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The Impact of Tank Size and Hormone Concentrations on Male-Male Claspings in

Xenopus laevis

Gabrielle P. Dufresne

Thesis Advisor: Heather J. Rhodes, Ph. D.

Denison University, Department of Biology, Granville, Ohio 43023

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Abstract

Male *Xenopus laevis* participate in a behavior known as male-male clasping. Previous research has suggested that male-male clasping may be a way for males to engage in sperm competition by increasing their likelihood of passing on their genes. To better understand the conditions under which this behavior occurs, we began pilot investigations on three questions. First, we examined whether the size of the tank impacted the frequency of clasping behavior. Then we measured the levels of testosterone and corticosterone in males to see if either hormone correlated with behavioral patterns. Finally, we looked at testes and larynx size to see if either of those correlated with behavioral patterns or hormone levels. Based off of previous knowledge in how hormones can affect dominance hierarchies, we hypothesized that males with higher concentrations of testosterone may show a higher frequency of male-female clasping and a lower frequency of male-male clasping, and those with higher concentrations of corticosterone may show a higher frequency of male-male clasping and a lower frequency of male-female clasping. We also hypothesized that testosterone levels would correlate to both testes and larynx size. The frogs were set up in triads, 2 males and 1 female, and time-lapse photography was used overnight to monitor the clasping behavior. Once the triads of frogs had gone through 2 non consecutive nights of recording, the male frogs were anesthetized to collect their testes, larynx, and a blood sample through a cardiac puncture. Two different ELISA tests were run on the plasma to determine the concentration of corticosterone and testosterone in the frogs. An initial behavioral analysis found the male-male clasping behavior occurred in 6 of the 8 trials, and the males showed similar patterns in clasping to small tank data, indicating that this behavior will still show up in the frogs given ample space, and was not an artifact of the small testing environments

in previous studies. Analysis of ELISA data suggests that testosterone may have an impact on clasping behavior with frogs having higher testosterone levels being the dominant frog. Our data also suggests that testes size also relates to clasping behavior with frogs with larger testes being the more dominant frog. Determining a possible relationship between *Xenopus laevis* hormone levels and the frequency of the clasping behavior will allow us to better understand the mechanisms behind their reproductive behaviors.

Introduction

The reproductive behaviors of anurans have been researched for decades. The majority of anurans use external fertilization and will release their respective gametes in a posture known as “amplexus” (Chuang et al., 2013). Amplexus involves one frog clasping another frog from behind. Typically, a male frog would be clasping a female frog in hopes to increase fertilization, however in certain species, males may clasp other males to gain proximity to a mating event and to contribute sperm (Rhodes et al., 2014; Chuang et al., 2013; Carvajal-Castro et al., 2020).

Competition between males tends to lead to the occurrence of male-male clasping. Size, density, reproductive success, and type of breeding pattern all contribute to an increase in the competition between males (Luo et al., 2016; Wells, 1977; Rhodes et al., 2014; Liao et al., 2010). There are two basic patterns of reproduction: explosive breeding and prolonged breeding (Wells, 1977). Explosive breeders tend to have a breeding period that lasts for only a few hours to a few weeks. As a consequence of this, dense aggregations will form resulting in the male frogs clasping any small moving objects around them, whether it is a female frog, a male frog, or something else (Arak, 1983). This leads to a great deal of male-male competition where aggressive behaviors may be observed towards other males for prime positioning on a female (Wells, 1977). For prolonged breeders, their breeding season will last longer than a month. The

consequence of this style of breeding is that unlike explosive breeders, where the males actively search for the females, the females will typically come to the males (Wells, 1977). In many species of prolonged breeders, we will see indirect competition between males (vocal competition), rather than the physical, direct competition between males seen in explosive breeders (Wells, 1977; Tobias et al., 2010).

The African clawed frog, *Xenopus laevis*, is one of the species that has been observed to engage in male-male clasping (Tobias et al., 2004; Rhodes et al., 2014; Wells 1977). *X. laevis* are a fully aquatic species that take part in a prolonged breeding season from July to December (Tobias et al., 2004). Due to the fact that they have this prolonged breeding season, it would be predictive that they would be more selective in their mates because time is not acting as a reproductive pressure. Research done by Rhodes et al., (2014) has shown that male *X. laevis* are able to distinguish between male and female frogs since they showed a sex preference to clasping female frogs over male frogs. This alluded to the idea that any male-male clasping that occurs is purposeful. Therefore, this idea that the male-male clasping behavior was no mistake led to the belief that this species of frog engages in sperm competition as an alternative tactic of reproduction (Rhodes et al., 2014).

Male-male interactions can also be examined through vocalizations (Tobias et al., 2004; Tobias et al., 2010). In *X. laevis* vocal communication is incredibly important in male-male interactions regarding territory, intermale spacing, chorusing and reproduction in general (Tobias et al., 2004; Wells, 1977). There are varying calls that both males and females will use to express their sexual receptiveness/readiness. In males, an advertisement call indicates that the male is sexually active and ready to mate. Tobias et al., (2004) placed two sexually active male frogs together in a tank and found that one frog would become the vocally dominant frog and produce

this advertisement call more than the other. In these duos, male-male clasping occurred from time to time, and they found that there were different calls associated with this behavior. The male clasping would produce either a chirp or amplexant call, while the male being clasped would produce a growl vocalization. The male clasping would also produce the advertisement call more often than the male being clasped. When placed with a sexually receptive female, they found that the female was more “attracted” to whoever was producing the advertisement call. This suggests that the vocally dominant male, would also become the sexually dominant male when placed in groupings with a female. Rhodes et al., (2014), found contradictory evidence to the idea that the vocally dominant male would also be the sexually dominant male. When looking strictly at behavior, and no calls were analyzed, it was found that the males who do not engage in clasping of other males tend to be more reproductively successful when a female is added (Rhodes et al., 2014).

Vocalizations in *X. laevis* have also been studied in relation to androgen concentrations. Male advertisement calling is known to be completely dependent on the presence of androgens (Wetzel and Kelley, 1983). If a male frog is castrated, and there are no androgens flowing in their bodies, they will not only cease to produce the advertisement call, but also cease to clasp altogether (Moore et al., 2005). Injecting a testosterone agonist into normal male frogs led to an increase in the amount of advertisement calls produced and injecting a testosterone agonist into castrated males led to the restoration of advertisement calling and the reappearance of clasping behavior (Hoffmann and Kloas, 2012; Moore et al., 2005; Wetzel and Kelley, 1983). In addition to androgen levels affecting the vocalizations of *X. laevis*, they also have a correlation to both larynx and testes size. The larynx in male *X. laevis* is much larger than in female *X. laevis*, and this can also be attributed to androgen levels in the frogs (Sassoon et al., 1986; Yamaguchi and

Kelley, 2000). Testes size has also been found to have a positive relationship with overall and maximum possible androgen levels within frogs (Emerson and Hess, 2001; Emerson, 1997).

Glucocorticoids, or more specifically, corticosterone, is another hormone that has been identified to impact vocalization and mating behaviors in frogs (Leary et al., 2006; Emerson and Hess, 2001). In other anuran species, the increase in corticosterone in males may induce a satellite reproductive tactic and decrease the production of reproductive calls (Leary et al., 2006; Emerson and Hess, 2001). A satellite reproductive tactic in frogs involves a non-calling male staying in relatively close proximity to a calling male in hopes of intercepting the female that is attracted to the calling males' vocalizations (Leary et al., 2004).

The purpose of our study was to run two pilot experiments to further investigate this male-male clasping behavior. Due to the fact that all of the previous studies done used small tanks to observe the clasping behavior, we wanted to see if placing the frogs in a larger tank environment would impact the presence of the behavior. We hypothesized that the tank size would not be an artifact in the presence of the male-male clasping behavior. The second question we looked at was if either testosterone or corticosterone had any relationship to the clasping behaviors observed between the frogs. Previous research has shown that those with higher testosterone levels produce more advertisement calls, and those who produce more advertisement calls tend to be the dominant male. In addition, higher levels of corticosterone have been associated with an increase in satellite reproductive behaviors as well as a decrease in vocal behavior. Based off of these reasons as well as previous knowledge on how hormones can affect dominance hierarchies, we hypothesized that males with higher concentrations of testosterone may show a higher frequency of male-female clasping and a lower frequency of male-male clasping, and those with higher concentrations of corticosterone may show a higher frequency of

male-male clasping and a lower frequency of male-female clasping. As a part of this question, we also wanted to look at testes size and larynx size to see if they had any correlation with hormone levels or behavioral patterns. For this we hypothesized that there would be a correlation between testosterone and testes size as well as testosterone and larynx size.

Methods

Ethics Statement and Animals

All animal handling and experiments were conducted in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and with approval and oversight from the Denison University Institutional Animal Care and Use Committee. 12 adult *Xenopus laevis* frogs were used: 4 pigmented females (avg mass = 145.65 g, avg length = 11.73 cm), 4 pigmented males (avg mass = 70.51 g, avg length = 8.85 cm), and 4 albino males (avg mass = 68.82 g, avg length = 8.46 cm). Frogs were purchased from Nasco (Fort Atkinson, Wisconsin) and were housed in large unisex group tanks, 5-10 frogs per tank, at room temperature (20-23°C) on natural light cycles. Experiments were conducted between September 2020-October 2020 in Granville, OH.

Investigation on Clasping Behavior

To investigate the frequency of male-male clasping triads of frogs were used. A pigmented male frog and an albino male frog were placed into a small 12 L tank to allow for a 24-hour acquaintance period (Night 0). The tank was filled with 6 L of treated tap water and was placed inside of a larger, 1m x 1m x .5m tank that was filled with 56 L of water which was equivalent to a water depth of 3 inches (Figure 1). The water temperature during each night of the experiment was controlled to be 18°C. The frogs were placed together anytime between 6:30pm-10:15pm.

The following night (Night 1), a Logitech webcam was placed over the tank containing the frogs from night 0. Using YAWCAM software, a picture was taken every minute throughout the night to observe the clasping behavior. This was done under low light conditions achieved by placing 4 LED lamps behind a Carolina Biological Supply red 650-filter about .5 inch from the sides of the larger tank. The recording period for night 1 typically started around 10 pm and ended at around 8:15 am. On this night, one pigmented female frog was separated from the housing tanks and placed alone in a small 10 L tank filled with 4 L of treated tap water. The female was not recorded and was separated as preparation for joining the males during night 2.

The two male frogs and female frog were taken out of their smaller tanks and placed into the larger tank the following night (Night 2) (Figure 1). The low light conditions and the time lapse photography set up remained the same on Night 2. Photos were taken every minute throughout the night to observe the frogs' behavior. The recording started between 7pm and 10:30 pm, and the recording was stopped around 8:20 am.

This experiment was repeated 3 additional times with 4 different triads of frogs being used in total. After each round, the frogs were returned to their group housing tanks. One month after the first experiment, the same triads were reassembled to have their behavior recorded again. Frogs were identified by spot patterns, weight, length, and home tank assignment. The same procedure done in the first round of behavioral data collection was repeated for this second round of data collection. The only difference was there was not a night of recorded observation for just the males. There was one night of just two males for an acclimation period, and then one night of recorded observation of the triad of frogs, two males and one female.

Blood and Tissue Collection

The following morning, after overnight observation, only the male frogs were dissected, and nothing was done to the females. The first male was injected with 1ml of a 0.05 M solution of MS222. Once the MS222 was injected, they would stay in a small 12 L tank until they were deeply anesthetized and unresponsive to a toe pinch; this typically took about 10 min. Once they were unresponsive, they were placed on ice on their dorsal side. In this position, a large incision was made to expose the heart. A 27-gauge needle was used to puncture the heart and draw blood out. Blood was continuously collected until we were unable to gather anymore. The blood samples were placed in microcentrifuge tubes and centrifuged for 6 min at 3000 rpm to separate the plasma. If the plasma was not completely separated, we would microcentrifuge them again until separated. The plasma was pipetted off into their own microcentrifuge tubes and stored in the freezer until they were needed. Organ tissues were also collected. After the blood draw, the testes and the larynx were removed, and their mass and length were recorded. Once the first male had its blood drawn and testes and larynx removed, the second male was injected with 1 ml of MS222 and underwent the same procedure.

ELISA Tests

Corticosterone and Testosterone ELISA kits were purchased through Cayman Chemical (Ann Arbor, Michigan). In brief, we extracted hormone from plasma samples with 4 additions of diethyl ether, evaporated under a gentle stream of nitrogen, and then dissolved into an ELISA buffer solution. Then the proper ELISA reagents were added to each well which came precoated with a mouse monoclonal anti-rabbit IgG. We ran each sample in triplicate and at 3 different dilutions: 1:1, 1:10, 1:100. Each sample contained 50 uL of plasma. If we did not obtain enough plasma, we used the limited amount for the testosterone kit rather than the corticosterone kit and didn't run it in triplicate.

Data Analysis

The overnight photos were coded into a spreadsheet and the behavior of both frogs was recorded for each image: not clasping, clasping male, clasping female, pseudo-clasping male, pseudo-clasping female, or unidentifiable. Only the first 8 hours of photos were coded for each trial run to make sure an equal amount of time was analyzed for each frog. To look at the effect of the size of the tank on the clasping behavior our data was compared to data reported in a Rhodes et al. 2014 paper. To look at the clasping behavioral patterns, who the males clasped across the two nights, and who they clasped throughout one night were compared separately. The frequency of how often they clasped was calculated by dividing the number of minutes they were reported clasping by the total 481 minutes analyzed (one picture equates one minute). Frogs were considered a winner if they clasped the female more than the other frog through the night, or they were considered a loser if they clasped the male more or did not clasp at all. To examine any possible relationship between hormone level and clasping behavior, results from the ELISA tests were compared to our frequency data with the winner and loser frogs.

Results

Experiment one was looking at if the size of the tank the frogs were placed in had any impact on the frequency of the clasping behavior. We found similar patterns in behavior regardless of the tank they were in. There are two behavioral patterns observed in both the larger tank used in our experiment, and in the small tank being used in a previous experiment by Rhodes et al., (2014). The first pattern seen is males who clasped males on night 1, were likely to clasp the male again on the second night, even with a female present (Figure 2). The second pattern that we see is on night two, the frog who clasps the female will clasps the female predominantly and either never clasp the male, or barely clasp the male. Similarly, the frog who

clasps the male will predominantly clasp the male, and either never clasp the female, or barely clasp the female (Figure 3).

For experiment two, we successfully collected data on corticosterone and testosterone concentrations for 6 of the 8 frogs. Data from 2 of the frogs was not included because of human error with hormone extraction that caused their concentration levels to not be a range that was consistent with previous research. Corticosterone concentrations ranged from 1323 pg/ml to 7512 pg/ml, and testosterone concentrations ranged from 350 pg/ml to 5443 pg/ml. There appeared to be a correlation between testosterone concentration and clasping behavior. We saw that the frogs who clasped the female more, the “winning” frogs, had higher testosterone concentrations than their partnered male (Table 1). There did not appear to be a correlation between corticosterone concentrations and clasping behavior (Table 2). Looking at the testes and larynx data, there does not appear to be a correlation between testes mass and testosterone concentration (Figure 4), or between larynx mass and testosterone concentration (Figure 5). Clasping behavior drastically dropped off in experiment two compared with experiment one (Figure 6), so we compared testes and larynx mass to the behavior from experiment one since their masses should remain more stable. We saw that the frogs with larger testes clasped the female more in experiment one suggesting a possible correlation between testes size and the clasping behavior (Figure 7). We did not see any correlation between larynx size and the clasping behavior (Figure 8).

Both of the experiments we ran were pilot studies. Even though no substantial conclusions can be drawn from the data we collected, we were able to identify noticeable patterns. Overall, we feel as though these pilot experiments were successful because we were

able to effectively collect data on the hormone levels and start to formulate a hypothesis that will need further investigation.

Discussion

We looked at three initial questions regarding the tank size, hormone levels, and testes and larynx size. Our first hypothesis was male-male clasping would not be an artifact of tank size. Based on the data we collected, the clasping behavioral patterns were very similar between the large tank and the small tank data (Rhodes et al., 2014) providing further support for our hypothesis, that the size of the tank doesn't dictate the clasping behavior. For the second question, we hypothesized that males with higher testosterone levels would engage in male-female clasping more, and males with higher levels of corticosterone would engage in male-male clasping more. Though we aren't able to make any formal conclusions from our data, we can see that testosterone appears to correlate with clasping behavior. We did not see any correlation with behavior and corticosterone levels. We also hypothesized that testes size and larynx size would correlate to testosterone levels. We did not find a correlation between testes size and testosterone or larynx size and testosterone, but our data suggests a possible correlation between testes size and clasping behavior.

Focusing on the effect of tank size on clasping behavior, we wanted to make sure that the clasping behavior that has been reported in the small tank (Rhodes et al., 2014), was not an artifact for the small environment they were placed in. The behavioral patterns that we saw in the small tank were replicated when placed in the larger tank, even with a small sample size. This was not surprising to see, as this behavior occurs in the wild for many anuran species, though it is not well documented in *X. laevis*. (Wells, 1977; Arak, 1983).

Shifting focus over to our pilot investigation on hormones and their possible correlations to clasping behavior, we were successful in proof of concept. We were able to successfully collect data on hormone concentrations in ranges that have been previously reported in *X. laevis* (Kang et al., 1995; Jaudet and Havey, 1984). Additionally, we were able to formulate hypotheses as to where correlations in our data may lie. A possible correlation between clasping behavior and testosterone levels can be seen. Testosterone levels in the males that were considered the winners, were higher than the “loser” frogs they were paired with. In the world of amphibians, there has been contradictory data on the effect of testosterone, or more generally androgens on male clasping behaviors. In studies where male frogs are castrated, which results in the disappearance of the clasping behavior, there is a split amongst amphibian species that, with an injection of testosterone some will either start to clasp again while others will remain absent of reproductive behaviors (Kelley and Pfaff, 1976; Wada and Gorbmen, 1977; Malacarne and Giacoma, 1980; Moore, 1978). *X. laevis* is one of the species where the injection of testosterone will induce the reappearance of clasping behaviors (Kelley and Pfaff, 1976; Wetzel and Kelley, 1983). This provides support for the correlations that we see with testosterone and clasping behavior because these studies indicate that androgen levels in *X. laevis* are important in the control of the clasping behavior.

With our data, we didn't see any correlation between corticosterone and clasping behavior, but that doesn't mean corticosterone doesn't have any impact on reproductive behaviors. Increased levels of corticosterone in various species of amphibians have been reported to inhibit advertisement calls, thus inhibiting the likelihood of being the “winning” frog (Moore and Miller, 1984; Leary et al., 2006; Emerson and Hess, 2001). However, similar to testosterone, there has been some contradictory evidence that in some species of anurans, it is actually the

males producing the advertisement call who have more corticosterone than the non-calling male (Leary et al., 2004). Studies on the impact of corticosterone on the reproductive behaviors of *X. laevis* specifically are lacking. This means that it's still unknown how exactly corticosterone would impact the clasping behavior of *X. laevis*.

One limitation that impacts the analysis of our ELISA data is for the second round of experiments, the clasping behavior significantly declined. In experiment one, the average time spent clasping amongst the males was around 67.95% of the night. In experiment 2, the average time spent clasping went down to only 2.47% of the night. We saw a 65.48% decrease in the average time spent clasping between experiment one and experiment two. This could be due to the fact that we did experiment two in mid to late October, and their sexual receptiveness/activeness was not at its peak time. The peak breeding period appears to be from July to September, the summer months (Kalk, 1960).

We did not see any correlations in our data between testes size and testosterone concentration. Based on previous research, we would have expected to see a correlation between testes size and testosterone concentration (Emerson 1997; Emerson and Hass, 2001), but our small sample size may have impacted our ability to see one. However, we did see a correlation between testes size and the clasping behavior from experiment one. There was a general trend where the longer a male would clasp a female throughout the night, the larger their testes size would be. We were able to analyze the testes size in relation to experiment one's behavior because in mature anurans, though testes size can vary between species, it remains relatively constant within individuals (Emerson, 1997). Emerson (1997) has also reported that testes size is positively correlated to testosterone levels. By linking the findings of Emerson's study, and the

correlation that we see in our data we can gather further support for our hypothesis that testosterone has an effect on clasping behavior.

No correlations were found between larynx size and testosterone concentration. Similar with testes size, we would have thought there to be a relationship between larynx size and testosterone concentration based on previous studies (Sassoon et al., 1986; Yamaguchi and Kelley, 2000; Tobias et al., 1991). The larynx, especially in *X. laevis*, is a highly, sexually dimorphic organ (Sassoon et al., 1986). One of the main components influencing the difference in size of the larynx between males and females is testosterone. Research has been done to show that injecting a female with testosterone will lead to the masculinization of the larynx and increase its size (Tobias et al., 1991). Again, for similar reasons to testes size, the small sample size may have impacted our ability to see any correlations between larynx size and testosterone concentrations.

These pilot experiments were important for laying out groundwork for future research. We were able to start to see possible correlations between hormone concentrations and clasping behavior. The small sample size and the decreased presence of clasping behavior in experiment two may have contributed to the differences we see compared to previous studies. Future research goals include conducting the experiments during the summer months to ensure the presence of the clasping behavior and collecting data on a much larger sample size. Achieving these goals will result in the ability to correlate the hormone levels with clasping behavior and provide a better understanding of the mechanisms driving this reproductive behavior in *X. laevis*.

Table 1. The testosterone concentrations within each frog. The pairings, as well as the winning and losing frog are depicted.

Frog Pairing	“Winner” Testosterone Level (pg/ml)	“Loser” Testosterone Level (pg/ml)
Pig 2 & Alb 12	5443	2551
Pig 3 & Alb 10	507	350
Pig 7 & Alb 11	1264	1135

Table 2. The corticosterone concentrations within each frog. The pairings, as well as the winning and losing frog are depicted from experiment two.

Frog Pairing	“Winner” Corticosterone Level (pg/ml)	“Loser” Corticosterone Level (pg/ml)
Fig 2 & Alb 12	3871	2038
Fig 3 & Alb 10	7512	2587
Fig 7 & Alb 11	1323	1592



Figure 1. Tank set up used during both experiment one and experiment two. The top image also depicts the size of the small tank used in previous experiments, while the bottom image shows the frogs in the larger tank used in our experiments.

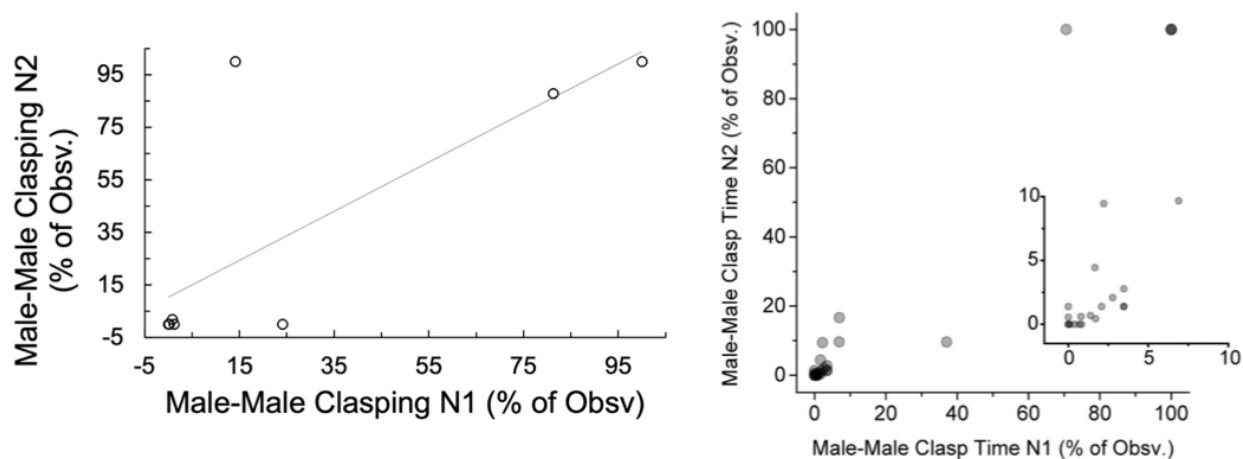


Figure 2. Clasping behavior within male-male pairs is consistent from night 1 to night 2. Both graphs show that males frogs who clasped the other male on night 1 were likely to clasp that male again on night two. **A:** Data points are from the big tank experiments run in the fall of 2020. **B:** This graph has data points taken from an experiment where smaller tanks were used (Rhodes et al., 2014).

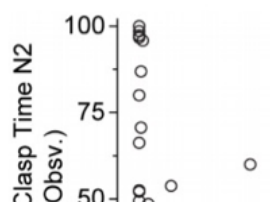
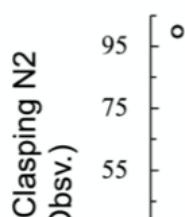


Figure 3. Frogs maintained consistency with who they would clasp for an entire night. The red points indicate the frog showed a preference towards the male. The black points indicate the frog showed a preference towards the female. The blue data points indicate that the frog showed no preference for neither male nor female. **A:** Data points are from the big tank experiments run in the fall of 2020. **B:** This graph has data points taken from an experiment where smaller tanks were used (Rhodes et al., 2014).

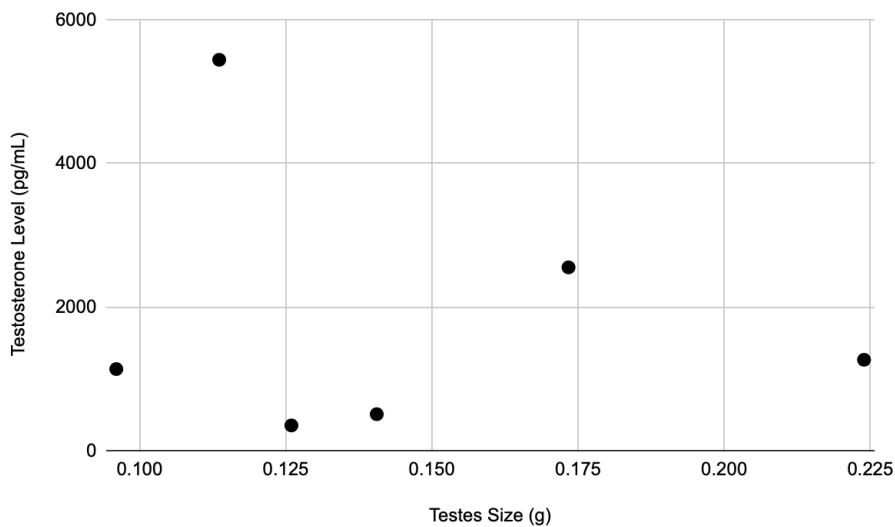


Figure 4. Scatterplot of testes size (g) and testosterone level (pg/mL). There was no correlation between testes size and testosterone level.

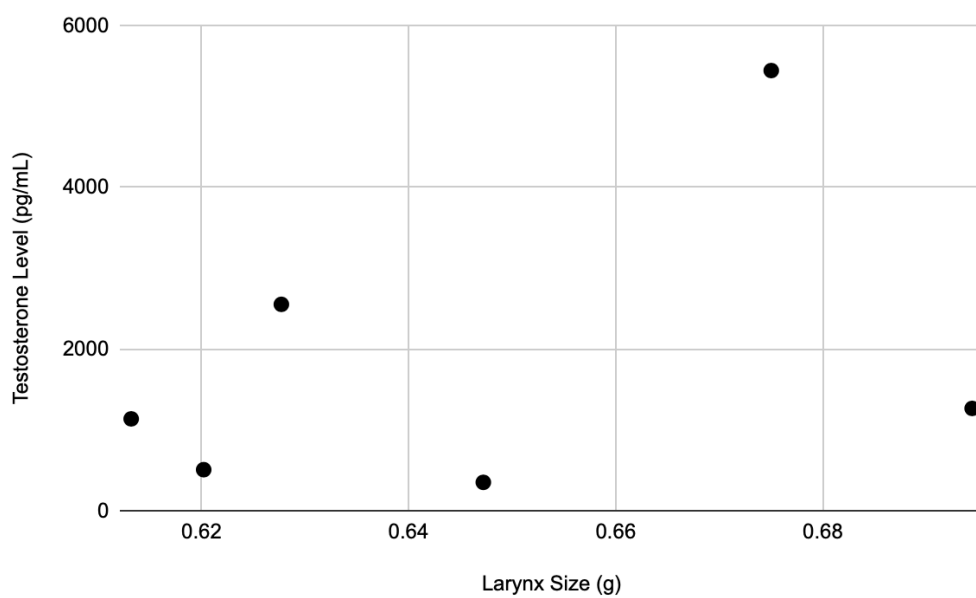


Figure 5. Scatterplot of larynx size (g) by testosterone level (pg/mL). No correlation was found between larynx size and testosterone level.

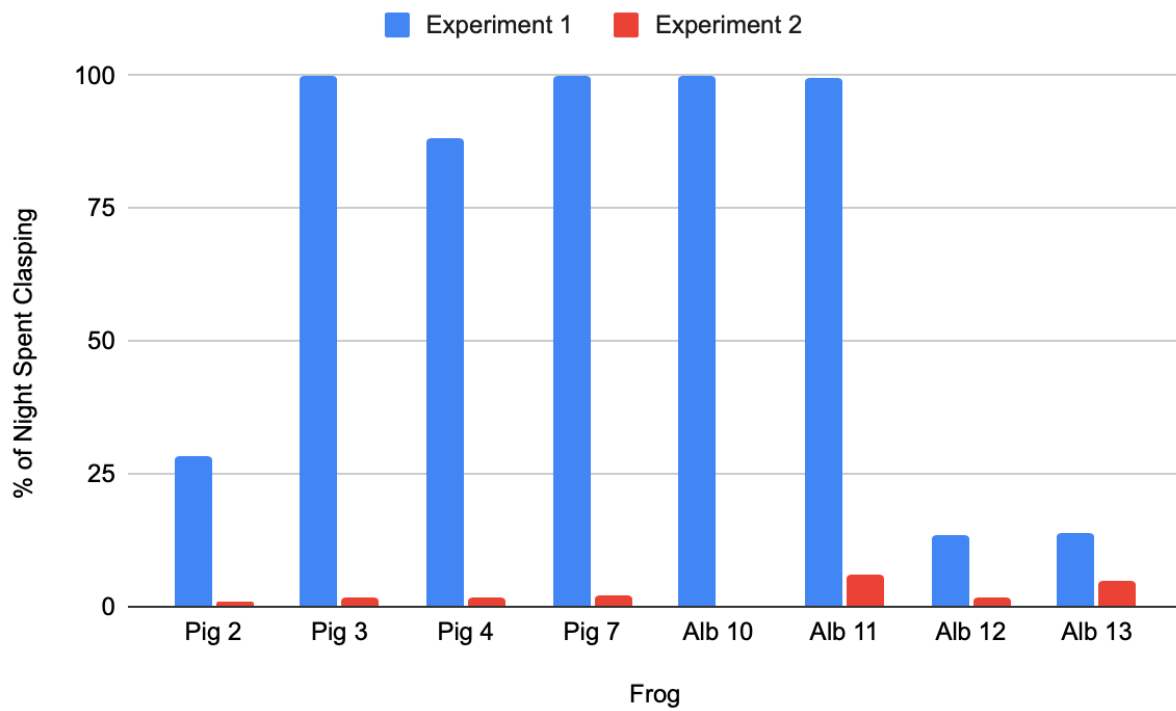


Figure 6. Clasping behavior in experiment 1 vs experiment 2. The blue bars indicate the total time each male spent clasping on night two during experiment one, and the red depicts the total time each male spent clasping during experiment two. There is a clear decline in the clasping behavior in experiment two compared to experiment one. The average time spent clasping across all frogs dropped 65.48% between the two experiments.

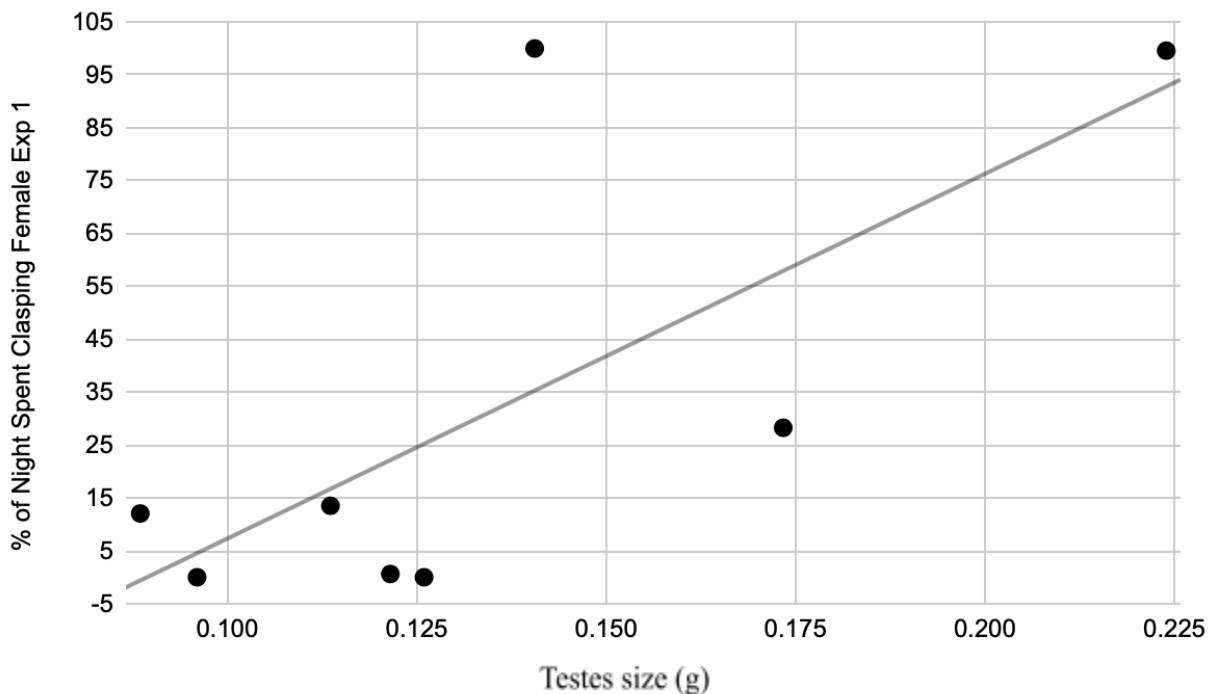


Figure 7. Relation between testes size and male-female clasping behavior in experiment 1. As testes size (g) increased, the time a male would spend clasping the female also increased. The

behavior plotted is the data recorded from experiment one where the presence of clasping was much higher.

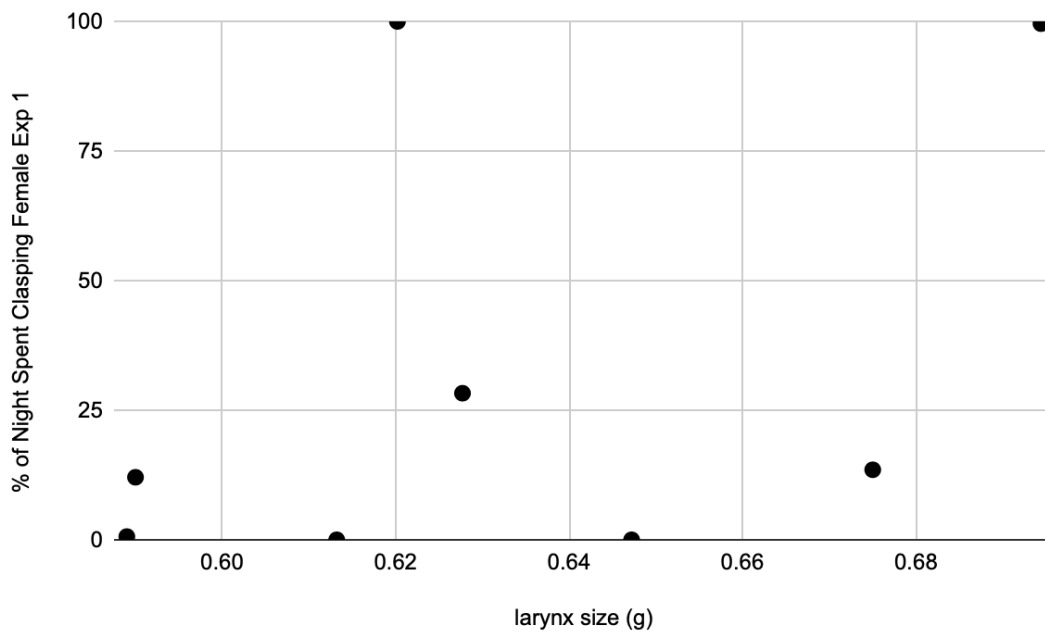


Figure 8. Relation between larynx size and male-female clasping behavior in experiment 1. No correlation was found between larynx size and male-female clasping behavior.

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