerged in water, the pivoting motion of the cylinder transferred an undulating motion to the silicone tail, simulating the hula behavior.

We used a series of electronic components to drive and program the oscillating motion of the silicone tail, including a power distribution channel, Victor SP speed controller, 104:1 NeveRest Sport gearbox, NeveRest gearmotor, and Thrifty Throttle 3/Cypress PSoC4™ microcontroller from AndyMark Inc. (Kokomo, IN) as well as a PSoC4™ MiniProg3 programmer (Infineon Technologies AG, Munich, Germany) and a potentiometer (DigiKey, Thief River Falls, MN). Additionally, we created a metal gimbal to control the lateral position of the tail. The electronics and gimbal were mounted to an acrylic sheet attached to a wooden frame elevated approximately 60 cm above the aquarium floor (Fig. 2.4). We wrote custom software in C (Kernighan & Ritchie, 2002) using PSoC™ Creator (Infineon Technologies AG, Munich, Germany) to control the speed and sweep angle of tail oscillation. The elevation angle of the silicone tail was changed manually.

Female behavioral responses to the Robotail

We determined the parameters for our behavioral tests by simplifying each of the 3 continuous variables of hula motions (sweep angle, hula speed, and elevation angle) into 3 discrete categories, which were based on the minimum, median, and maximum values of each parameter (Table 2.1); thus, we condensed our data set into 27 different combinations of parameters. We then created a series of triple-axis matrices to assess the occurrence rate of each motion combination; we discarded any hula bouts in which we were unable to measure all 3 parameters and then counted which combinations occurred “often” (occurred more often than the median value), “sometimes” (occurred less often than the median value), or “never” (not occurring). Importantly, the matrices allowed us to determine which combinations were natural (i.e., those that male axolotls

perform) and which were contrived (i.e., those that males were unlikely or unable to exhibit).

We programmed the Robotail to perform each of the 27 motion combinations to assess female behavioral responses to both natural and contrived hula motion patterns; thus, we tested sweep angles of 10°, 30°, and 90°, speeds of 0.5 Hz, 1.0 Hz, and 1.5 Hz, and elevation angles of 0°, 20°, and 55°. In total, we staged 162 encounters with the Robotail by testing each motion combination with 6 different females. All trials were conducted after the lights in our animal facility turned off for the evening; between trials, we wore headlamps with red lights to minimize disrupting our subjects. All trials were recorded from an overhead angle using a Lorex Night Vision Security Camera system (model: HDIP82W). We placed a female in the test aquarium (filled with HF at the same concentration as their home aquarium) with the robot and began each trial with a 5 min acclimation period, after which we turned the robot on and allowed the female to interact with it for 15 min. We quantified behavioral responses to the robotic axolotl tail in BORIS by measuring locomotor behaviors (walk, swim, pause), hula behaviors, time spent near the robot (i.e., within a circular area around the tail, with the tail's length being equal to its radius) orientation behaviors, physical contacts with the robot (nudge base, nudge tip), as well as aggressive behaviors (nip, bite, attack). Full definitions for all behaviors are provided in Table 2.2.

In preliminary trials, female axolotls attacked the robot unless male odorants were present in the aquarium. In the absence of male odorants, it is possible that the Robotail resembled prey more than a male conspecific; thus we included male odorants throughout the experiment. We collected whole-body odorants from male axolotls by

placing 3 males into plastic bowls each filled with 1 L of HF roughly 24 hr before the start of a trial. The following day, we combined the HF solution from all 3 buckets into a single solution to minimize effects of individual variation. Odorants were delivered into the aquarium through a silicone tube mounted near the base of the robot; a peristaltic pump delivered odorant solution at a rate of approximately 33 mL/min while the robot was on. We replaced the HF solution in the aquarium between trials to remove male odorants as well as any odorants that the female test subject may have released.

Statistical analyses

We analyzed female behavioral responses to the Robotail (locomotor behaviors, physical contacts with the Robotail, time spent near the robot, and aggressive behaviors) using general linear mixed-effects models in R (R Core Team, 2020); we utilized the packages "lme4" (version 1.1.35.3; Bates et al., 2015) and "lmerTest" (version 3.1.3; Kuznetsova et al., 2017). The three motion hula parameters (sweep angle, speed, and elevation, angle) were treated as fixed effects, with female ID as a random effect because females were repeated in the dataset an unequal number of times. Analyses were run on models alone and in combination. We set the restricted maximum likelihood (REML) to false because we compared models with different fixed effects (Bolker, 2015). Zeros were removed for total durations and average bout durations (i.e., total duration divided by count) but retained for count variables. Additionally, we compared models of increasing complexity by calculating corrected Akaike information criterion (AICc) values using the R package "glmulti" (version 1.0.8). Models with a delta AICc of 3 or less were considered equally weighted models, and among weighted models we considered the simplest model to be the best fitting model.

RESULTS

Hula motion parameters

Male axolotls performed the hula behavior using moderate sweep and elevation angles and speeds and rarely moved their tails at the upper and lower extremes of their motion range. Among close hula combinations, males sometimes used moderate (30°) to wide (90°) sweep angles, moderate speeds (1.0 Hz), and moderate elevation angles (20°). Additionally, we found that males performed two close hula combinations the most often: 30° sweep/1.0 or 1.5 Hz/20° elevation (Table 2.3A). In contrast, distant hula bouts that fell into the “sometimes” category generally featured narrow to moderate (10°-30°) sweep angles, moderate speeds (1.0 Hz), and low to moderate (0°-20°) elevation angles. Males performed 4 combinations of distant hula motions most often; 10° sweep/1.5 Hz/0° elevation, 30° sweep/1.0 Hz/0° elevation, 30° sweep/1.5 Hz/0° elevation, and 30° sweep/1.0 Hz/20° elevation (Table 2.3B). Thus, the motion patterns of close hula bouts were typically wider and higher than distant hula bouts, but males tended to hula with moderate speeds regardless of their proximity to a female.

When we pooled all hula bouts (close plus distant) and examined the combinations that occurred sometimes, we found that males tended to perform all sweep angles, but only moderate speeds (1.0 Hz) and low to moderate (0°-20°) elevation angles. We additionally found 3 motion combinations that males performed most often overall; 30° sweep/1.0 Hz/0° or 20° elevation and 30° sweep/1.5 Hz/20° elevation (Table 2.3C). Six of the hula motion combinations we tested in our experiment were never performed by males, including 1.5 Hz/55° elevation at any sweep angle (10°, 30°, or 90°). Additionally, males did not exhibit 0.5 Hz/55° elevation at the

extremes of their sweep angle range (10° or 90°), nor did they move their tails with a combination of 90° sweep/0.5 Hz/0° elevation. Overall, males did not lift their tails high if they were moving at the extremes of their speed range (0.5 or 1.5 Hz) or lower their tails if they were moving slowly and widely.

Female behavioral responses to the Robotail

Locomotor behaviors

We found that female axolotls initiated and terminated bouts of locomotion more often when the Robotail was oscillating quickly or at a wide angle. Females walked for significantly shorter bouts of time as the sweep angle increased, with a sweep angle of 90° being significantly different from 10° ($t = -2.91$, $p = 0.004$; Fig. 2.5). Females also walked for shorter bouts of time when the tail was moving at its fastest speed of 1.5 Hz compared to the slowest speed of 0.5Hz ($t = -2.27$, $p = 0.03$; Fig. 2.6). Females exhibited shorter bouts of swimming as the sweep angle increased (10° vs 30°; $t = -2.21$, $p = 0.03$; 10° vs 90°; $t = -2.21$, $p = 0.03$; Fig. 2.7) and they also exhibited shorter bouts of locomotion (walking + swimming) as the sweep angle increased (10° vs 90°; $t = -2.15$, $p = 0.03$; Table 2.4; Fig. 2.8). The Robotail parameters we tested did not affect the total durations of time that female axolotls spent performing walking, swimming, and pausing behaviors.

The sweep angle of the Robotail best explained the duration of bouts of walking and swimming; however, the duration of pausing bouts was not explained by a single best model. Additionally, the overall amounts of time that females spent walking, swimming, and pausing were not explained by a single best model. Every variable of total locomotion duration had multiple, equally weighted models (i.e., within 3 AICc units

of the top ranked model); in all cases, the equally weighted models included a single parameter (sweep angle, speed, or elevation angle), so parsimony could not be used to select a best model (Table S2.2).

Time spent near the Robotail

We found that females spent significantly more time overall near the Robotail when it was moving at its fastest speed of 1.5 Hz ($t = 2.63$, $p = 0.009$; Fig. 2.9). In contrast, females spent less time per visit to the tail when it was moving at its widest angle of 90° ($t = -2.20$, $p = 0.03$; Fig. 2.10). Additionally, we found a marginal negative effect of elevation angle on the number of times that a female oriented toward the tail (0° vs 20° ; $t = -1.92$, $p = 0.06$; 0° vs 55° ; $t = -1.92$, $p = 0.06$; Table 2.5).

The statistical model that best described the total amount of time female axolotls spent near the tail included the speed of the Robotail as the only predictor, whereas the duration of each visit to the tail was not explained by a single best model. Accordingly, the statistical model that best described orient count included elevation angle only (Table S2.3). Thus, speed played an important role in determining how long females stayed near the Robotail overall, the sweep angle impacted how long each visit to the tail lasted, and elevation angle best explained how often females directed themselves towards it.

Physical contacts with the Robotail

Female axolotls touched the tip of the Robotail with their snouts more often as the speed increased but made fewer physical contacts when the tail was moving at a moderate sweep angle or was positioned at a steep elevation angle. Females nudged

the tip of the tail significantly more often when the tail was moving at its fastest speed ($t = 2.11$, $p = 0.04$) but less often at its highest elevation ($t = -2.85$, $p = 0.005$; Table 2.6). Additionally, we found that females nudged the base of the tail marginally less often when the sweep angle was increased from 10° to 30° ($t = -1.89$, $p = 0.06$).

The number of times that females nudged the base of the robot was best described by the model with only sweep angle as a predictor, whereas the model that best explained the number of tip nudges included only the elevation angle. We found that four models for the total number of nudges (count of nudges anywhere on the Robotail) were equally weighted, and rules of parsimony did not distinguish among models (Table S2.4).

Female aggression

Females behaved less aggressively towards the Robotail as the sweep angle and elevation angles were increased. Specifically, females nipped at the tail significantly less often as the sweep angle of the Robotail increased (10° vs 30° ; $t = -3.00$, $p = 0.003$; 10° vs 90° ; $t = -2.55$, $p = 0.01$) and marginally less often when the tail was elevated to 20° ($t = -1.73$, $p = 0.09$; Table 2.7). Additionally, female axolotls attacked the tail slightly less often as the sweep angle increased (10° vs 30° ; $t = -1.77$, $p = 0.08$; 10° vs 90° ; $t = -1.77$, $p = 0.08$) and as the elevation angle increased (0° vs 20° ; $t = -1.77$, $p = 0.08$; 20° vs 55° ; $t = -1.77$, $p = 0.08$; Table 2.7).

The statistical model that best described nip count included only sweep angle, however bite count and attack count did not have a single best model; bite and attack

count both had multiple models with equal weighting and rules of parsimony did not distinguish among these models (Table S2.5).

Locomotion transitions per minute (combination analysis)

Female axolotls transitioned between locomotive states per minute more often when the Robotail performed motion combinations featuring moderate to wide sweep angles and moderate to fast speeds. Females transitioned significantly more frequently when presented with a combination of 90°/1.0 Hz ($t = 2.19$, $p = 0.03$), 30°/1.5 Hz ($t = 2.37$, $p = 0.02$), or 90°/1.5 Hz ($t = 4.01$, $p = 9.51\text{e-}05$; Table 2.8).

DISCUSSION

Hula motion parameters

Although male axolotls are capable of performing the hula behavior at the extremes of their sweep angle, speed, and elevation angle ranges, we found that they generally did not hula in this fashion. Additionally, males did not combine fast hula speeds with high tail elevation angles, regardless of the sweep angle. Thus, we posit that fast speeds paired with high elevation angles may be too energetically costly for males to sustain during courtship, no matter the degree of side-to-side motion. Additionally, the males in our study only combined slow speeds with high elevation angles if the sweep angle was moderate (30°), but not if the sweep angle was very narrow or very wide, suggesting that males may have been more likely to perform a motion combination if at least one of the parameters had a moderate value. Lastly, we never observed males performing a motion pattern of 90° sweep combined with 0.5 Hz

and 0° elevation, suggesting that males may not be able to achieve their widest sweep angle at the slowest speed and lowest elevation angle.

Similarly to axolotls, male Korean salamanders (*Hynobius leechii*) generate vibrational signals with their bodies and tails to communicate with both males and females during mating. Kim et al. (2009) demonstrated that *H. leechii* males exhibit two types of vibration behaviors during mating: body undulations, which involve a side-to-side motion of the tail and the posterior region, and tail undulations, which feature a waving motion of only the distal third of the tail. *H. leechii* males performed body undulations at an average speed of 0.64 Hz to communicate with both females and other males; in contrast, tail undulations (performed on average at 1.7 Hz) are typically only displayed during aggressive interactions between males. Although male axolotls are known to court other males and deposit spermatophores when housed in all-male aquaria (Gresens, 2004), the function of these behaviors is currently unknown. It is possible that male axolotls perform slower hula motions in the presence of a female they are attempting to court and reserve faster tail motions for communicating with other males.

Close hula bouts featured somewhat wider sweep angles and higher elevation angles than distant hula bouts, but males tended to hula with a moderate speed of 1.0 Hz regardless of their proximity to a female (Table 2.3). Moderate vibration speeds play an important communicative role in other aquatic species as well. In landlocked red salmon, (*Oncorhynchus nerka*) moderate whole-body vibration speeds generated by a female-like model induced significantly greater numbers of spawning behaviors (approach and spawning acts) in males compared with extreme speeds (Satou et al.,

1994). However, lower frequencies resulted in male salmon performing greater numbers of courtship behaviors, suggesting that an increase in the female's vibration speed may indicate a transition from courtship to spawning. Although we did not test how male hula parameters directly related to the preliminary and mating phases (see Chapter 1) of axolotl pairs, we did find that males were slightly more likely to hula at slow speeds of 0.5 Hz if the female was in close proximity. Thus, males may modulate their hula speeds depending on the which phase of courtship they are in, and this idea warrants further investigation. Overall, we found that male axolotls generally hula'd with moderate angles and speeds and tended to modulate their sweep and elevation angles, but not the speed of tail motions, depending on their proximity to a female. The fact that males mainly performed moderate behaviors either suggests that males are unable to perform the extreme behaviors, or that it is costly; alternatively, this may suggest that females do not respond (or respond negatively) to extreme behaviors and therefore there is no reason for males at the extremes of their motion ranges. To evaluate these hypotheses, we used the Robotail to test female response to single hula parameters as well as combinations of sweep angles and speeds; we excluded elevation angle from our combination analyses because females did not exhibit meaningful responses to changes in this parameter.

Female behavioral responses to the Robotail

Locomotor behaviors

Female axolotls modulated their locomotor behaviors in response to changes in the sweep angle and speed of the Robotail. Specifically, females altered the rate at which they started and stopped locomoting, but they did not change the overall amounts

of time that they spent walking, swimming, and pausing. Females exhibited shorter bouts of walking and swimming as the sweep angle widened to 90° (Fig. 2.5, Fig. 2.7) and exhibited shorter bouts of walking as the speed increased to 1.5 Hz (Fig. 2.6). Accordingly, when we examined female responses to combinations of sweep angles and speed, we found that females transitioned between locomotive states more frequently if the sweep angle was moderate to wider and the speed was moderate to fast (Table 2.8).

The average duration of time spent walking and time spent swimming in each bout were both explained best by the model that included only the sweep angle parameter. However, the total amount of time spent in all locomotion categories (walking, swimming, and combined) all had multiple models that were equally weighted (i.e., within 3 AICc units of the top ranked model) and were not nested models (Table S2.2). Thus, the sweep angle and the speed of the Robotail influenced the duration on individual bouts of walking and swimming, but the overall amounts of time that females spent locomoting were not influenced by any single parameter.

The male axolotls in our study were slightly more likely to hula at 1.5 Hz when they were at a distance from a female, but more likely to exhibit a 90° sweep angle when they were in close proximity. We found that female axolotls in a courtship setting paused significantly more often and for longer periods of time when they were closer to a hula-ing male (see Chapter 1). Interestingly, in our experiments with the Robotail, females responded to both fast speeds and wide sweep angles by starting and stopping locomotion bouts more frequently. We recognize that, in a courtship setting, males and females can move independently of one another, whereas in our current experiment the

Robotail was stationary and only the female was free to move around the aquarium. Thus, a faster Robotail speed may not have been indicative of a distant male, just like a wider sweep angle may not have suggested the presence of a nearby male. We speculate that these behaviors may have represented an increased search effort to localize the source of hydrodynamic stimuli, given that females spent more time near the Robotail when it was moving at 1.5 Hz.

Time spent near the Robotail

Female axolotls spent less time per visit to the tail when it was moving at a wider angle (Fig. 2.10) but spent more time near the Robotail overall when it was moving at a faster pace (Fig. 2.9). Additionally, females tended to avoid orienting towards the tail when it was raised at higher elevation angles. Wider sweep angles occupy more physical space in the designated “near” zone around the Robotail than narrower angles, which may have made it difficult for females to enter or stay in the zone for extended periods of time. Accordingly, the statistical model that best explained the total duration of time spent near included only speed. However, the average duration of time spent near had multiple models that were equally weighted (i.e., within 3 AICc units of the top ranked model; Table S2.3), suggesting that while speed encouraged more time spent in close proximity, the time spent for each visit near the robotail was not influenced by any particular set of variables that we tested (Table S2.3).

The male axolotls in our experiment were slightly more likely to hula at a speed of 1.5 Hz when they were at a distance from a female, suggesting that a faster undulation speed may serve to draw females towards a courting male. Although research on female preferences for the speed of hydrodynamic cues is limited, some

female insects are known to prefer faster rates of substrate-borne vibration signals during courtship. For example, female meadow katydids (*Conocephalus nigropleurum*) prefer male tremulation signals with shorter inter-pulse intervals (i.e. a faster rate of tremulation) because shorter intervals correspond to larger males; large males typically produce larger spermatophores, which help to increase female fecundity (De Luca & Morris, 1998). Thus, faster hula speed could potentially be indicative of a higher quality potential mate.

In contrast, a moderate or high elevation angle had a repelling effect; female axolotls oriented toward the tail marginally less often when the tail was raised to 20° or 55°. Correspondingly, the statistical model that best explained orient count included elevation angle as the only predictor (Table S2.3). Although the male axolotls in our study did not commonly raise their tails to the highest elevation angle, we still observed males holding their tails aloft during courtship, particularly during process of spermatophore transfer. However, females may not have equated a high elevation angle with a courting male, given that many of the factors that contribute to mating, such as courtship-specific pheromones (Maex et al., 2016) and tactile feedback in the form of the “male bump” behavior (see Chapter 1), were absent from our experiment. Thus, moderate or high elevation angles may have repelled the females in our study because the Robotail’s undulations may have been too dissimilar to an actual male while performing these elevation angles.

Physical contacts with the Robotail

Female axolotls were more likely to nudge the robot’s tail tip if it was moving at a fast speed, but less likely to make physical contact in general if it was positioned at a

high elevation angle or moving at a moderate sweep angle. Importantly, we did not differentiate physical contacts (nudge tip or nudge base) that were initiated by the female from occurrences in which the robot hit the female in the snout; thus, the relationship between speed and tip nudges could be a simple consequence of a faster rate of motion leading to more opportunities for the Robotail to collide with the female's snout. In contrast, females nudged the tip of the tail significantly less often when the tail was raised to its highest elevation angle of 55°; appropriately, the statistical model that best explained the number of tip nudges only included elevation angle (Table S2.4). At its highest elevation angle, the tip of the silicone tail can only be accessed by females while they are swimming, meaning that tip nudges at this elevation may have been coincidental. Thus, it seems likely that the physical contacts that we observed between the female's snout and the tail tip happened accidentally.

Females nudged the base of the tail marginally less often when the tail was moving at a moderate sweep angle of 30°. Accordingly, the statistical model that best described the number of base nudges included only sweep angle as a predictor (Table S2.4). The “nudge base” behavior is important for coordinating spermatophore transfer between male and female ambystomatid salamanders (Shoop, 1960), yet none of the hula parameters that we tested significantly influenced the number of times that females nudged the base of the Robotail with their snouts. This is likely a result of the fact that our experimental design omitted the tactile interactions that are necessary for successful courtship and mating, such as “pushing” and “male bump” behaviors (see Chapter 1). Additionally, although we delivered male whole-body odorants near the base of the robotail, which was intended to mimic odorant secretions from the cloacal

region (Maex et al., 2016), females did not perform the nudge base behavior to the same degree that they might in an actual courtship encounter. Given that we designed the Robotail to only deliver hydrodynamic stimuli and male whole-body odorants (rather than courtship-specific pheromones), females axolotls in our study were not provided with all the appropriate sensory stimuli to perform their entire suite of courtship behaviors.

Female aggression

Female axolotls nipped at the Robotail significantly less often as the sweep angle widened and attacked the tail marginally less often as the sweep and the elevation angles increased. The statistical model that best explained nip count included only sweep angle, whereas bite and attack count had multiple equally weighted models (Table S2.5). A moderate or wide sweep angle may have discouraged female axolotls from nipping or attacking the tail simply because the tail should have been easier for the female to secure in her mouth while it was moving at a narrower angle. Similarly to the “nudge tip” behavior, when the tail was raised to a moderate or high elevation angle it could only be accessed by a female while she was swimming; perhaps females are not physically able to coordinate aggressive behaviors during bouts of swimming. Thus, a narrower sweep angle and a lower elevation angle may have led females to behave more aggressively toward the tail simply because these parameters would have made the tail more accessible to the female’s mouth.

We found that the adding a solution of male whole-body odorants to the aquarium during our experiments drastically reduced the severity of aggression that female axolotls exhibited, compared to our pilot trials in which no male odorants were

present. An experiment conducted by Proctor (1991) demonstrated a similar phenomenon in the courtship ritual of the water mite, *Neumania papillator*. Male water mites vibrate their first and second pairs of legs during courtship (called “trembling”) when they approach a female. Females often respond to trembling with prey-seeking behaviors unless the male has deposited a spermatophore, which may be embedded with pheromones; at this point, the female begins to respond to the male as a potential mate instead. Thus, the combination of vibrational stimuli and spermatophore odorants enables female water mites to recognize the presence of male conspecifics.

Given that the addition of male axolotl odorants did not completely eliminate nipping, biting, and attacking behaviors in our experiment, it is possible that female axolotls may have perceived the Robotail as a potential prey item while it was moving with particular motion combinations. For example, the lateral line system of the surface-feeding topminnow, *Aplocheilichthys lineatus*, is particularly sensitive to vibration frequencies between 70 and 120 Hz, which correspond to the hydrodynamic stimuli generated by flying insects that fall onto the water’s surface (Topp, 1983). Although male axolotls hula at much slower speeds (≤ 1.5 Hz), axolotls are known to strike at earthworm pieces dropped in front of the mouth (Lauder & Shaffer, 1985). Additionally, we have observed females infrequently nipping at males’ tails during actual courtship encounters, particularly when the tail glanced against the females’ snout. Thus, the amount of nipping, biting, and attacking we observed in our experiment may reflect a typical response to a moving object near the snout.

CONCLUSION

Our study demonstrated that courting male axolotls modulated their hula motions depending on female proximity, such that they exhibited wider sweep angles, slower undulations, and higher elevations when they were close to a female. Notably, wider sweep angles (performed by the Robotail) caused females to exhibit shorter bouts of walking and swimming as well as longer bouts of spending time near the Robotail, and reduced aggressive behaviors from the female. Similarly, faster speeds led females to shorten their bouts of walking and spend more time overall near the Robotail. When we examined female responses to combinations of sweep angle and speeds, we found that females transitioned between locomotive states more frequently when the Robotail was operating at moderate to wide sweep angles combined with moderate to fast speeds. Overall, our experiments demonstrated that the sweep angle and speed of male hula motions are important aspects of axolotl courtship dynamics, whereas elevation angle plays a more minor role. Importantly, we found that while male axolotls are more likely to exhibit moderate hula patterns, female axolotls exhibit stronger behavioral responses to more extreme hula parameters.

TABLES

	Raw data value	Bin
Sweep (°)	$< 30^\circ$	10°
	$\geq 30^\circ - 60^\circ \leq$	30°
	$\geq 60^\circ$	90°
Frequency (Hz)	$< 0.6 \text{ Hz}$	0.5 Hz
	$\geq 0.6 \text{ Hz} - 1.1 \text{ Hz} \leq$	1.0 Hz
	$\geq 1.1 \text{ Hz}$	1.5 Hz
Elevation (°)	$< 20^\circ$	0°
	$\geq 20^\circ - 55^\circ \leq$	20°
	$\geq 55^\circ$	55°

Table 2.1: Binning criteria for tail motion matrices. For each of the 3 tail motion parameters (sweep angle, frequency, and elevation angle), we organized raw data values into 3 bins. Bin values represent the minimum, median, and maximum angles for each parameter that male axolotls demonstrated during hula behaviors.

Behavior	Description
Walk	Female walks forward or backward at least 3 steps, with all four legs actively moving.
Swim	Female swims forward using her tail only, with legs pressed against the body.
Pause	Female is not walking or swimming.
Hula	Female sways hips and posterior legs while undulating tail.
Time Spent Near	Female is within the designated zone around the tail. Female must have at least her head (up to the gills) within the zone.
Orient	Female is within the designated zone and turns her head to face the tail.
Nudge Base	Female makes contact with the proximal half of the tail with her snout.
Nudge Tip	Female makes contact with the distal half of the tail with her snout.
Nip	Female bites the tail and immediately releases it.
Bite	Female bites the tail and briefly keeps jaws engaged.
Attack	Female bites the tail and thrashes her head side to side.

Table 2.2: Ethogram of female behaviors. We measured female behavioral responses to the robot by quantifying locomotor behaviors, instances of hula-ing, time spent near the robot, physical contact with the robot, and aggressive behaviors.

A) Close Bouts

		Sweep Angle									Occurrences	
Elevation Angle	Hz ↘	10°			30°			90°				
	0°	0.5	1.0	1.5	0.5	1.0	1.5	0.5	1.0	1.5		Never
	20°	0.5	1.0	1.5	0.5	1.0	1.5	0.5	1.0	1.5		Sometimes
	55°	0.5	1.0	1.5	0.5	1.0	1.5	0.5	1.0	1.5	Often	

B) Distant Bouts

		Sweep Angle								
Elevation Angle	Hz ↘	10°			30°			90°		
	0°	0.5	1.0	1.5	0.5	1.0	1.5	0.5	1.0	1.5
	20°	0.5	1.0	1.5	0.5	1.0	1.5	0.5	1.0	1.5
	55°	0.5	1.0	1.5	0.5	1.0	1.5	0.5	1.0	1.5

C) All Bouts

		Sweep Angle								
Elevation Angle	Hz ↘	10°			30°			90°		
	0°	0.5	1.0	1.5	0.5	1.0	1.5	0.5	1.0	1.5
	20°	0.5	1.0	1.5	0.5	1.0	1.5	0.5	1.0	1.5
	55°	0.5	1.0	1.5	0.5	1.0	1.5	0.5	1.0	1.5

Table 2.3: Matrices displaying the occurrence rate of tail motion combinations.

Each hula bout (**A**; Close, **B**; Distant, **C**; All) was binned (Table 1) and then categorized into one of the 27 motion combinations. Combination frequencies above the median value were deemed as “often”, and values below the median were labeled as “sometimes”, and values of 0 were labeled as “never”.

Dependent Variable	Model	Fixed Effects	t statistic	p - value
Walk Total Duration	Sweep	30° sweep	1.64	0.10
		90° sweep	0.81	0.42
	Speed	1.0 Hz	0.72	0.48
		1.5 Hz	0.18	0.86
	Elevation	20° elevation	0.47	0.64
		55° elevation	-1.02	0.31
Walk Average Duration	Sweep	30° sweep	-0.20	0.84
		90° sweep	-2.91	0.004 **
	Speed	1.0 Hz	-0.23	0.82
		1.5 Hz	-2.27	0.03 *
	Elevation	20° elevation	1.34	0.18
		55° elevation	0.97	0.34
Swim Total Duration	Sweep	30° sweep	-0.84	0.40
		90° sweep	0.03	0.98
	Speed	1.0 Hz	0.62	0.54
		1.5 Hz	-0.48	0.63
	Elevation	20° elevation	0.42	0.68
		55° elevation	0.80	0.43
Swim Average Duration	Sweep	30° sweep	-2.21	0.03 *
		90° sweep	-2.21	0.03 *
	Speed	1.0 Hz	0.62	0.54
		1.5 Hz	-0.78	0.44
	Elevation	20° elevation	0.31	0.76
		55° elevation	0.63	0.53

Table 2.4: General linear mixed-effects models for female locomotor data (continued on next page).

Dependent Variable	Model	Fixed Effects	t statistic	p - value
Locomote Total Duration	Sweep	30° sweep	0.58	0.56
		90° sweep	0.64	0.53
	Speed	1.0 Hz	1.22	0.22
		1.5 Hz	-0.34	0.74
	Elevation	20° elevation	0.61	0.54
		55° elevation	-0.04	0.97
Locomote Average Duration	Sweep	30° sweep	-1.58	0.12
		90° sweep	-2.15	0.03 *
	Speed	1.0 Hz	0.78	0.44
		1.5 Hz	-0.97	0.34
	Elevation	20° elevation	0.62	0.54
		55° elevation	0.88	0.38
Pause Total Duration	Sweep	30° sweep	-0.57	0.57
		90° sweep	-0.61	0.54
	Speed	1.0 Hz	-1.14	0.26
		1.5 Hz	0.38	0.71
	Elevation	20° elevation	-0.52	0.60
		55° elevation	0.09	0.93
Pause Average Duration	Sweep	30° sweep	-0.70	0.49
		90° sweep	-1.39	0.17
	Speed	1.0 Hz	-1.47	0.15
		1.5 Hz	-1.79	0.08
	Elevation	20° elevation	0.76	0.45
		55° elevation	0.57	0.57

Table 2.4 (continued): Changes in female locomotor behaviors in response to the 3 levels of each hula motion parameter. Average durations of walk, swim, and locomote bouts differed significantly with sweep angle, speed, or both. Female ID number was used as the random effect in all analyses; all t statistics and p-values were rounded to 2 decimals, except when the p-value was less than 0.01.

Dependent Variable	Model	Fixed Effects	t statistic	p - value
Time Spent Near Total Duration	Sweep	30° sweep	-0.51	0.61
		90° sweep	-1.38	0.17
	Speed	1.0 Hz	1.48	0.14
		1.5 Hz	2.63	0.009 **
	Elevation	20° elevation	-1.08	0.28
		55° elevation	-1.30	0.20
Time Spent Near Average Duration	Sweep	30° sweep	-1.04	0.30
		90° sweep	-2.20	0.03 *
	Speed	1.0 Hz	0.43	0.67
		1.5 Hz	1.90	0.06
	Elevation	20° elevation	-0.29	0.77
		55° elevation	-0.09	0.93
Orient Count	Sweep	30° sweep	-1.14	0.26
		90° sweep	-1.14	0.26
	Speed	1.0 Hz	0	1
		1.5 Hz	0.38	0.71
	Elevation	20° elevation	-1.92	0.06
		55° elevation	-1.92	0.06

Table 2.5: General linear mixed-effects models for time spent near the Robotail. Changes in duration and count of times that the female spent near the tail, as well as orient count, in response to the 3 levels of each hula motion parameter. Duration of time spent near (total and average) differed significantly with speed or sweep angle. Female ID number was used as the random effect in all analyses; all t statistics and p-values were rounded to 2 decimals, except when the p-value was less than 0.01.

Dependent Variable	Model	Fixed Effects	t statistic	p - value
Nudge Base Count	Sweep	30° sweep	-1.89	0.06
		90° sweep	-1.49	0.14
	Speed	1.0 Hz	-0.48	0.63
		1.5 Hz	0.45	0.65
	Elevation	20° elevation	-0.59	0.56
		55° elevation	-0.54	0.59
Nudge Tip Count	Sweep	30° sweep	0.50	0.61
		90° sweep	0.25	0.80
	Speed	1.0 Hz	0.58	0.57
		1.5 Hz	2.11	0.04 *
	Elevation	20° elevation	-1.43	0.15
		55° elevation	-2.85	0.005 **
Total Nudge Count	Sweep	30° sweep	-1.56	0.12
		90° sweep	-1.26	0.21
	Speed	1.0 Hz	-0.35	0.72
		1.5 Hz	0.77	0.44
	Elevation	20° elevation	-0.86	0.39
		55° elevation	-1.09	0.28

Table 2.6: General linear mixed-effects models for physical contacts with the Robotail. Changes in the number of times females physically interacted with the tail, relative to the 3 levels of each hula motion parameter. Nudge tip count, but not nudge base count or the total number of nudges performed, differed significantly with speed and elevation angle. Female ID number was used as the random effect in all analyses; all t statistics and p-values were rounded to 2 decimals, except when the p-value was less than 0.01.

Dependent Variable	Model	Fixed Effects	t statistic	p - value
Nip Count	Sweep	30° sweep	-3.00	0.003 **
		90° sweep	-2.55	0.01 *
	Speed	1.0 Hz	0.25	0.80
		1.5 Hz	0.15	0.88
	Elevation	20° elevation	-1.73	0.09
		55° elevation	0.58	0.56
Bite Count	Sweep	30° sweep	-1.24	0.22
		90° sweep	-1.24	0.22
	Speed	1.0 Hz	1.24	0.22
		1.5 Hz	0	1
	Elevation	20° elevation	-1.24	0.22
		55° elevation	-1.24	0.22
Attack Count	Sweep	30° sweep	-1.77	0.08
		90° sweep	-1.77	0.08
	Speed	1.0 Hz	-0.87	0.38
		1.5 Hz	0	1
	Elevation	20° elevation	-1.77	0.08
		55° elevation	-1.77	0.08

Table 2.7: General linear mixed-effects models for female aggression data.

Changes in female aggressive behaviors relative to the 3 levels of each hula motion parameter. The number of times females nipped at the tail, but not the number of bites or attacks differed significantly with sweep angle. Female ID number was used as the random effect in all analyses; all t statistics and p-values were rounded to 2 decimals, except when the p-value was less than 0.01.

Dependent Variable	Random Effect	Model	Fixed Effects		t statistic	p - value
			Sweep (°)	Speed (Hz)		
Locomotion transitions per minute	Female ID	Combo	30	0.5	0.52	0.61
			90	0.5	1.69	0.09
			10	1.0	0.69	0.49
			30	1.0	1.79	0.08
			90	1.0	2.19	0.03 *
			10	1.5	-0.28	0.78
			30	1.5	2.37	0.02 *
			90	1.5	4.01	9.51e-05 ***

Table 2.8: General linear mixed-effects models for locomotion transitions.

Changes in the number of times that females transitioned between locomotive states per minute relative to each combination of sweep angle and speed that the Robotail performed. Females locomoted significantly more often if the Robotail was programmed with a combination of 90°/1.0 Hz, 30°/1.5 Hz, or 90°/1.5 Hz compared to a combination of 10°/1.5 Hz. Female ID number was used as the random effect in this analysis; all t statistics and p-values were rounded to 2 decimals, except when the p-value was less than 0.01.

FIGURES

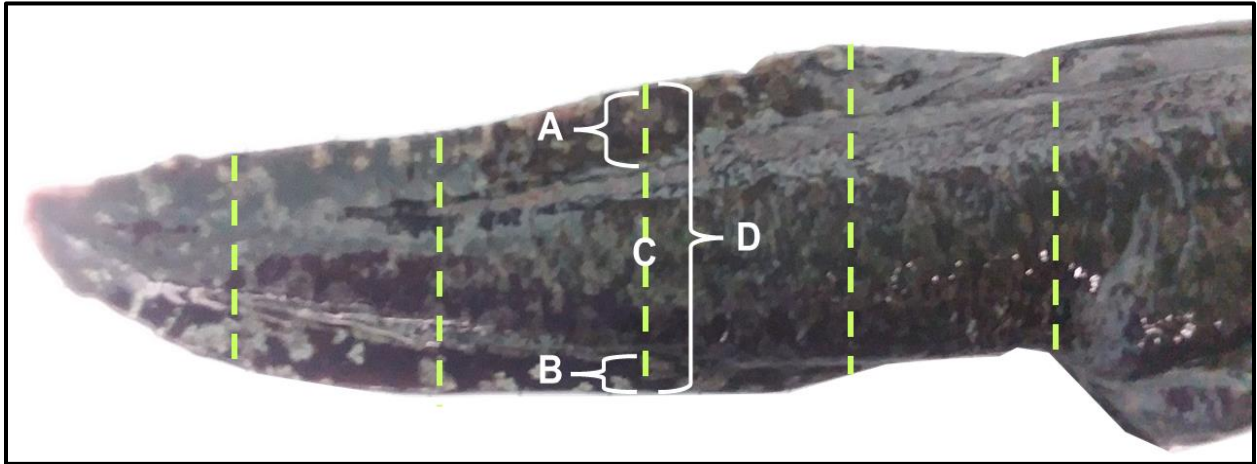


Figure 2.1: Measurement parameters for male axolotl tails. Green dashed lines indicate the 5 positions along the tail at which measurements were taken to create the Robotail. At each position, we measured the width and height of the dorsal fin (A), the width and height of the ventral fin (B), the width at the middle of the tail (C) and the total height (D).

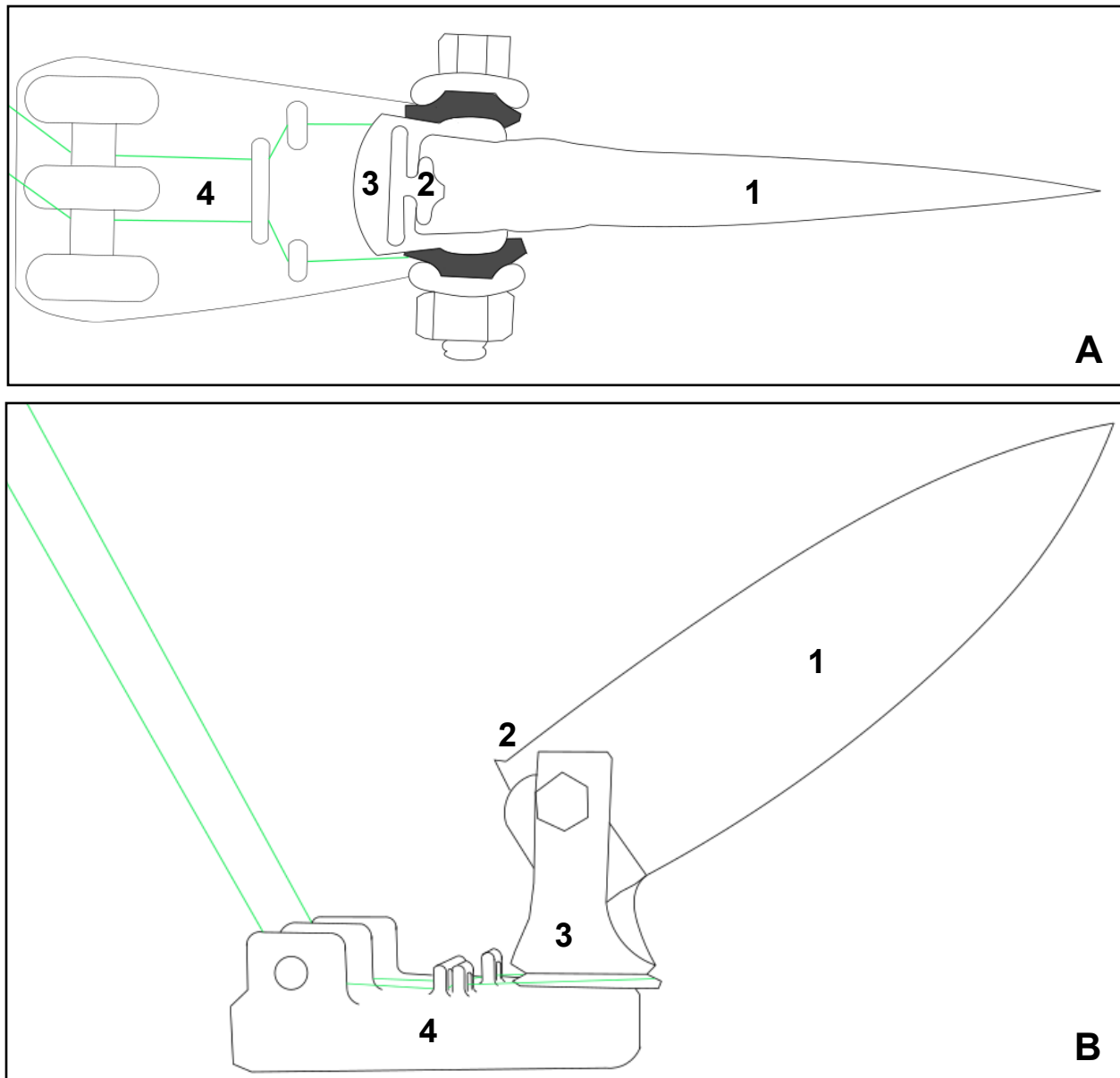


Figure 2.2: Diagram of the silicone tail and mounting components. Overhead (A) and side (B) views of the tail and mounting components; parts consisted of (1) the silicone tail (2) the clip embedded in the tail (3) the pivoting cylinder and (4) the triangular base. The embedded clip slid into a slot on the pivoting cylinder and was held in place by a nylon bolt and nut and 2 rubber washers. Green lines indicate the position of the fishing lines that were used to turn the pivoting cylinder from side to side.

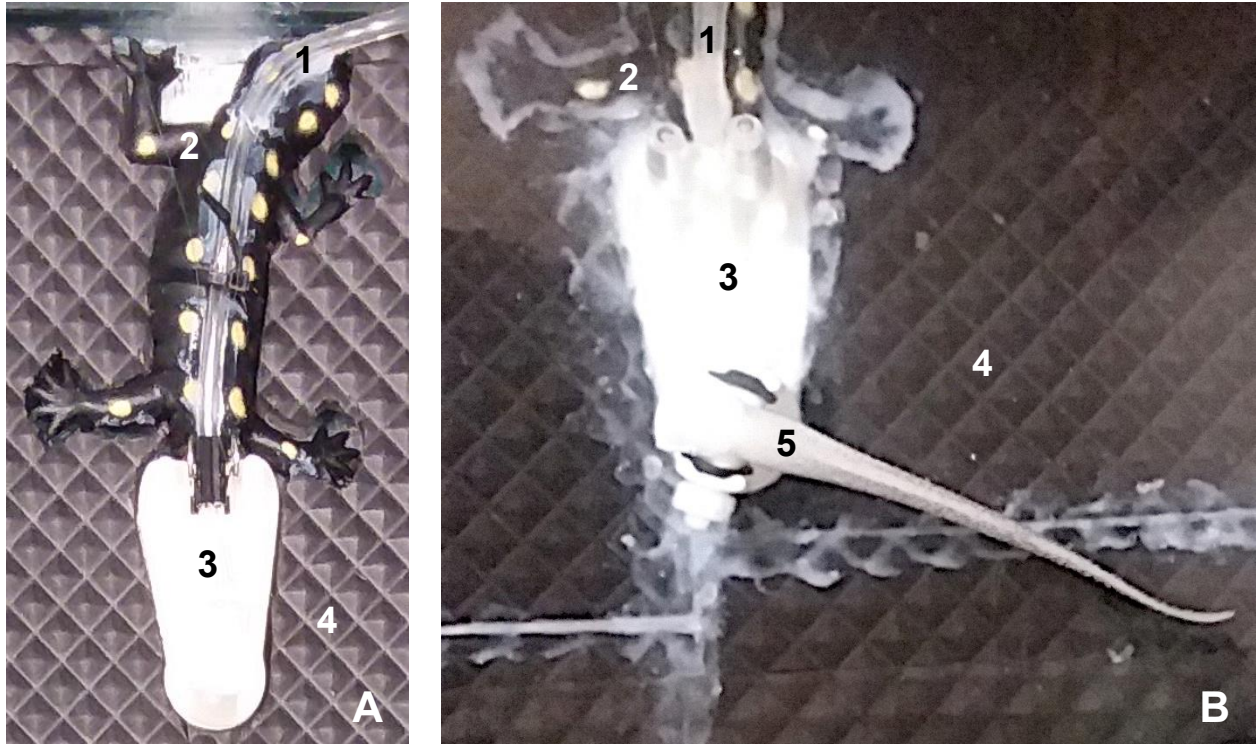


Figure 2.3: Robotic components contained within the aquarium. The Robotail with dry components (A) and with the components submerged in water (B). Components attached to the aquarium floor included tubing carrying male odorants (1), the silicone salamander toy (2), triangular base plate (3), and EVA foam tiles (4); the silicone tail (5) was attached to the base plate via a pivoting cylinder and is shown in panel B only.

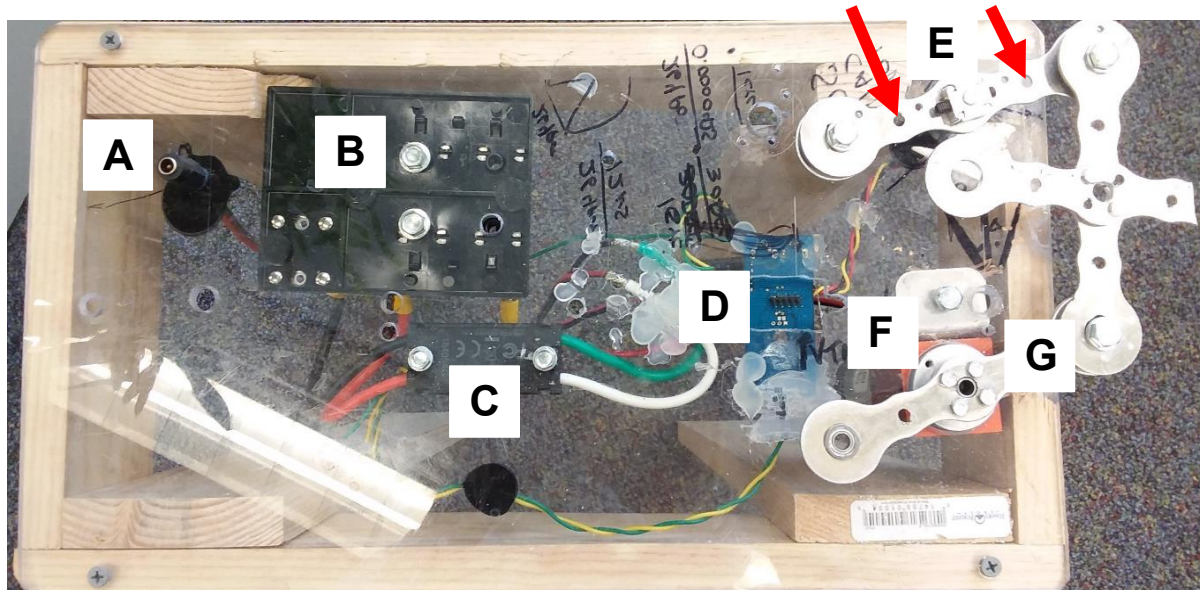


Figure 2.4: Electronic components of the Robotail. Components consisted of (A) power source (B) power distribution channel (C) Victor SP speed controller (D) Thrifty Throttle 3/Cypress PSOC4™ microcontroller (E) potentiometer (F) 104:1 NeveRest Sport gearbox and NeveRest gearmotor and (G) custom gimbal. The gimbal swiveled to the left and right to drive the position of the silicone tail; red arrows indicate the attachment points on the gimbal for the fishing lines.

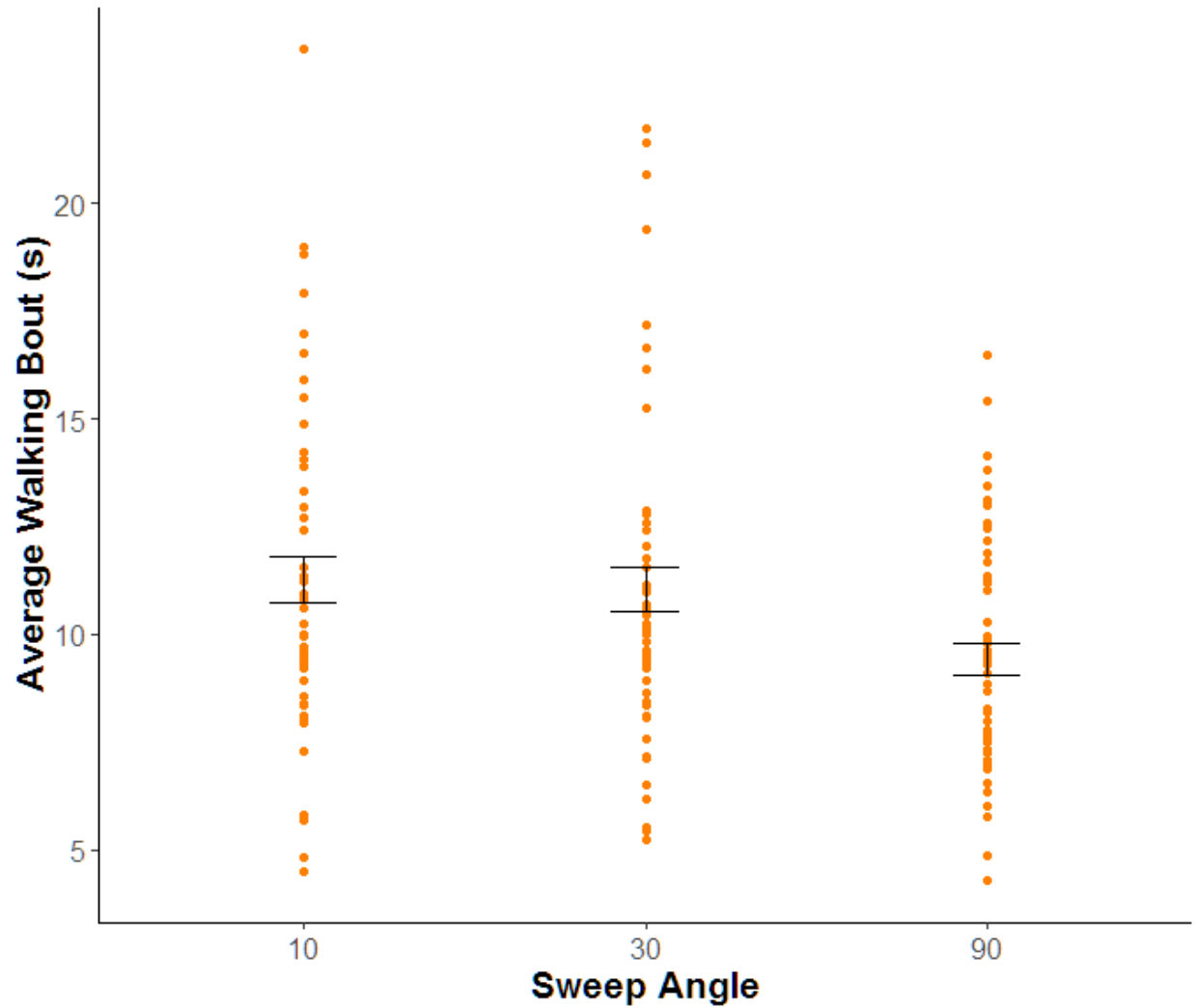


Figure 2.5: Average walk duration of females in response to sweep angle. Female axolotls exhibited shorter bouts of walking when stimulated by the widest sweep angle of 90° (mixed model analysis, $t = -2.91$, $p = 0.004$). Each dot represents the average duration of a walking bout for a given trial, and bars represent the standard error of each group.

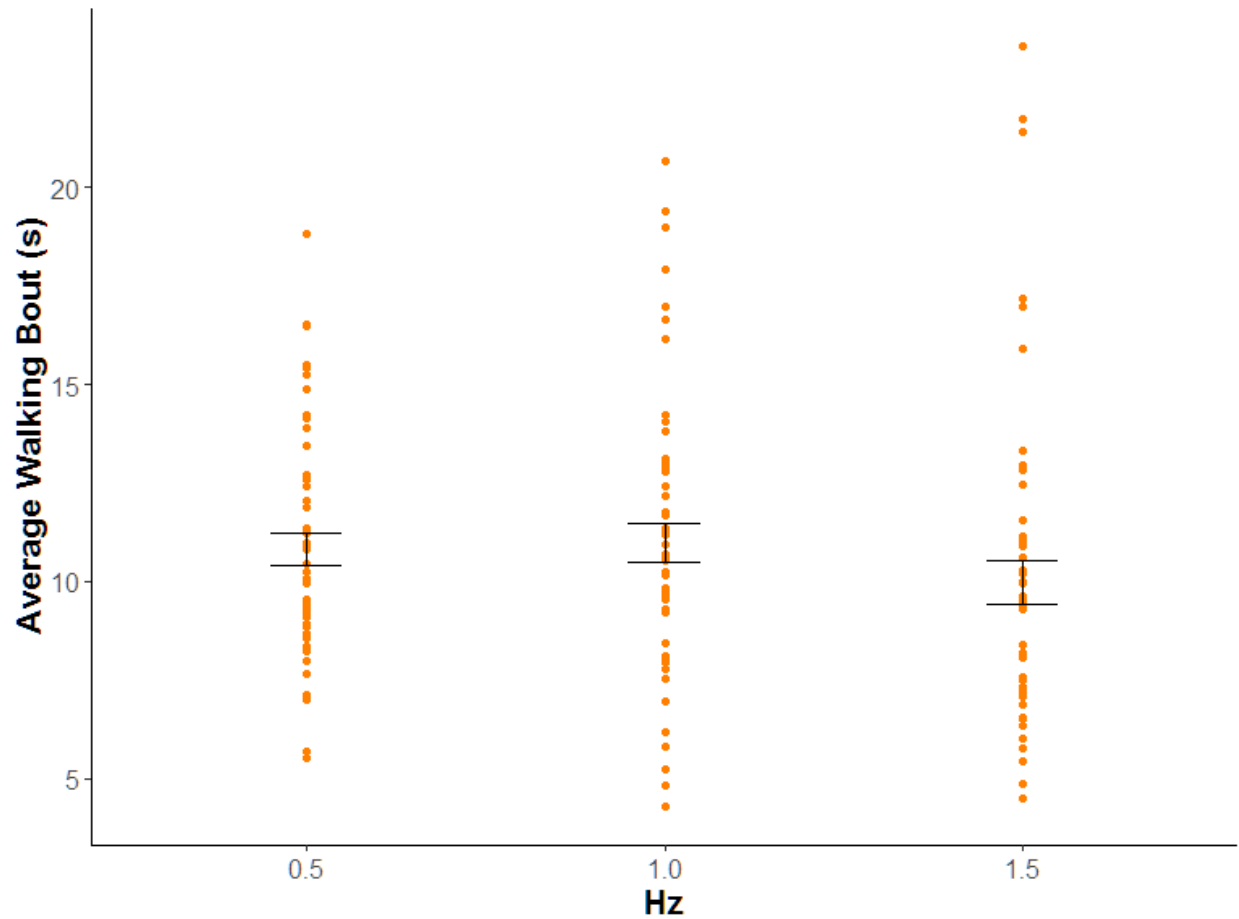


Figure 2.6: Average walk duration of females in response to speed. Female axolotls exhibited shorter bouts of walking when stimulated by the fastest speed of 1.5 Hz (mixed model analysis, $t = -2.27$, $p = 0.03$). Each dot represents the average duration of a walking bout for a given trial, and bars represent the standard error of each group.

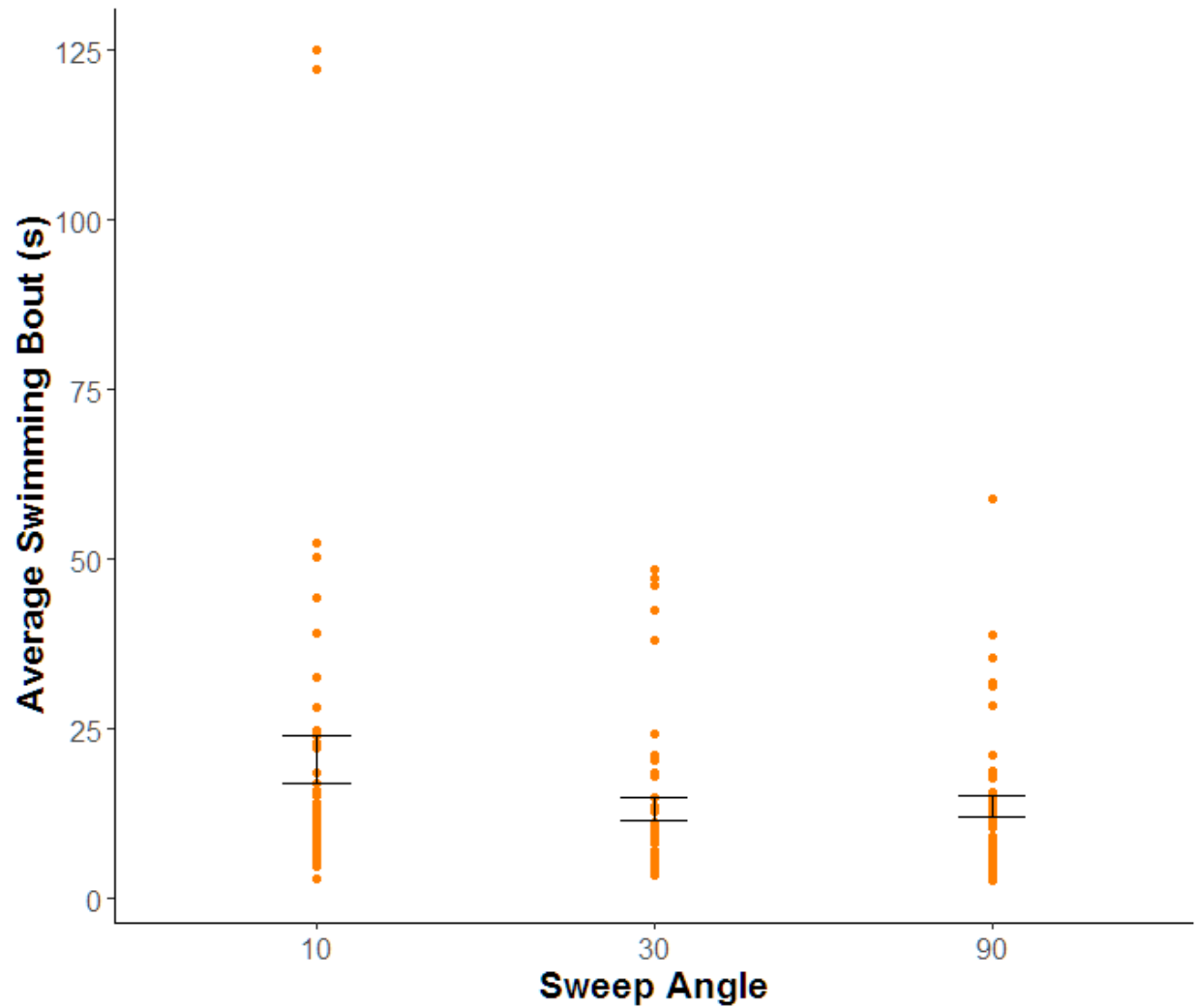


Figure 2.7: Average swim duration of females in response to sweep angle. Female axolotls exhibited shorter bouts of walking as the sweep angle of the Robotail increased (mixed model analysis, 10° vs 30° , $t = -2.21$, $p = 0.03$, 10° vs 90° , $t = -2.21$, $p = 0.03$). Each dot represents the average duration of a swimming bout for a given trial, and bars represent the standard error of each group.

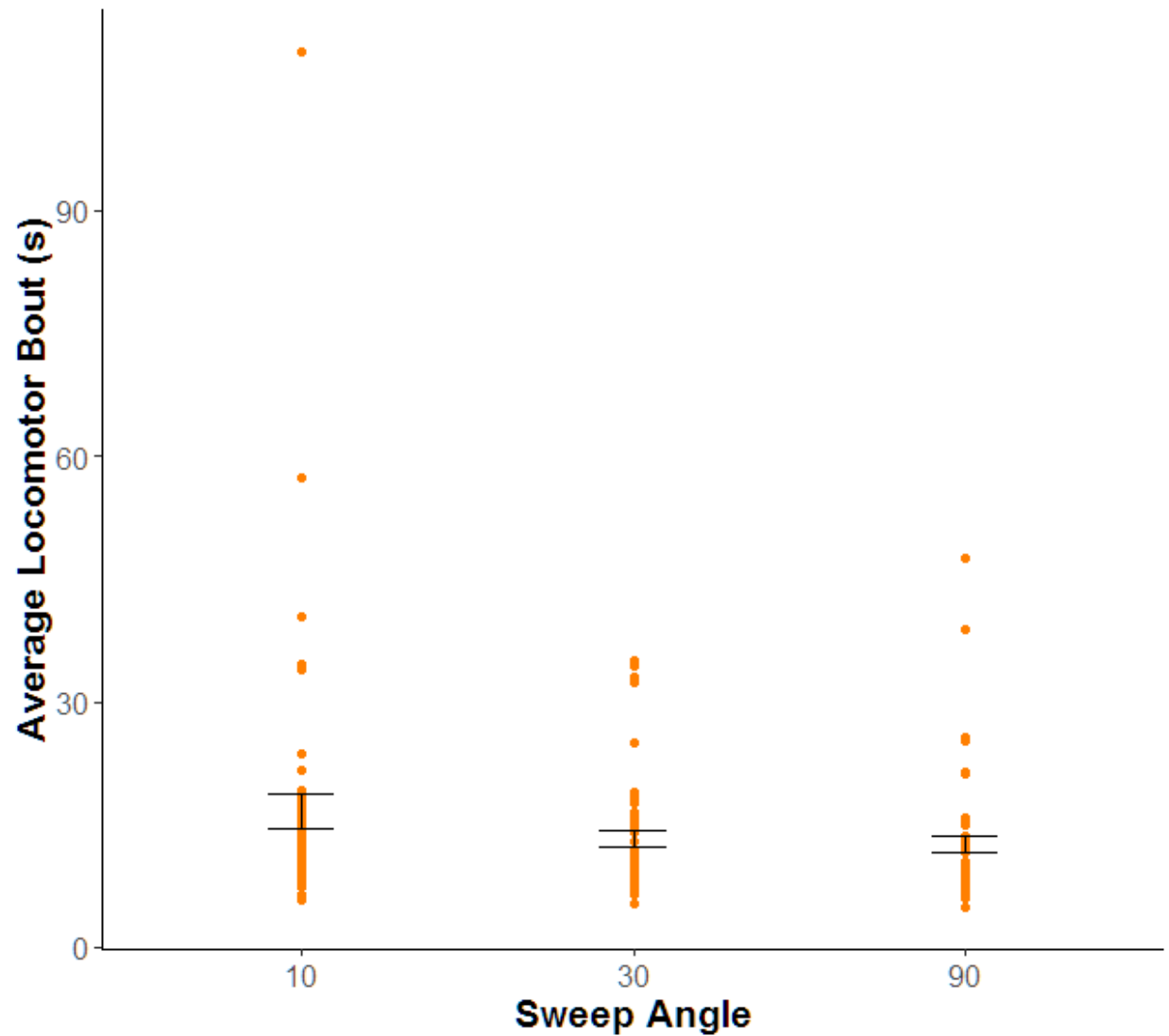


Figure 2.8: Average locomotion duration of females in response to sweep angle. Female axolotls exhibited shorter bouts of locomotion (walking plus swimming) when stimulated by the widest sweep angle of 90° (mixed model analysis, $t = -2.15$, $p = 0.03$). Each dot represents the average duration of a locomotor bout for a given trial, and bars represent the standard error of each group.

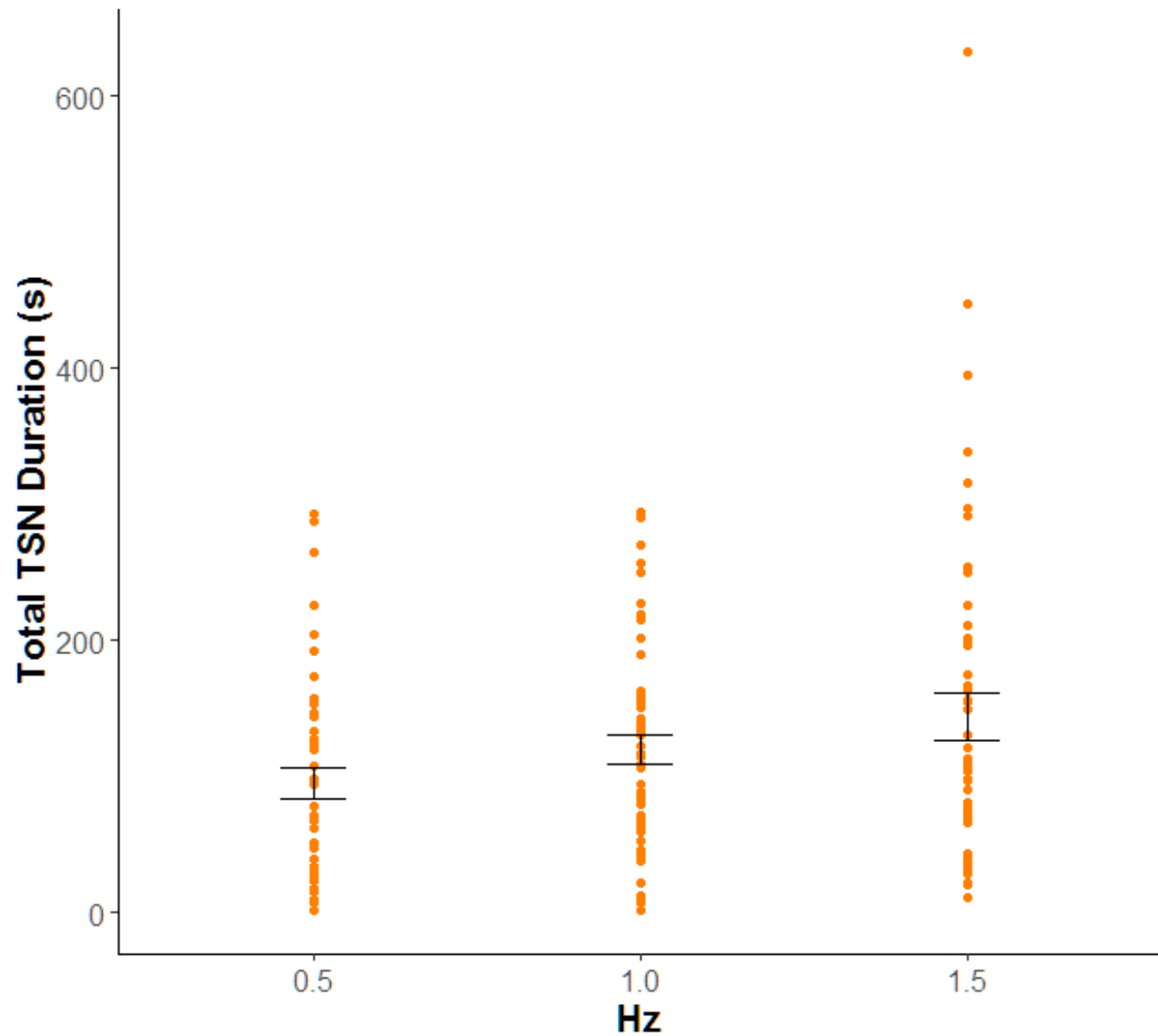


Figure 2.9: Total duration of time spent near for females in response to speed. Female axolotls exhibited more time near the Robotail overall when stimulated by the fastest speed of 1.5 Hz (mixed model analysis, $t = 2.63$, $p = 0.009$). Each dot represents the total duration of TSN for a given trial, and bars represent the standard error of each group.

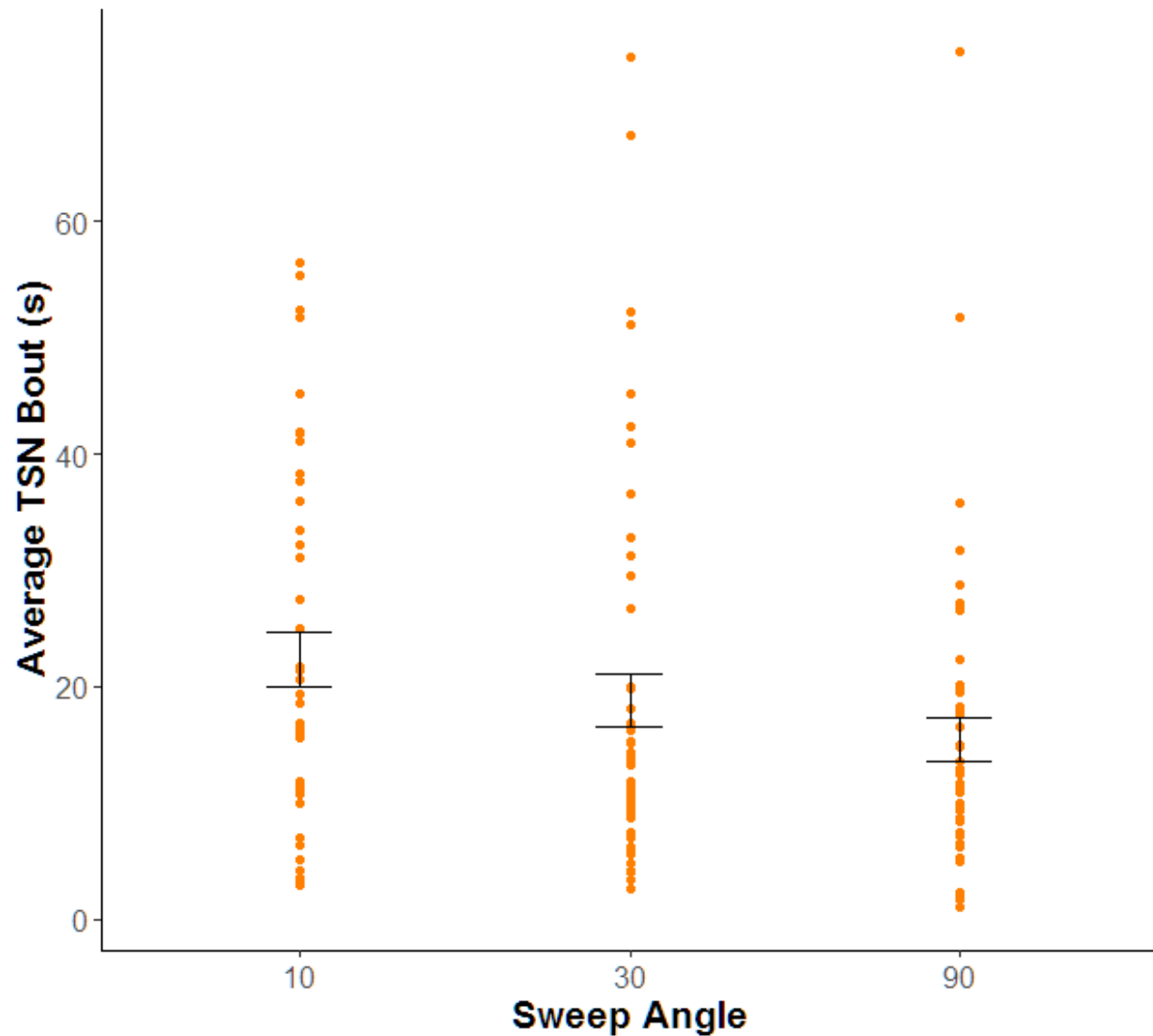


Figure 2.10: Average duration of time spent near for females in response to sweep angle. Female axolotls exhibited shorter bouts of spending time near the Robotail when stimulated by the widest sweep angle of 90° (mixed model analysis, $t = -2.20$, $p = 0.03$). Each dot represents the average duration of a TSN bout for a given trial, and bars represent the standard error of each group.

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APPENDIX

A) Close Bouts

		Sweep Angle									Elevation Angle	
Hz ↘		10°			30°			90°				0 males
0°		0.5	1.0	1.5	0.5	1.0	1.5	0.5	1.0	1.5		1 male
20°		0.5	1.0	1.5	0.5	1.0	1.5	0.5	1.0	1.5		2 males
55°		0.5	1.0	1.5	0.5	1.0	1.5	0.5	1.0	1.5		3 males
												4 males
												5 males

B) Distant Bouts

		Sweep Angle								
Hz ↘		10°			30°			90°		
Elevation Angle	0°	0.5	1.0	1.5	0.5	1.0	1.5	0.5	1.0	1.5
	20°	0.5	1.0	1.5	0.5	1.0	1.5	0.5	1.0	1.5
	55°	0.5	1.0	1.5	0.5	1.0	1.5	0.5	1.0	1.5

C) All Bouts

		Sweep Angle								
Hz ↘		10°			30°			90°		
Elevation Angle	0°	0.5	1.0	1.5	0.5	1.0	1.5	0.5	1.0	1.5
	20°	0.5	1.0	1.5	0.5	1.0	1.5	0.5	1.0	1.5
	55°	0.5	1.0	1.5	0.5	1.0	1.5	0.5	1.0	1.5

Table S2.1: Matrices displaying the number of males that performed each tail motion combination. We organized hula bouts by proximity (**A**; Close, **B**; Distant, **C**; All), and counted the total number of males that performed each of the 27 motion combinations. No single combination was performed by all 6 males.

Dependent Variable	Model	AICc	Δ AICc
Walk Total Duration	Sweep	2053.646	0
	Elevation	2053.983	0.337
	Sweep+Elevation	2055.664	2.018
	Speed	2055.758	2.112
	Speed+Sweep	2057.400	3.754
	Speed+Elevation	2057.749	4.103
	Speed+Sweep+Elevation	2059.512	5.866
Walk Average Duration	Speed+Sweep	837.4518	0
	Sweep	839.0042	1.5524
	Speed+Sweep+Elevation	839.752	2.3002
	Sweep+Elevation	841.1188	3.667
	Speed	843.0461	5.5943
	Speed+Elevation	845.5645	8.1127
	Elevation	847.1814	9.7296
Swim Total Duration	Speed	1981.949	0
	Sweep	1982.210	0.261
	Elevation	1982.516	0.567
	Speed+Sweep	1985.427	3.478
	Speed+Elevation	1985.694	3.745
	Sweep+Elevation	1985.867	3.918
	Speed+Sweep+Elevation	1989.221	7.272
Swim Average Duration	Sweep	1238.13	0
	Speed+Sweep	1241.036	2.906
	Sweep+Elevation	1242.004	3.874
	Speed	1242.577	4.447
	Elevation	1244.118	5.988
	Speed+Sweep+Elevation	1245.051	6.921
	Speed+Elevation	1246.567	8.437

Table S2.2: AICc rankings of mixed-effects models for female locomotion data (continued on next page).

Dependent Variable	Model	AICc	Δ AICc
Locomote Total Duration	Speed	2152.368	0
	Elevation	2154.483	2.115
	Sweep	2154.522	2.154
	Speed+Sweep	2156.163	3.795
	Speed+Elevation	2156.181	3.813
	Sweep+Elevation	2158.361	5.993
	Speed+Sweep+Elevation	2160.126	7.758
Locomote Average Duration	Sweep	1230.631	0
	Speed+Sweep	1232.01	1.379
	Speed	1232.531	1.9
	Sweep+Elevation	1234.137	3.506
	Elevation	1234.72	4.089
	Speed+Sweep+Elevation	1235.631	5
	Speed+Elevation	1236.07	5.439
Pause Total Duration	Speed	2153.560	0
	Sweep	2155.540	1.980
	Elevation	2155.563	2.003
	Speed+Sweep	2157.388	3.828
	Speed+Elevation	2157.469	3.909
	Sweep+Elevation	2159.468	5.908
	Speed+Sweep+Elevation	2161.442	7.882
Pause Average Duration	Speed	1499.972	0
	Sweep	1501.661	1.689
	Speed+Sweep	1502.495	2.523
	Elevation	1502.95	2.978
	Speed+Elevation	1503.736	3.764
	Sweep+Elevation	1505.337	5.365
	Speed+Sweep+Elevation	1506.325	6.353

Table S2.2 (cont'd): Seven statistical models were built for each locomotion variable and their corresponding AICc values calculated to assess which model(s) best explained our data set. Equally weighted models (i.e., within 3 AICc values of the top ranked model) are indicated by gray shading. The model that best described each behavioral variable, which is the simplest model with the lowest AICc value, is indicated by bold text.

Dependent Variable	Model	AICc	Δ AICc
Time Spent Near Total Duration	Speed	1764.445	0
	Speed+Sweep	1766.572	2.127
	Speed+Elevation	1766.768	2.323
	Speed+Sweep+Elevation	1769.153	4.708
	Sweep	1769.252	4.807
	Elevation	1769.294	4.849
	Sweep+Elevation	1771.826	7.381
Time Spent Near Average Duration	Speed+Sweep	1210.355	0
	Sweep	1210.456	0.101
	Speed	1211.277	0.922
	Sweep+Elevation	1214.821	4.466
	Speed+Sweep+Elevation	1214.856	4.501
	Elevation	1215.148	4.793
	Speed+Elevation	1215.587	5.232
Orient Count	Elevation	377.085	0
	Sweep+Elevation	379.641	2.557
	Sweep	380.211	3.127
	Speed+Elevation	381.230	4.145
	Speed	381.752	4.668
	Speed+Sweep+Elevation	383.899	6.814
	Speed+Sweep	384.360	7.276

Table S2.3: AICc rankings of mixed-effects models for time spent near (TSN) and orient data. Seven statistical models were built for the duration and frequency of TSN as well as orient count, and their corresponding AICc values calculated to assess which model(s) best explained our data set. Equally weighted models (i.e., within 3 AICc values of the top ranked model) are indicated by gray shading. The model that best described each behavioral variable, which is the simplest model with the lowest AICc value, is indicated by bold text.

Dependent Variable	Model	AICc	Δ AICc
Nudge Base Count	Sweep	1119.697	0
	Speed	1122.730	3.033
	Speed+Sweep	1123.090	3.393
	Elevation	1123.168	3.471
	Sweep+Elevation	1123.644	3.947
	Speed+Elevation	1126.653	6.956
	Speed+Sweep+Elevation	1127.155	7.458
Nudge Tip Count	Speed+Elevation	599.138	0
	Elevation	599.704	0.566
	Speed	602.893	3.755
	Sweep+Elevation	603.750	4.612
	Speed+Sweep+Elevation	603.320	4.182
	Speed+Sweep	606.974	7.836
	Sweep	607.319	8.182
Total Nudge Count	Sweep	1148.241	0
	Elevation	1149.645	1.404
	Speed	1149.646	1.405
	Speed+Sweep	1151.174	2.933
	Sweep+Elevation	1151.306	3.065
	Speed+Elevation	1152.691	4.450
	Speed+Sweep+Elevation	1154.357	6.116

Table S2.4: AICc rankings of mixed-effects models for physical contacts with the Robotail. Seven statistical models were built for nudge base, nudge tip, and total nudges and their corresponding AICc values calculated to assess which model(s) best explained our data set. Equally weighted models (i.e., within 3 AICc values of the top ranked model) are indicated by gray shading. The model that best described each behavioral variable, which is the simplest model with the lowest AICc value, is indicated by bold text.

Dependent Variable	Model	AICc	Δ AICc
Nip Count	Sweep+Elevation	472.933	0
	Sweep	474.195	1.263
	Speed+Sweep+Elevation	477.322	4.390
	Speed+Sweep	478.446	5.514
	Elevation	478.664	5.732
	Speed+Elevation	482.955	10.022
	Speed	484.277	11.345
Bite Count	Sweep	-357.098	0
	Speed	-357.098	0
	Elevation	-357.098	0
	Sweep+Elevation	-354.806	2.292
	Speed+Sweep	-354.806	2.292
	Speed+Elevation	-354.806	2.292
	Speed+Sweep+Elevation	-352.426	4.672
Attack Count	Elevation	-247.894	0
	Sweep	-247.894	0
	Sweep+Elevation	-247.759	0
	Speed	-244.808	3.086
	Speed+Elevation	-244.593	3.301
	Speed+Sweep	-244.593	3.301
	Speed+Sweep+Elevation	-244.372	3.522

Table S2.5: AICc rankings of mixed-effects models for aggression data. Seven statistical models were built for measures of aggression and their corresponding AICc values calculated to assess which model(s) best explained our data set. Equally weighted models (i.e., within 3 AICc values of the top ranked model) are indicated by gray shading. The model that best described each behavioral variable, which is the simplest model with the lowest AICc value, is indicated by bold text.

CHAPTER 3: Lateral Line Responses to the Male Hula Behavior

ABSTRACT

Many physical features of the communication signals exchanged between animals are governed by a hypothesis known as sender-receiver matching. This hypothesis posits that communication signals generated by senders are shaped by the tuning properties of the receiver's corresponding sensory system. While sender-receiver matches have been extensively documented within auditory communication, it is currently unclear if this hypothesis also pertains to vibratory communication strategies within mechanosensory systems. We aimed to determine whether sender-receiver matching occurs within the mechanosensory lateral line system specifically, and we leveraged the courtship ritual of the axolotl, *Ambystoma mexicanum*, a fully aquatic salamander, to achieve this aim.

Male axolotls reliably perform a courtship behavior known as the “hula”, which features a swaying motion of the hips paired with an undulation of the tail; this behavior generates disturbances in the surrounding water, which likely stimulate the lateral line systems of female receivers. We used a custom device called the “Robotail” that mimics the male hula behavior to deliver a range of hydrodynamic stimuli to our test subjects and recorded multi-unit activity from the peripheral aspect of the anterodorsal lateral line nerve (ADLLn). This nerve innervates all neuromasts (mechanically sensitive hair cells) on the anterior dorsal surface of the axolotl's head; given that females position their heads underneath the hula-ing male's tail at several key points during the courtship ritual, this cluster of neuromasts is likely to be highly stimulated by the hula behavior.

We found that the ADLLn of female axolotls exhibited excitatory responses to all sweep angles (i.e., angle of side-to-side motion) and speeds that we tested and exhibited the strongest responses to moderate angles and fast speeds. In contrast, the ADLLn of male axolotls exhibited inhibitory responses to narrow angles and slow speeds, but excitatory responses to all other parameters that we tested. In our previous research, we determined that male axolotls most often hula'd during courtship using moderate sweep angles; thus, the results of our current experiment act as preliminary evidence that sender-receiver matching may occur within the vibratory communication modality in axolotl courtship.

INTRODUCTION

Animals exchange countless communication signals with conspecifics to coordinate social processes like aggressive contests (Butler & Maruska, 2015), cooperative behaviors (Noë, 2006), and mating (Denoël & Doellen, 2010). The sender-receiver matching hypothesis seeks to identify and explain physical constraints on communication signals and their detection (Capranica & Moffat, 1983); this hypothesis posits that senders generate communication signals that are tuned to the sensory systems of receivers and vice versa, such that receivers also play a role in shaping signal characteristics (Henry et al., 2016). Sender-receiver matching has been most notably demonstrated in the acoustic communication modality (Gall et al., 2012; Henry et al., 2016; Mhatre et al., 2011), but it also occurs in electric organ discharges and their detection by the lateral line system of *Apteronotus* (Allen & Marsat, 2019).

However, considerably less research has been conducted on sender-receiver matching that potentially occurs in the mechanosensory aspect of the lateral line

system. Although Satou et al. (1994) demonstrated that the lateral line is responsible for mediating vibratory communication during the courtship ritual of the himé salmon (*Oncorhynchus nerka*), little evidence demonstrates that the lateral line system is physiologically tuned to hydrodynamic signals that are generated by conspecifics. Here, we aim to investigate the degree of sender-receiver matching that occurs in the mechanosensory lateral line system as it specifically relates to communication signals generated by conspecifics in courtship scenarios.

Salamanders that court in aquatic environments are useful model organisms for understanding the sensory tuning properties of the mechanosensory lateral line system. Many male salamanders perform a courtship behavior that involves an oscillating motion of the pelvic region and/or tail, such as the “tail fan” behavior, which generates mechanical stimuli (Green, 1989). We leveraged the courtship ritual of the fully aquatic axolotl, *Ambystoma mexicanum*, to examine how both senders and receivers shape the physical properties of mechanosensory signals. Male axolotls reliably perform a behavior known as the “hula”, a swaying motion of the hips paired with tail undulations, throughout their courtship ritual (Arnold, 1976; Park et al., 2004). The hula behavior generates hydrodynamic stimuli in the form of water disturbances, which likely act as a communication signal during mating encounters by stimulating the mechanosensory lateral line system of female axolotls.

Importantly, axolotls maintain the functionality of their lateral line systems throughout their life cycles, unlike amphibians that undergo metamorphosis and typically lose their lateral line functions upon transitioning to a terrestrial environment (Wahnschaffe et al., 1987). Hydrodynamic stimuli likely play an important role for female

axolotls during courtship, as they may indicate the male's location as well as his quality as a potential mate. Additionally, these stimuli may aid the female in coordinating sperm transfer with the male. Thus, axolotl courtship provides a unique opportunity to study both sender and receiver dynamics of mechanical signals.

Hydrodynamic signals generated by conspecifics can be highly complex; we previously captured multiple aspects of the hula by measuring the sweep angle (i.e., degree of side-to-side motion), oscillation speed, and the elevation angle of tail movements. Using these data, we built a programmable device called the "Robotail", which mimics the motions of the male hula (see Chapter 2 for methods). Importantly, the Robotail allowed us to evaluate how factors influencing components of vibrational communication (including sweep angle, speed, elevation) signals are received by mechanosensory lateral line systems. We tested 3 sweep angles (10°, 30°, 90°) paired with 3 speeds (0.5 Hz, 1.0 Hz, 1.5 Hz) for a total of 9 motion combinations; because hula elevation angle did not impact female behavior significantly (see Chapter 2), we excluded this parameter from our experiment. Using multi-unit recordings, we measured the firing rates of the anterodorsal lateral line nerve (ADLLn) in response to the 9 different hula motion combinations.

We chose to study the ADLLn specifically because it innervates dozens of neuromasts (i.e., mechanically sensitive hair cells) on the dorsal surface of the axolotls' head (Northcutt, 1992); given that female axolotls position their heads directly underneath the male's tail during spawning (Salthe, 1967), these neuromasts are likely to be highly stimulated during courtship. We created a semi-natural setting for our recordings by 1) positioning the recording preparation behind the raised Robotail,

mimicking the orientation of the female relative to the male during courtship, and 2) recording inside an aquarium filled with several inches of water, well above the top of the recording preparation; typical recording dishes hold less than a centimeter of fluid. Although we were primarily focused on studying female responses to hydrodynamic stimuli, we also tested males because their responses may differ from females, providing additional insight into sender-receiver matching and potentially into male-male competition in axolotls (Park et al., 2008).

We found that the ADLLn of female axolotls exhibited an excitatory response to all sweep angles and all speeds; however, it responded most strongly to moderate sweep angles (30°) and moderate speeds (1.0 Hz). In contrast, the ADLLn of males exhibited inhibitory responses to narrow sweep angles (10°) and slow speeds (0.5 Hz), but excitatory responses to all other hula parameters, suggesting that the tuning of the lateral line systems in the two sexes may differ. Furthermore, we found that males most often hula with moderate combinations of motions (i.e., 30° paired with 1.0 Hz) during courtship (see Chapter 2), indicating that a high level of alignment exists between the hydrodynamic cues generated by males and the tuning properties of the female lateral line. Our experiments thus suggest that sender-receiver matching occurs within the mechanosensory lateral line system of axolotls.

MATERIALS AND METHODS

Subjects

Adult axolotls (*Ambystoma mexicanum*) were obtained from the Ambystoma Genetic Stock Center at the University of Kentucky. Animals were housed in ~114 L aquaria in 40-100% Holtfreter's (HF) solution (Armstrong et al., 1989) supplemented

with Replenish™ solution at temperatures between 18 and 22°C; animals were separated by sex and each aquarium contained 1-3 animals. We programmed the lights in our facility to match the natural sunrise and sunset of Mexico City, Mexico (the native habitat of axolotls) with monthly updates to the axolotls' photoperiod. Housing protocols and experiments were conducted with the approval of the Institutional Animal Care and Use Committee of Michigan State University (approval numbers: PROTO201800106, PROTO202100239).

Electrophysiology

We used the “Robotail” (Chapter 2) to assess responses of the anterodorsal lateral line nerve to a range of mechanosensory stimuli similar to those produced by male axolotls during the hula behavior. We tested 3 sweep angles (10°, 30°, 90°) paired with 3 speeds (0.5 Hz, 1.0 Hz, 1.5 Hz) for a total of 9 motion combinations and rotated the order of stimulus presentation across animals. We used a constant elevation angle of ~45° because in earlier work we found that the elevation angle of the Robotail did not affect female behavior (Chapter 2); additionally, at lower angles the silicone tail interfered with the preparation and electrodes. The testing protocol, which we ran in triplicate for each combination, included a preparation period of 15 sec followed by a stimulus period of 30 sec, with an intertrial interval, or recovery period, of 2 min. Our preparations generally remained viable for a period of 3-4 hr; we analyzed data from recordings with robust action potentials (**APs**; generally above 1 mV) and stopped recording when nerve activity dropped below 0.5 mV. Using these criteria, we obtained viable recordings from 14 females and 5 males in total.

Experiments were conducted in a glass aquarium (71 cm x 22 cm x 18 cm) that held approximately 20 L of HF solution (~15 cm depth) at the same concentration as the animals' home aquaria; the aquarium was housed inside a Faraday cage to shield our recordings from electrical noise. The mounting plate for the Robotail's silicone tail was attached to the aquarium floor with waterproof silicone and the electronics were housed outside the Faraday cage; the tail was controlled via fishing lines fed through openings in the cage wall (Fig. 3.1). Importantly, we fabricated all submerged components of the robot from non-metallic materials to ensure that the Robotail did not generate electrical stimuli that might stimulate the electrosensory receptors innervated by the ADLLn.

We performed rapid decapitation and exposed the peripheral aspect of the right ADLLn on all subjects by removing a ~1cm² piece of skin posterior to the eye. The preparation was secured with pins to a Petri dish lined with Sylgard™ compound (Dow Inc., Midland, MI), which we affixed to the aquarium floor with dental wax. The preparation was placed underneath the silicone tail such that the anterior edge of the axolotl's snout was ~4 cm from the mounting plate and ~8 cm from the tail tip (Fig. 3.2) to mimic courtship postures and to maximize stimulation of the neuromasts innervated by the ADLLn. We fabricated a pair of waterproof electrodes with long shafts that allowed us to record electrical activity deep under water; an Ag/AgCl wire suction electrode with a borosilicate glass pipette tip was used for recording and an Ag/AgCl pellet electrode acted as the reference electrode. We performed multiunit recordings (Fig. 3.3) by lowering the recording electrode onto the surface of the nerve as posteriorly as possible and drawing gentle suction, moving the recording site anteriorly

when the signal deteriorated. The reference electrode was placed in the HF solution approximately 1 cm away from the tip of the suction electrode.

Signals were amplified through a differential amplifier (DP-304, Warner Instruments LLC, Holliston, MA), high-pass filtered (60 Hz), and digitized using a Digidata® 1550A digitizer (Molecular Devices LLC, San Jose, CA). We used pCLAMP™ 10 software (Molecular Devices LLC, San Jose, CA) with Clampex and Clampfit programs (version 10.7.0.3) for data recording and analysis, respectively. Automated spike data extraction was performed using MATLAB code (MathWorks Inc, Natick, MA, version R2023b), which was modified from a classroom handout created by Wagenaar & Wright (2008) for the Neural Systems and Behavior Course at the Marine Biological Laboratory in Woods Hole, MA.

Statistical analyses

We calculated summary statistics (mean, standard deviation, minimum, maximum) for female (Table 3.1) and male (Table 3.2) firing rates (**FR**; i.e., the number of action potentials per second) using the “psych” package (version 2.4.3; Revelle, 2009) in R (R Core Team, 2020). We also calculated the “percent change” (**PC**) for each trial, which is the percent change in mean firing rate between the preparation and stimulation periods (Table 3.3). To calculate the PC, we used the following equation: $((\text{Stimulation FR} - \text{Preparation FR}) / \text{Preparation FR}) * 100$. For example, a trial with a Preparation FR of 5.93 APs/sec and a Stimulation FR of 10.2 APs/sec would yield a PC value of ~72%. Furthermore, a positive PC value represents an excitatory response from the nerve, whereas a negative value represents an inhibitory response. We chose to standardize our data in this fashion to reduce variation due to individual differences in

baseline nerve activity, and because males typically had higher firing rates than females. PC values lent context to our FR summary statistics and acted as the dependent variable in our statistical models. Summary statistics are written as mean($\pm$ SD) throughout the rest of this chapter.

Because females and males were used an unequal number of times, we also evaluated percent change in the nerve FR with general linear mixed-effects models (Table 3.4) using the R packages "lme4" (version 1.1.35.3; Bates et al., 2015) and "lmerTest" (version 3.1.3; Kuznetsova et al., 2017). Sweep angle and speed were treated as fixed effects. In models testing for differences between the sexes, animal ID was included as a random effect (Table 3.5). Due to limited power and unbalanced sample sizes between males and females ($n = 5,14$ respectively) we assessed responses to sweep angles and speeds within each sex and used either female ID or male ID (as appropriate) as a random effect. We set the restricted maximum likelihood (REML) to false because we compared models with different fixed effects (Bolker, 2015).

RESULTS

Responses of the female ADLLn to individual parameters

Responses to sweep angles

The ADLLn of female axolotls exhibited excitatory responses (i.e., a positive percent change in FR) to all 3 sweep angles that we tested, but displayed the largest PC when stimulated with a moderate sweep angle of 30°. Our summary statistics indicate that a stimulus of 10° caused the firing rate (FR) of the ADLLn to increase from

4.33($\pm$ 4.57) APs/sec during the preparation period (i.e., before the Robotail was turned on) to 5.35($\pm$ 5.88) APs/sec during the stimulation period (i.e., while the Robotail was in operation). A 30° stimulus caused the FR to increase from 6.57($\pm$ 9.39) to 12.23($\pm$ 19.52) APs/sec, and a 90° stimulus resulted in an increase in FR from 6.46($\pm$ 5.64) to 9.98($\pm$ 7.67) APs/sec (Table 3.1). Additionally, the PC (i.e., change in FR from preparation to stimulus period) for female ADLLNs exposed to a sweep angle of 10° was 75.42($\pm$ 272.48)%; a stimulus of 30° resulted in a PC of 203.75($\pm$ 461.37)%, whereas a stimulus of 90° resulted in a PC of 111.05 ($\pm$ 223.11)% (Table 3.3; Fig. 3.4). The results of our general linear mixed-effects models corroborated these findings; the FR exhibited a significantly greater increase (i.e., we observed a higher PC) when the Robotail was moving at a sweep angle of 30° compared to 10°, but showed no difference when we presented our preparation with extreme (90°) angles (10° vs 30°, $t = 2.001$, $p = 0.049$; 10° vs 90°, $t = 0.686$, $p = 0.494$; Table 3.4; Fig. 3.4).

Responses to speed

The female ADLLN exhibited excitatory responses to all 3 speeds that we tested and displayed the greatest increase in FR when stimulated by a moderate speed of 1.0 Hz. A stimulus of 0.5 Hz caused the FR to increase from 5.82($\pm$ 6.59) APs/sec during the preparation period to 8.01($\pm$ 10.61) APs/sec during the stimulation period; 1.0 Hz resulted in an increase in FR from 6.01($\pm$ 6.59) to 9.80($\pm$ 12.02) APs/sec, whereas 1.5 Hz resulted in an increase of 5.59($\pm$ 7.26) to 9.91($\pm$ 14.56) APs/sec (Table 3.1). Additionally, the PC value for female ADLLN stimulated by a speed of 0.5 Hz was 94.05($\pm$ 317.70)%; a stimulus of 1.0 Hz resulted in a PC of 196.24($\pm$ 423.06)% , whereas a stimulus of 1.5 Hz resulted in a mean PC of 90.80($\pm$ 196.39)% (Table 3.3; Fig. 3.5). Although female

nerve responses to the 3 speeds did not differ significantly, we observed a slightly elevated PC when presented with a stimulus speed of 1.0 Hz compared to 0.5 Hz ($t = 1.653$, $p = 0.100$; Table 3.4; Fig. 3.5).

Responses of the male ADLLn to single parameters

Responses to sweep angle

ADLLn activity in males was inhibited when stimulated with a sweep angle of 10° , but we observed excitatory responses when we tested sweep angles of 30° and 90° . Specifically, a stimulus of 10° resulted in a decrease in FR from $12.35(\pm 14.50)$ APs/sec during the preparation period to a FR of $10.45(\pm 12.97)$ APs/sec during the stimulation period. In contrast, a 30° stimulus caused the FR to increase from $8.50 (\pm 11.77)$ to $9.54(\pm 13.46)$ APs/sec, and a 90° stimulus resulted in an increase from $33.90(\pm 18.68)$ to $40.57(\pm 20.57)$ APs/sec (Table 3.2). Furthermore, the PC value for male ADLLn exposed to a sweep angle of 10° was $-7.07(\pm 90.14)\%$, whereas 30° resulted in a PC of $33.00(\pm 91.78)\%$ and 90° resulted in a PC of $5.71(\pm 63.05)\%$ (Table 3.3; Fig. 3.6). We found no significant differences in male ADLLn PC responses to the 3 sweep angles that we tested, but the nerve did display a slightly elevated response to a sweep angle of 30° ($t = 1.642$, $p = 0.106$; Table 3.4; Fig. 3.6).

Responses to speed

In males, the ADLLn exhibited an inhibitory response to a slow speed of 0.5 Hz, but excitatory responses to moderate and fast speeds. A speed of 0.5 Hz caused a decrease in nerve activity from a FR of $14.16(\pm 18.48)$ APs/sec during the preparation period to a FR of $13.92(\pm 16.68)$ APs/sec during the stimulation period; 1.0 Hz resulted

in an increase in FR from 15.89($\pm$ 17.47) to 16.38($\pm$ 20.19) APs/sec, whereas 1.5 Hz resulted in an increase in FR from 14.12($\pm$ 15.56) to 17.12($\pm$ 20.20) APs/sec (Table 3.2). Accordingly, male ADLLn stimulated by 0.5 Hz exhibited a PC value of 26.41($\pm$ 95.16)%, a stimulus 1.0 Hz resulted in a PC of 6.48($\pm$ 97.46)%, whereas a stimulus of 1.5 Hz resulted in a PC of 4.69($\pm$ 69.35)% (Table 3.3; Fig. 3.7). We did not find any significant differences in male PC responses to the 3 speeds that we tested (Table 3.4; Fig. 3.7).

Female vs male responses to single parameters

Overall, PC values were lower in the ADLLn of male axolotls compared with that of females ($t = -2.095$, $p = 0.056$). Additionally, when presented with a moderate sweep angle or faster speeds, the ADLLn of males exhibited a marginally lower PC than females (30° , $t = -1.81$, $p = 0.108$; 1.5 Hz, $t = -1.914$, $p = 0.071$; Table 3.5).

Female responses to combinations of sweep angles and speeds

The ADLLn of female axolotls exhibited a significantly higher PC value when stimulated by a combination of $30^\circ/1.0$ Hz, which represented a moderate sweep angle and speed, compared to a combination of $10^\circ/0.5$ Hz ($t = 2.962$, $p = 0.004$). Additionally, the female ADLLn displayed a marginally higher PC to a combination of $10^\circ/1.0$ Hz ($t = 1.467$, $p = 0.144$; Table 3.6).

Male responses to combinations of sweep angles and speeds

We found no significant differences in response of the ADLLn of males to the 9 different combinations of sweep angles and speeds that we tested (Table 3.7). However, our ability to detect differences in nerve responses among our male subjects is likely limited by our sample size ($N = 5$). Additionally, the ADLLn response to the

Robotail stimulus was inhibitory when presented with narrow sweep angle parameters or slow speeds, but was excitatory when presented with other sweep angles and speeds; therefore, combination effects may be obscured in the overall PC.

Comparison of the male hula to female behavioral and physiological responses

We found a high level of alignment between male hula behaviors and responses of the female ADLLn to the Robotail. During courtship, male axolotls most often hula'd with a combination of 30°/1.0 Hz, which represents both a moderate sweep angle and speed, and 30°/1.5 Hz, which represents a moderate sweep angle combined with a relatively high speed. We also found that female axolotls exhibited heightened ADLLn responses to a hula combination of moderate speed and sweep angle. When we stimulated the female ADLLn using the Robotail, we found that the nerve exhibited a significantly higher PC to a combination of 30°/1.0 Hz (compared to 10°/0.5 Hz). We also observed a marginally higher female ADLLn response to 10°/1.0 Hz, which males only performed “sometimes” (Fig. 3.8).

In contrast, we observed a moderate level of alignment between male hula patterns and female behavioral responses to the Robotail. Female axolotls transitioned between locomotive states (i.e., walking, swimming, and pausing) significantly more times per minute when the Robotail was oscillating with a pattern of 30°/1.5 Hz or 90°/1.5 Hz compared to 10°/0.5 Hz (see Chapter 2). Additionally, females transitioned between locomotive states marginally more often when the Robotail operated with a pattern of 30°/1.0 Hz ($t = 1.785$, $p = 0.076$) or 90°/0.5 Hz ($t = 1.693$, $p = 0.093$; Fig. 3.8) compared to 10°/0.5 Hz. Thus, we found a moderate level of alignment between male and female behavior for the combination of 30°/1.0 Hz; male axolotls performed this

pattern often during courtship, but females only exhibited a slightly higher rate of locomotion transitions, which may indicate a reduced searching effort by the female to locate the source of mechanical stimuli. Although we observed a greater level of alignment between the male hula and female behavioral response for the combination of 30°/1.5 Hz, female axolotls also transitioned between locomotive states more rapidly when the Robotail was moving at wider angles, which male axolotls only performed “sometimes” during courtship.

DISCUSSION

Female ADLLn responses to single parameters

The ADLLn of female axolotls exhibited excitatory responses to all sweep angles and speeds that we tested but displayed the largest increase in FR when exposed to moderate sweep angles (30°) and moderate speeds (1.0 Hz; Table 3.1). Behaviorally, male axolotls generally hula using intermediate sweep angles during courtship (Chapter 2); thus, we found a high level of matching between the sweep angle of the male’s hula and the physiological response of the female AD LL nerve. Interestingly, in our behavioral experiments (Chapter 2), we also observed that females responded to wide sweep angles by initiating bouts of locomotion more frequently, compared to narrower angles; we speculate that this result may represent an increased search effort by the female to localize the male. One potential explanation is that a wider sweep angle may deflect the neuromast hair cells at an intermittent rate, whereas a narrower sweep angle likely results in more consistent stimulation, which may partially explain our behavioral results.

Based on the work of Mogdans & Bleckmann (1999) in goldfish (*Carassius auratus*) lateral line nerves, we expected the firing rate of the ADLLn to increase as the stimulus amplitude increased; instead, we observed a more subdued response when the sweep angle was increased to 90°. However, Mogdans & Bleckmann (1999) used a small vibrating sphere that moved in a single axis to generate their stimuli, whereas we used a flexible robot with greater degrees of freedom in its motion patterns, and so there were perhaps other hydrodynamic factors at play in our experiment that may explain the discrepancy in our results. Additionally, the mechanical stimuli that arise from the Robotail are naturalistic, so we may have observed an aspect of sender-receiver matching occurring, given that the female ADLLn generally responds more strongly to motions that males display often (i.e., 30° sweep or 1.0 Hz) compared to wider sweep angles.

Similarly, the female ADLLn exhibited a larger percent change in FR when we stimulated the nerve with a moderate speed of 1.0 Hz, and a smaller PC when tested the fastest speed of 1.5 Hz (Table 3.3). Thus, we also observed a high level of alignment between male hula behaviors and female neurophysiological responses within the speed parameter, given that male axolotls generally hula'd with moderate speeds of 1.0 Hz (see Chapter 2). In contrast, our previous behavioral research demonstrated that female axolotls spent more time near the Robotail when it was moving at 1.5 Hz (Chapter 2). It is unclear why female axolotls behaviorally preferred faster hula speeds but the female ADLLn responded with a faster FR when presented with a moderate speed stimulus, although we speculate that faster hula speeds may be indicative of a higher quality mate or may serve to draw the female closer to the male

during courtship. Meadow katydid (*Conocephalus nigropleurum*) females also prefer male substrate-borne vibrational signals with shorter inter-pulse intervals (i.e., faster frequencies), which are indicative of a larger, and likely higher-quality, potential mate. Additionally, our results from Chapter 2 suggest that male axolotls are somewhat more likely to hula at 1.5 Hz when they are at a distance from a female, thus, it is possible that faster hula speeds may serve to draw the female towards the male during courtship.

Male ADLLn response to single parameters

The ADLLn of male axolotls exhibited inhibitory responses to narrow sweep angles (10°) and slow speeds (0.5 Hz), but excitatory responses to all other sweep angles and speeds. Although male ADLLn responses did not differ significantly with sweep angle, we found that the nerve displayed a marginally elevated FR when stimulated by a sweep angle of 30° (Table 3.4). Male axolotls, when hula-ing at a distance from a female, are more likely to perform the hula with a narrow sweep angle of 10° (see Chapter 2). Romer (1993) proposed that male insects in the order Orthoptera (i.e., grasshoppers, locusts, crickets) experience an inhibitory effect in the auditory pathway when other calling males are nearby. Thus, an inhibitory response of the male ADLLn to 10° may represent the presence of another male that is attempting to advertise to a nearby female. In contrast, male axolotls hula with a slightly wider sweep angle when they are closer to females; thus, a heightened FR within the male ADLLn may indicate that the male should engage in competition with the rival male rather than avoid him.

Female vs male responses to single parameters

Overall, the ADLLn of male axolotls exhibited significantly smaller increases in FR (i.e., between preparation and stimulus periods) than that of female axolotls. In the blue-spotted fantail stingray (*Taeniura lymma*), females possess a greater number of electrosensory axons within the anterior lateral line nerve than males do (Kempster et al., 2013). Given that the lateral line system in axolotls is composed of both mechanosensory and electrosensory modalities (Northcutt, 1992), female axolotls may possess higher numbers of mechanically sensitive axons within the ADLLn, which could contribute to the higher PCs that we observed in females. Alternatively, females exhibited lower FRs overall than males did, so this result may be explained by the fact that there is a greater potential for an increase in female FR compared to males.

Although we did not detect any significant differences between female and male nerve responses when we examined specific parameters, we found that the female ADLLn exhibited a marginally higher increase in FR than the male ADLLn when stimulated by a moderate angle of 30° or a fast speed of 1.5 Hz. A tail oscillation speed of 1.5 Hz may indicate the presence of a particularly robust male, whereas a sweep angle of 30° may represent a male that is preferable for females. The male ADLLn may experience an inhibitory effect when stimulated by these parameters. Within bushcrickets, male *Hemisaga denticulata* produce a discontinuous song, whereas males of the sympatric species *Mygalopsis marki* sing continuously. The auditory pathway of *H. denticulate* exhibits an inhibitory response when stimulated by the song of *M. marki*; additionally, researchers have observed that discontinuously singing bushcrickets reduce their singing activity in the presence of heterospecific crickets that

are singing continuously (Romer, 1993). Although we did not examine behavioral interactions between different salamander species, it is possible that the hula behavior in male axolotls may inhibit activity in the ADLLn of rival males and possibly hula-ing as well; this effect may allow males to conserve time and energy during courtship.

Female responses to combinations of sweep angles and speeds

The results of our experiments revealed a high level of alignment between male hula motion patterns and female physiological responses to those motions. The firing rate within the female ADLLn was significantly elevated when stimulated with a hula combination of 1.0 Hz and a 30° sweep angle (Table 3.6). This finding is corroborated by our single-parameter general linear mixed-effects statistical models; the female ADLLn exhibited a significantly greater increase in FR when stimulated by 30°, and a marginally higher response when stimulated by 1.0 Hz (Table 3.4). 30°/1.0 Hz is also one of the hula combinations that males most often display during actual courtship (Fig. 3.8). The female ADLLn also exhibited a marginal increase in FR when stimulated by 10°/1.0 Hz, although this particular hula combination was only “sometimes” displayed by the males in our behavioral experiments. Our experimental results suggest that hydrodynamic stimuli play an important role in axolotl courtship, and that females are attuned to detect mechanical communication signals that arise from the male hula behavior.

Male responses to combinations of sweep angles and speeds

We found no marginal or significant differences the responses of the male ADLLn to the 9 different combinations of sweep angles and speeds that we tested. The male

ADLLn may be more sensitive to changes in single hula parameters, given that the nerve showed a slightly elevated response to a 30° sweep angle (Table 3.4). These results could be partially due to our small sample size (5 males). Alternatively, given that the male ADLLn exhibited both excitatory and inhibitory responses, it is possible that any differences we observed with single parameters may have been “canceled out” when we examined responses to combinations of parameters.

Comparison of the male hula to female behavioral and physiological responses

We observed a high level of alignment between male hula behaviors and female ADLLn responses to simulated hula motions generated by the Robotail. In contrast, we found moderate alignment between the male’s hula and female behavioral responses to the Robotail. During courtship, male axolotls performed a combination of 30°/1.0 Hz “often”; the female ADLLn exhibited a significantly elevated PC to this combination, and females behaviorally responded by exhibiting a marginally elevated number of locomotion transitions per minute. We speculate that more frequent locomotion transitions (e.g., switching between walking, swimming, and pausing) may represent more frequent search attempts by the female to localize the stimulus source, but we did not test this hypothesis directly. Males also performed a combination of 30°/1.5 Hz “often”; we found that with this combination of stimuli females exhibited a significantly higher number of locomotion transitions per minute, but we found no significant physiological responses to this particular combination of sweep angle and speed.

Female axolotls also exhibited more frequent locomotion transitions in response to combinations that males only performed “sometimes” during courtship. We speculate that female axolotls exhibit broad behavioral responses but more narrowly tuned ADLLn

responses to our hula combinations because the male hula is likely associated with other sensory stimuli, such as sex pheromones, which may cause females to respond with more complex behaviors; in contrast, the mechanosensory lateral line only functions to detect hydrodynamic stimuli and may be more sensitive to hula combinations that males perform often. Interestingly, females always transitioned between locomotive states more frequently (either significantly or marginally) when the Robotail was moving at the widest sweep angle of 90° . A wider sweep angle is likely to displace more water during the hula behavior than a narrower sweep angle would, which may deflect the hair cells of the lateral line neuromasts more drastically; this phenomenon may contribute to the stronger behavioral responses exhibited by females. Besides the marginally elevated female nerve response to a motion pairing of $10^\circ/1.0$ Hz, females did not show heightened behavioral or physiological responses to any of the other combinations that featured a narrow sweep angle of 10° .

Male axolotls may avoid moving their tails at the extremes of their sweep angle and speed ranges because these motions could be too energetically costly to sustain over the course of an entire courtship ritual. However, more vigorous tail motions from the Robotail were effective at eliciting an increased behavioral response from female axolotls through more frequent locomotion transitions. In contrast, the female ADLLn exhibited heightened, excitatory responses to more moderate hula combinations (i.e., $30^\circ/1.0$ Hz or $10^\circ/1.0$ Hz). More extreme hula motions performed in short bursts may therefore serve to draw females toward the courting male, whereas more moderate combinations of motions may confirm the presence of a conspecific male. Our results thus suggest that sender-receiver matching occurs within the mechanosensory aspect

of the lateral line system. To our knowledge, our suite of experiments is the first to demonstrate this neuroethological phenomenon within the mechanosensory lateral line.

CONCLUSION

Our electrophysiology experiment demonstrated that the ADLLn of female and male axolotls exhibited different physiological responses to various sweep angles and speeds associated with the hula behavior. We observed excitatory responses within the female ADLLn to all sweep angles and speeds that we tested, and we found that the female ADLLn responded most strongly to moderate sweep angles and moderate speeds. In contrast, the male ADLLn exhibited inhibitory responses to narrow sweep angles, but excitatory responses to all other parameters that we tested. Additionally, we found a high level of alignment between actual male hula behaviors and female ADLLn responses, and a moderate level of alignment between the male hula and female behavioral responses. Importantly, these experiments indicate that sender-receiver matching occurs within the mechanosensory lateral line system, such that the female ADLLn is highly tuned to the hydrodynamic stimuli generated by males during courtship.

TABLES

Stimulus	Preparation FR	Stimulation FR	Recovery FR
10°	4.33(4.57) 0.13-18.67	5.53(5.88) 0-23.25	2.94(4.08) 0-20.32
30°	6.57(9.39) 0.13-29.53	12.23(19.52) 0-63.63	4.64(7.86) 0-23.33
90°	6.46(5.64) 0.13-20.93	9.98(7.67) 0-27.82	3.97(5.12) 0-21.11
0.5 Hz	5.82(6.59) 0.13-23.33	8.01(10.61) 0-44.85	4.61(6.76) 0.03-21.48
1.0 Hz	6.01(6.59) 0.13-25.33	9.80(12.02) 0-55.04	3.92(5.60) 0.01-23.25
1.5 Hz	5.59(7.26) 0.13-29.53	9.91(14.56) 0-63.63	2.96(4.92) 0-23.33

Table 3.1: Summary statistics for female ADLLn firing rates. The ADLLn of female axolotls were stimulated using 3 sweep angles and 3 speeds; we then quantified the firing rates (FRs) during the preparation, stimulus, and recovery periods of each trial. FR is defined here as the number of action potentials per second, and values are displayed as “mean(SD) min-max”.

Stimulus	Preparation FR	Stimulation FR	Recovery FR
10°	12.35(14.50) 0-35.87	10.45(12.97) 0-33.14	9.02(11.32) 0-24.23
30°	8.50(11.77) 0.13-34.73	9.54(13.46) 0-40.25	5.78(8.87) 0-27.17
90°	33.90(18.68) 0.13-51.33	40.57(20.57) 0-57.40	31.64(15.71) 0-40.42
0.5 Hz	14.16(18.48) 0.07-51.33	13.29(16.68) 0-44.78	10.83(14.53) 0.03-39.74
1.0 Hz	15.89(17.47) 0-49.67	16.38(20.19) 0-57.40	11.55(14.93) 0-39.92
1.5 Hz	14.12(15.56) 0.13-45.67	17.25(20.20) 0-54.41	13.16(15.38) 0-40.42

Table 3.2: Summary statistics for male ADLLn firing rates. The ADLLn of male axolotls were stimulated using 3 sweep angles and 3 speeds; we then quantified the FRs during the preparation, stimulus, and recovery periods of each trial. FR is defined here as the number of action potentials per second, and values are displayed as “mean(SD) min-max”.

Stimulus	♀ PC (%)	♂ PC (%)
10°	75.24(272.48) -100.00-1787.22	-7.07(90.14) -100.00-250.00
30°	203.75(461.37) -100.00-1585.80	33.00(91.78) -100.00-303.12
90°	111.05(223.11) -100.00-1454.31	5.71(63.05) -100.00-119.42
0.5 Hz	94.05(317.70) -100.00-1832.33	26.41(95.16) -100.0-205.00
1.0 Hz	196.24(423.06) -100.00-1858.80	6.48(97.46) -100.00-303.12
1.5 Hz	90.80(196.39) -100.00-1038.14	4.69(69.35) -100.00-127.27

Table 3.3: Summary statistics for female and male ADLLn PCs. The ADLLn of female and male axolotls were stimulated using 3 sweep angles and 3 speeds, and PC of the nerve to each stimulus was then quantified. PC is defined here as the percent change in firing rate between the preparation and stimulus phase of each trial; for example, a PC of 75% means that the FR of the ADLLn nerve increased by 75% from the preparation to the stimulus phase. Values are displayed as “mean(SD) min-max”.

Dependent Variable	Random Effect	Model	Fixed Effects	t statistic	p - value
Percent Change	Female ID	Sweep	30° sweep	2.001	0.049 *
			90° sweep	0.686	0.494
		Speed	1.0 Hz	1.653	0.100
			1.5 Hz	-0.034	0.973
Percent Change	Male ID	Sweep	30° sweep	1.642	0.106
			90° sweep	0.413	0.681
		Speed	1.0 Hz	-0.725	0.471
			1.5 Hz	-0.79	0.433

Table 3.4: General linear mixed-effects models for female and male PCs. We evaluated percent changes (PC) in female and male ADLLn firing rate in response to hula motion parameters (sweep angle and speed). PC reflects the percent change in firing rate from the preparation period to the stimulation period. Females exhibited a significantly higher PC to a sweep angle of 30° compared to 10°.

Dependent Variable	Random Effect	Model	Data Set	t statistic	p - value
Percent Change	Animal ID	Sex	All	-2.095	0.056*
			10°	-1.207	0.256
			30°	-1.81	0.108
			90°	-1.55	0.149
			0.5 Hz	-0.663	0.517
			1.0 Hz	-1.387	0.187
			1.5 Hz	-1.914	0.071

Table 3.5: General linear mixed-effects models for female vs. male PCs. We evaluated percent changes (PC) in ADLLn firing rate between males and females in response to combinations of stimuli across all 3 sweep angles and all 3 speeds. Overall, male axolotls exhibited lower PCs compared to females. Additionally, males displayed marginally lower PCs to a moderate sweep angle of 30° and fast speed of 1.5 Hz, compared to females.

Dependent Variable	Random Effect	Model	Fixed Effects		t statistic	p - value
			Sweep(°)	Speed(Hz)		
Percent Change	Female ID	Combo	30	0.5	1.023	0.308
			90	0.5	1.171	0.243
			10	1.0	1.467	0.144
			30	1.0	2.962	0.004**
			90	1.0	0.788	0.432
			10	1.5	0.111	0.911
			30	1.5	0.171	0.243
			90	1.5	0.904	0.368

Table 3.6: General linear mixed-effects models for female responses to combinations of sweep angles and speeds. We evaluated percent changes (PC) in female ADLLn firing rate in response to 9 combinations of sweep angles and speeds. Females exhibited a significantly higher PC to a combination of 30° and 1.0 Hz, and a marginally higher PC to a combination of 10° and 1.0 Hz.

Dependent Variable	Random Effect	Model	Fixed Effects		t statistic	p - value
			Sweep(°)	Speed(Hz)		
Percent Change	Male ID	Combo	30	0.5	0.088	0.930
			90	0.5	-0.66	0.512
			10	1.0	-1.23	0.223
			30	1.0	-0.282	0.779
			90	1.0	0.061	0.952
			10	1.5	-1.309	0.196
			30	1.5	0.517	0.607
			90	1.5	-0.714	0.478

Table 3.7: General linear mixed-effects models for male responses to combinations of sweep angles and speeds. We evaluated percent changes (PC) in male ADLLn firing rate when presented with 9 combinations of sweep angles and speeds. We found no significant differences in male nerve responses.

FIGURES

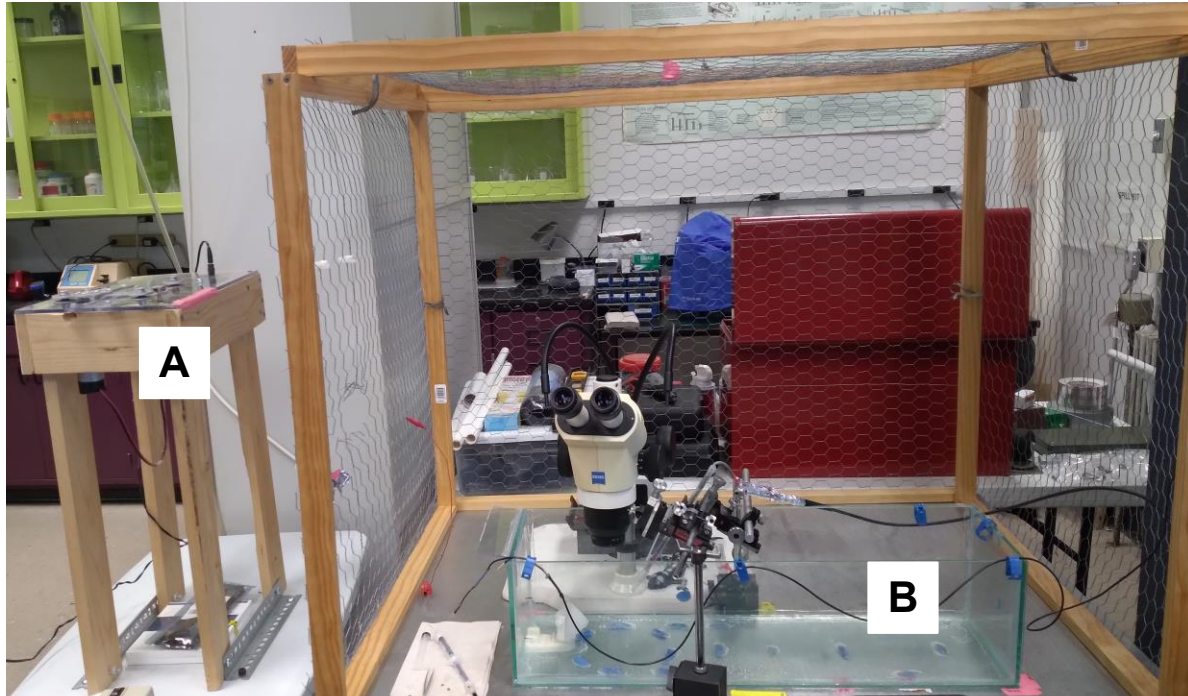


Figure 3.1: Electrophysiology setup. Photograph showing the position of the Robotail electronic components (A) relative to the aquarium (B), which was inside a Faraday cage. Nylon fishing lines were fed through openings in the cage wall to connect the robot gimbal to the silicone tail within the aquarium.

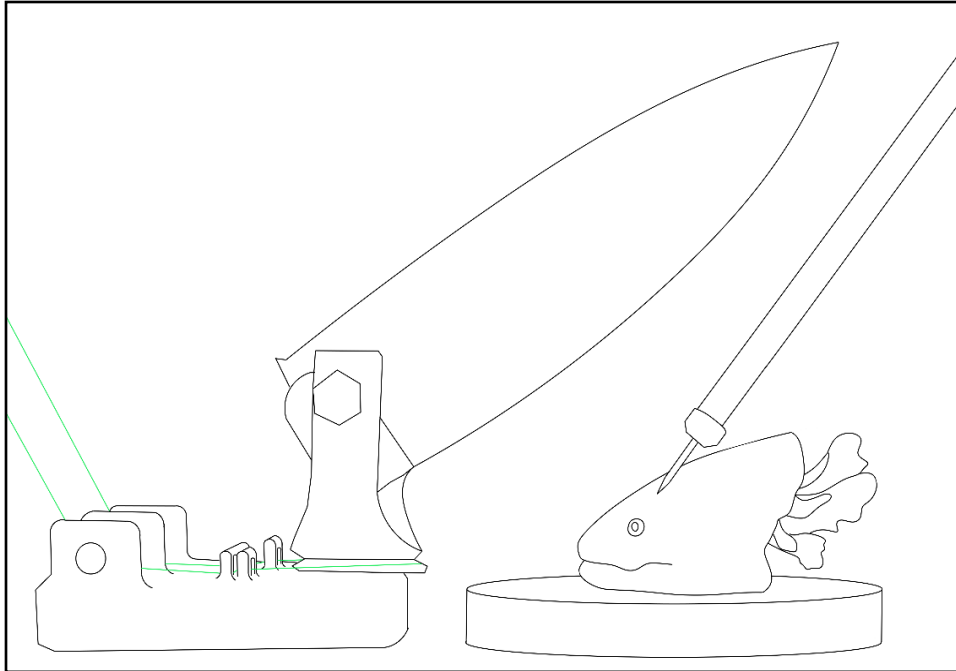


Figure 3.2: Diagram of preparation with Robotail. The preparation was placed on a Petri dish underneath the silicone tail to to mimic the position of the female relative to the male during mating and to maximize stimulation of neuromasts on the surface of the head. The recording electrode was lowered onto the right anterodorsal lateral line nerve and angled so that the tail did not interfere with the electrode while the robot was in operation.

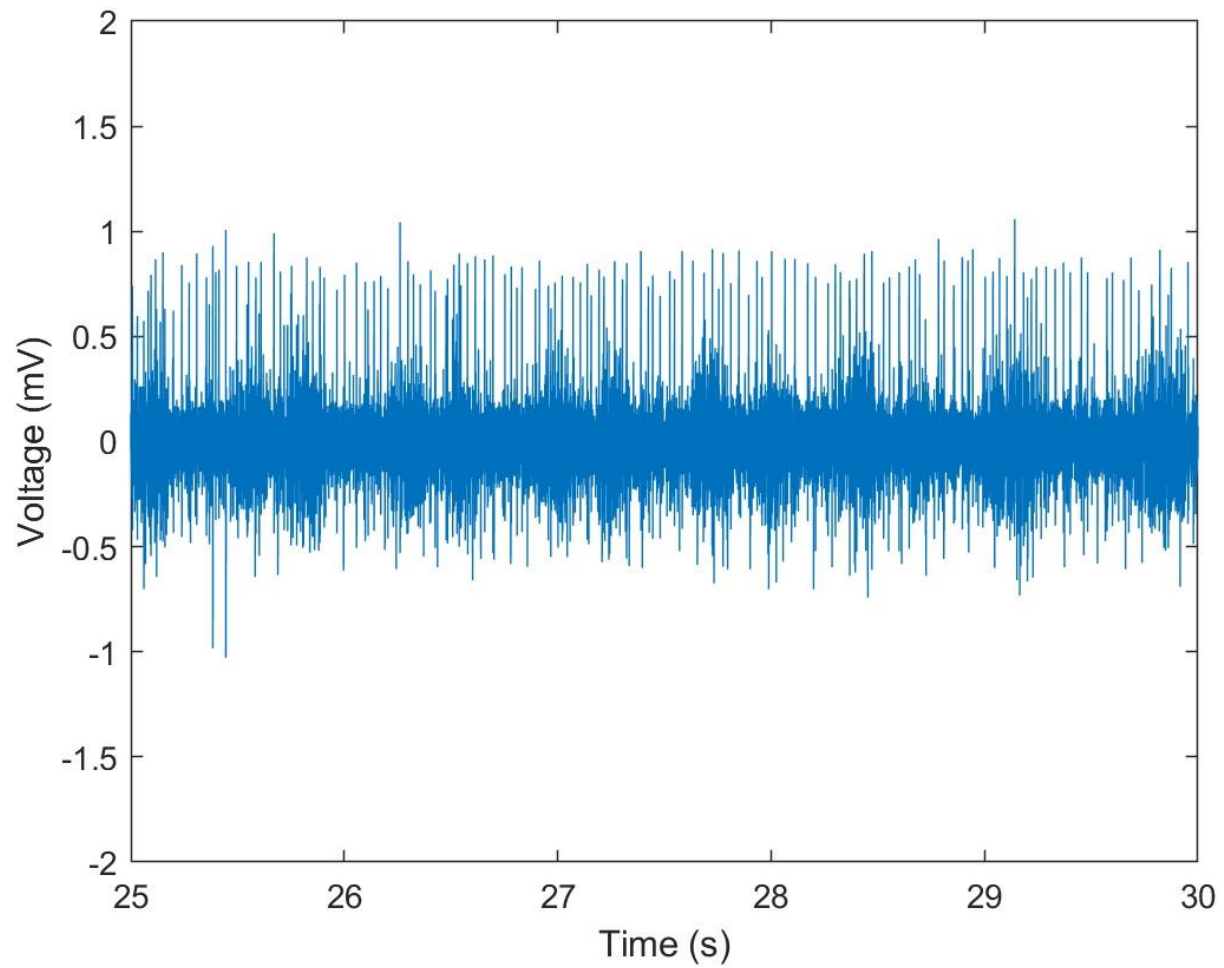


Figure 3.3: Multi-unit recording from the ADLLn stimulated with a 90° sweep angle and a 1.5 Hz speed. Action potentials were recorded from multiple fibers in the ADLLn using a suction electrode.

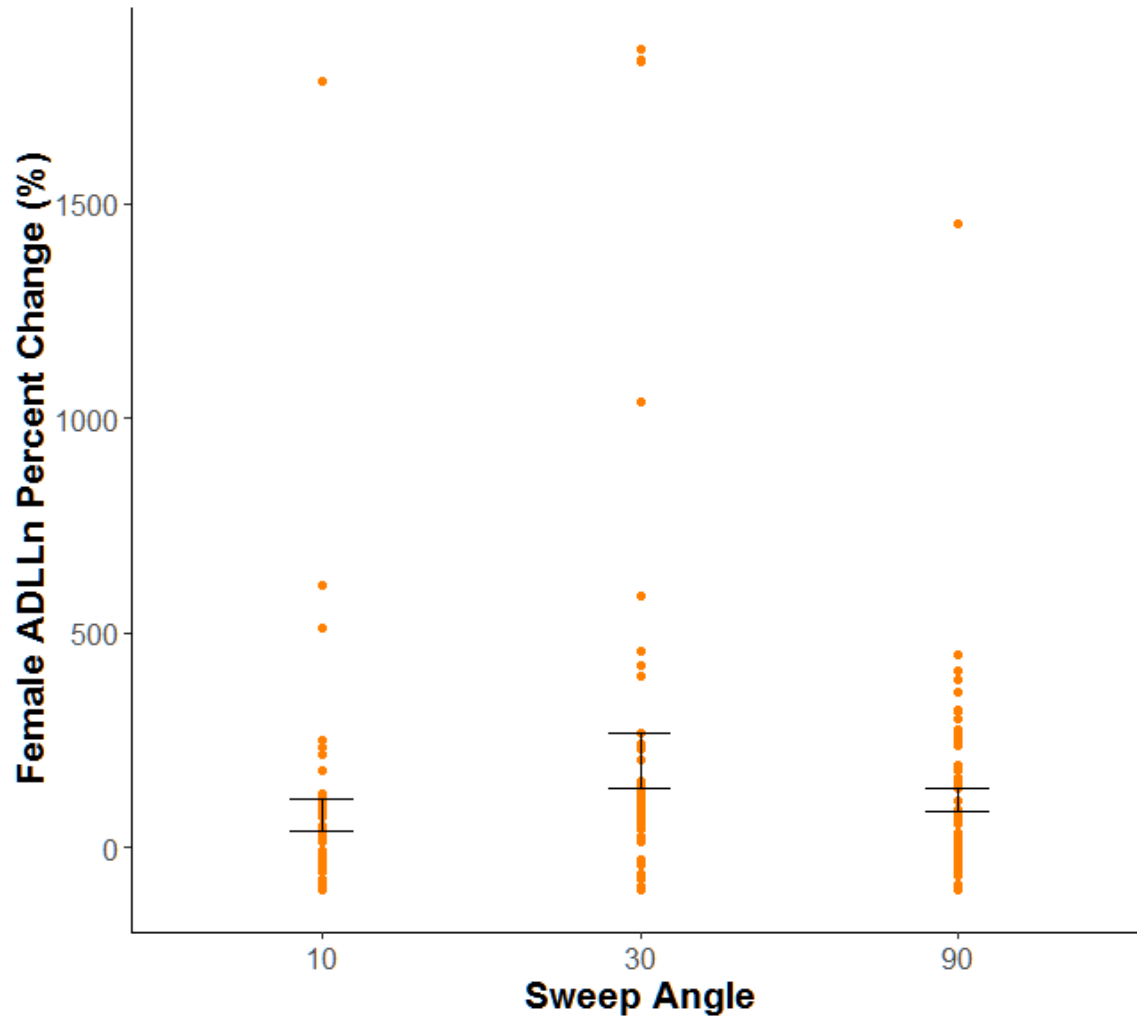


Figure 3.4: Firing rate in the female ADLLn in response to stimulation with different sweep angles. A significantly higher excitatory response from the female ADLLn was observed when presented with a moderate sweep angle of 30° compared with a narrow angle of 10° (mixed model analysis, $t = 2.001$, $p = 0.049$). Each dot represents the percent change in FR (between preparation and stimulus periods) for a given trial and bars represent the standard error of each group.

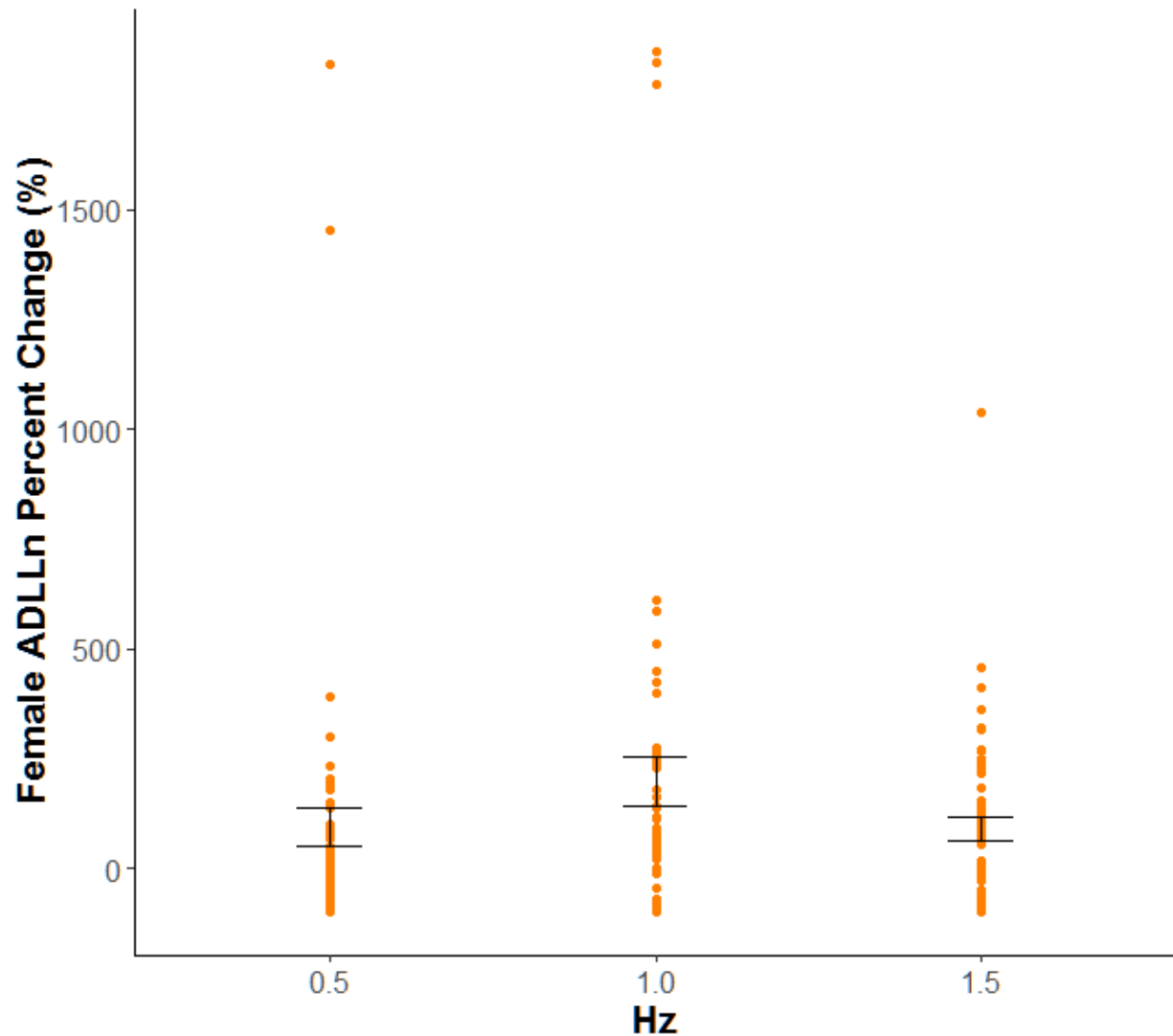


Figure 3.5: Firing rate in the female ADLLn in response to stimulation with different speeds. Our mixed model analysis revealed no significant differences in female ADLLn responses when stimulated with different hula speeds. Each dot represents the percent change in FR (between preparation and stimulus periods) for a given trial and bars represent the standard error of each group.

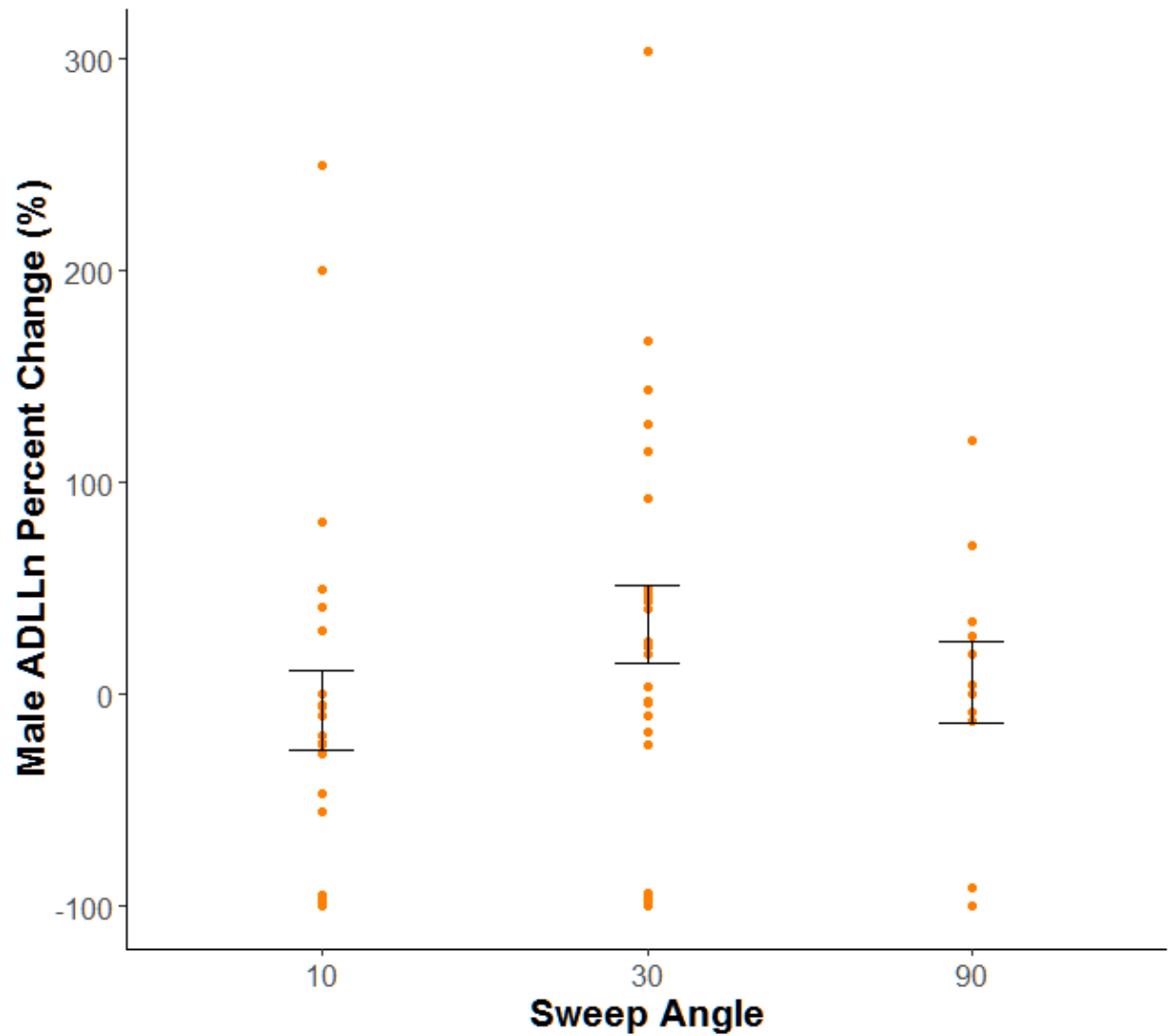


Figure 3.6: Firing rate in the male ADLLn in response to stimulation with different sweep angles. Our mixed model analysis revealed no significant differences in male ADLLn responses to different sweep angles. Each dot represents the percent change in FR (between preparation and stimulus periods) for a given trial and bars represent the standard error of each group.

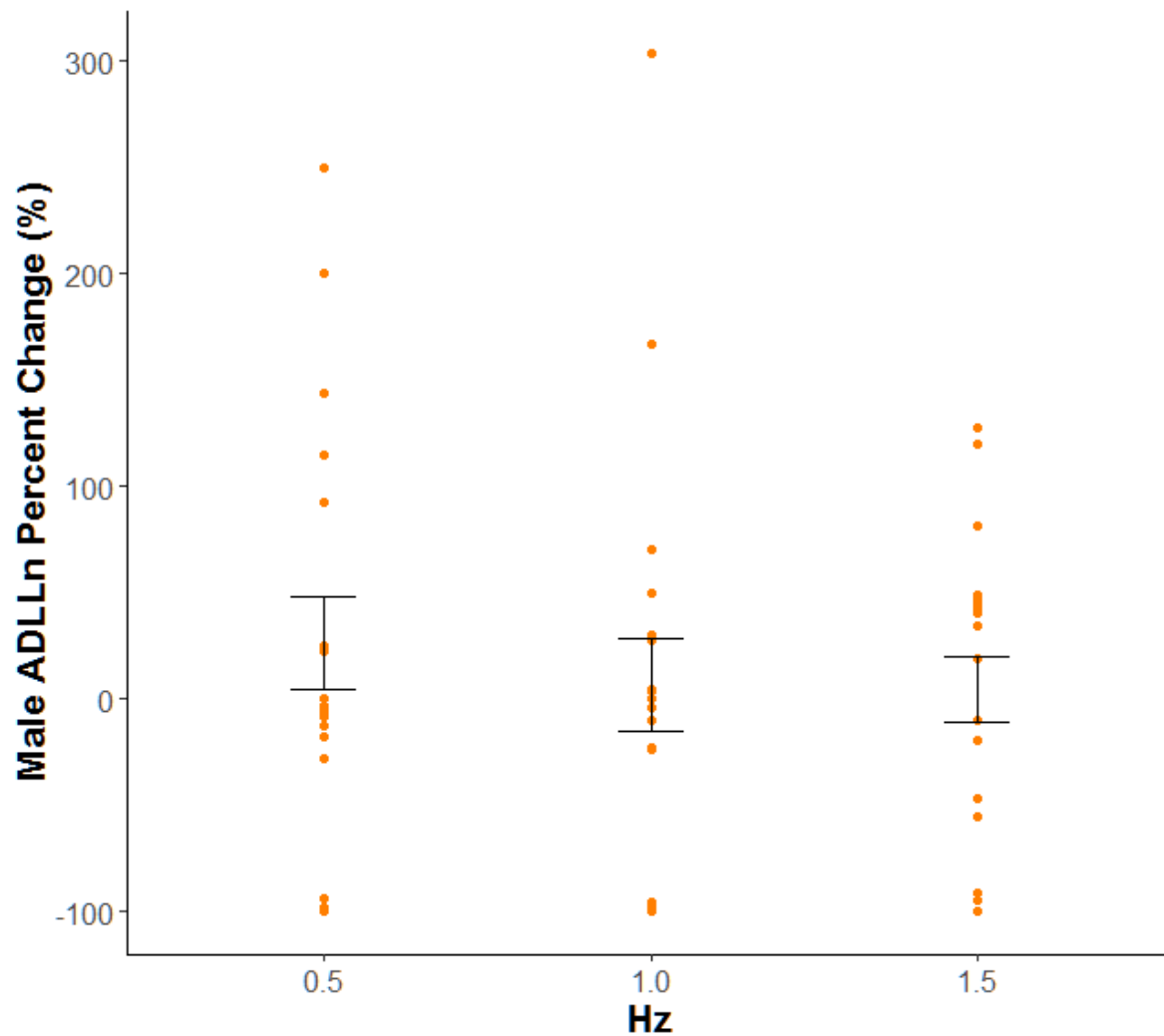


Figure 3.7: Firing rate in the male ADLLn in response to stimulation with different speeds. Our mixed model analysis revealed no significant differences in male ADLLn responses to different hula speeds. Each dot represents the percent change in FR (between preparation and stimulus periods) for a given trial and bars represent the standard error of each group.

		Sweep Angle					
		10°		30°		90°	
Speed	0.5 Hz	♂ Hula: Sometimes		♂ Hula: Sometimes		♂ Hula: Sometimes	
		♀ Behavior	♀ Physiology	♀ Behavior	♀ Physiology	♀ Behavior	♀ Physiology
		N/A	N/A	t = 0.516 p = 0.607	t = 1.023 p = 0.308	t = 1.693 p = 0.093 .	t = 1.171 p = 0.243
	1.0 Hz	♂ Hula: Sometimes		♂ Hula: Often		♂ Hula: Sometimes	
		♀ Behavior	♀ Physiology	♀ Behavior	♀ Physiology	♀ Behavior	♀ Physiology
		t = 0.694 p = 0.489	t = 1.467 p = 0.144 .	t = 1.785 p = 0.076.	t = 2.962 p = 0.004 **	t = 2.187 p = 0.030 *	t = 0.788 p = 0.432
	1.5 Hz	♂ Hula: Sometimes		♂ Hula: Often		♂ Hula: Sometimes	
		♀ Behavior	♀ Physiology	♀ Behavior	♀ Physiology	♀ Behavior	♀ Physiology
		t = -0.278 p = 0.782	t = 0.111 p = 0.911	t = 2.372 p = 0.019 *	t = 1.171 p = 0.243	t = 4.014 p = 9.51e-05 ***	t = 0.904 p = 0.368

Male Hula
Sometimes
Often

Female Behavior
Not significant
Marginally significant
Significant

Female Physiology
Not significant
Marginally significant
Significant

Figure 3.8: Comparison of male hula behaviors with female behavioral and neurophysiological responses. Alignment between male hula behaviors, female behavioral responses to the hula behavior, and responses of the female ADLLn to hula parameters. We found a high level of alignment between male behaviors and female physiological responses, especially within the 30°/1.0 Hz combination. In contrast, females exhibited a broader behavioral response to the hula combinations we tested, such that females transitioned between locomotive states more often when exposed to wider sweep angles and faster speeds.

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