The Significance of Estrogen in Reproductive Copulatory Success in African-clawed Frog (*Xenopus laevis*)

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ABSTRACT

Hormones are a vital component of reproductive processes, intimately acting on both sexes through proteins of pituitary origin that affect the gonads, and steroids of gonadal origin that affect the accessory organs and secondary sexual characters. Reproductive behavioral success is the capability of an organism to outcompete other organisms for a mate and to achieve copulation. Aside from its crucial role in courtship dynamics, testosterone works with its counterpart estrogen to regulate these behaviors. Once released by the testes, testosterone is converted by the aromatase enzyme into estradiol, which then acts similarly in its effect on male behavior. Although the influences of testosterone and estrogen are known in male African clawed frogs (*Xenopus laevis)*, little is known about the minimum amount of estrogen needed to inhibit testosterone and its effects on frog mating behavior. I performed breeding assays to determine how estrogen treatment of male frogs influenced reproductive behavior in both noncompetitive and competitive environments. I found that male frogs treated with estrogen were significantly less likely than non-treated frogs to copulate in a non-competitive setting. However, I found that treated and untreated male frogs achieved reproductive success at equal rates in a competitive setting. Thus, these unexpected results call into question the role of concentrating a specific amount of hormone in understanding how successful reproductive copulation can be achieved. Determining the threshold level of estrogen is necessary to understand the sensitivity of the amphibian endocrinology system and vital to addressing populations drastically affected by minute traces of endocrine disruption compounds.

KEYWORDS

environmental health, endocrine disruption, amphibian reproduction, behavioral sciences, reproductive endocrinology, toxicology

INTRODUCTION

Hormones are vital components of reproductive processes, intimately acting on both sexes through proteins of pituitary origin that affect the gonads, and steroids of gonadal origin that affect the accessory organs and secondary sexual characters (Burdick 2010). These two main groups of sex hormones are responsible for more than just the physiological changes of reproduction; their function is also significant for reproductive behaviors that are vital to a species' survival (Steinman et al. 2010). While most protein hormones act through genomic mechanisms that take some time, steroid hormones bind to receptors in the cell membrane that produce a cascade of rapid changes within seconds to minutes (Steinman et al. 2010). These non-genomic mechanisms can influence a wide variety of behaviors in the short term. For example, testosterone has been documented to act over several days to promote male courtship and mating behaviors (Steinman et al. 2010).

Reproductive behavioral success (hereafter simply "reproductive success") is the capability of an organism to outcompete other organisms for a mate and to achieve copulation (Hayes 2012). Such behavioral interactions between potential mates often require complex courtship dynamics that are dictated by steroid hormone levels. High testosterone concentrations in adult males across a number of animal species have been shown to correlate with a higher frequency of reproductive behaviors, such as the frequency of males approaching females (Lord et al. 2009). For example, upon encountering ovulating females, male goldfish show a rapid rise in plasma testosterone (Kobayashi *et al*. 1986). Testosterone has also been found to be critical for male copulatory behavior in African clawed frogs (*Xenopus laevis*), being associated with aggression and competition over mates (Hayes et al. 2010). Aside from its crucial role in courtship dynamics, testosterone works with its counterpart estrogen to regulate these behaviors.

Once released by the testes, testosterone is converted by the aromatase enzyme into estradiol, which then acts similarly in its effects on male behavior. Because of this, aromatase inhibitors can block the conversion of testosterone into estradiol and prevent testosterone from exerting its typical effects on male approach behavior in mating (Lord et al. 2009). High concentrations of estrogen can also inhibit testosterone production through feedback loops and prevent successful copulation in males. For example, male fathead minnows, when exposed to estrogen, were found to have reduced fitness due to the suppression of their reproductive behaviors (Martinović et al. 2007). As a result, successful reproduction was less likely to occur as they were unable to outcompete others for mates in large populations.

While mating behavior normally is governed by the endogenous hormones testosterone and estrogen, certain man-made compounds in the environment pose potential disruption risks. Endocrine disruption compounds (EDCs) have the potential to disrupt hormone-controlled physiological processes such as reproduction, because they can affect the synthesis, transport, binding, action, and degradation of a wide variety of hormones (Hayes et al. 2010). EDCs can affect reproductive parameters including sex differentiation, secondary sexual characteristics, oogenesis, spermatogenis, and even sexual maturation (Crisp et al. 1998). Commonly known EDCs such as DDT, atrazine, and bisphenol A function as weak estrogen agonists and are especial concern to fetuses in early development (Crisp et al 1998). Experimental work in multiple species has been critical in linking these endocrine disrupting compounds to their adverse effects (Wiemeyer 1975). For example, exposure to DDT has been linked to abnormal reproductive behavior alongside compromised fertility in populations of wildlife such as the bald eagle (Guillette et al. 2001). Atrazine exposure has been confirmed to affect larval development by chemically castrating males and demasculinizing adult African clawed frogs *Xenopus laevis* (Hayes et al. 2010).

Although the influences of testosterone and estrogen are known in male *Xenopus laevis*, little is known about the minimum amount of estrogen needed to inhibit testosterone or the minimum amount of testosterone required for males to exert reproductive behavior. Thresholds for both testosterone and estrogen levels are pertinent to assessing the sensitivity of amphibians' endocrinology system to such EDCs and understanding how populations can be affected by different concentrations of EDCs. This study will examine the effects of environmental estrogens on amphibian reproductive behavior. I predict that male *X. laevis* treated with higher concentrations of estrogen will be less successful in copulation in competition with control frogs.

METHODS

Study species

My study uses the African clawed frog (*Xenopus laevis*), specifically the Golden Gate population for its resilience. *Xenopus laevis* are originally native to South Africa and were brought to the US in the 1940s for use as a standard amphibian in laboratory studies and human pregnancy testing. The Golden Gate population's origin is from the Golden Gate Park in San Francisco and is one of the sites throughout California where there are established populations. They are accustomed to living in a variety of territories, from warm and stagnant grassland ponds to streams located in arid and semi-arid regions. With toleration to a wide range of water pH, *Xenopus laevis* are almost fully aquatic and only leave when forced to migrate. Often relatively inactive, they can lie dormant for up to a year but have the ability to swim long distances in all directions given their well-developed lungs and little cutaneous respiration (Garvey 2000).

The African clawed frog can breed up to four times a year; this can take place during any time of the year but is most common in spring. With fewer disturbances in the evening, mating occurs at night when males are able to vocalize and attract females as part of their pre-mating behavior. After this mating call is heard, the female responds with either an acceptance call (rapping sound) or a rejection call (slow ticking sound). Males have mating pads that develop on the underside of their forearms and hands that are used in their pelvic mating embrace, the amplexus. During this 3-4 hour mating event, females are known to release several hundreds of sticky eggs that attach to plants and other anchors individually as well as in clumps. While animals in the wild are known to reach 15 to 16 years old, animals in captivity can live as long as 20 years (Garvey 2000).

Housing & treatment

Xenopus laevis (12 males and 12 females) were housed individually in a laboratory basement at room temperature in plastic boxes 0.40m x 0.50m x 0.30m and ¾ filled with water. Animals were exposed to an artificial light cycle from 7am-7pm and a dark cycle from 7pm-7am at stable room temperatures around 22°C. A routine of care was maintained that consisted of a 3 day cycle interval, alternating each day between a feed day, a change of water day, and a rest day. Animals were kept and studied humanely, as permitted by University of California ACUC #210-5186.

Estrogen water treatment

Males were treated in 2 separate groups: an estrogen group and a control group. There were 4 animals in each treatment, with 8 animals in total. Treatment of a concentration well below 0.003mg/L (3 ppb) was made from powdered industrial grade estrogen dissolved in ethanol as a vehicle of transmission. Both the treated set and control set had a form of treatment added to their ¾ filled water tank every feed and rest day to control for their exposure levels. Treatment for the control set was only ethanol while the treated set received the estrogen dissolved ethanol over a treatment course of three months.

Breeding assays

Non-competitive: "motel 6"

The "Motel 6" breeding test sequence was used to assess how hormone treatments or other experimental conditions influenced reproductive behavior in a noncompetitive environment. Eight female frogs injected intramuscularly with 500 μL of Human Chorionic Gonadotrophin (HCG) induced ovulation prior to being placed into their individual tanks (about 0.40 x 0.50 x 0.30 meters in size) with the presence of one male frog. There were a total of 8 pairs: four males from the estrogen treatment group and four males from the control group each paired with one female. These tanks were kept at room temperature in a plastic tank (size) ¾ filled with water in a laboratory setting at stable room temperatures around 22°C. Frogs were left in their respective tanks for 12 hours overnight from 6pm to 6am in dark conditions to replicate the natural conditions of mating. The capability of males to be receptive to females was measured by the presence of clasping, or amplexus at the 6am assessment; this was used as the endpoint for evaluating reproductive behavior. Presence of amplexus was conclusive of reproductive success while the absence of amplexus indicated reproductive failure.

Competitive: "pool party"

The "Pool Party" breeding assay was used to assess if reproductive function of treated amphibians would affect their ability to be successful in a competitive environment. Males from the estrogen treated group and control group were surgically sutured the morning of the procedure with distinct colored thread per treatment group. These sutures were placed at either front left, front right, back left, or back right positions per animal in each treatment group. Green thread was used to distinguish the estrogen treated group while orange thread was to distinguish the control group. Four female frogs injected intramuscularly with 500 μL Human Chorionic Gonadotrophin (HCG) induced ovulation as per the methods discussed above. Within a half hour of injection, four females were placed alongside four estrogen treated males and four control group males in a 0.9m x 0.6m x 0.3m small pool filled with water. Results of their behavior was observed and noted after a 12 hour cycle from 6pm to 6am in dark conditions at stable room temperatures around 22°C to replicate the natural conditions of mating. The capability of males to be receptive to females as well as competitiveness against other males was measured by the presence of clasping, or amplexus. Clasping behavior of males was used as the endpoint to evaluate reproductive success at the behavioral level. Presence of amplexus was conclusive of reproductive success while absence of amplexus indicated reproductive failure for an individual male.

Blood collection

Animals had blood samples collected for assessment of estrogen levels. After each breeding assay amplexus check, no more than 5mls of blood were collected into centrifuge tubes from male X. *laevis*. Blood samples were centrifuged to collect plasma that was stored in the freezer at -18°C for future use.

RESULTS

Breeding assays

Non-competitive: "Motel 6"

After three trials of the motel 6 experiment, I found the majority of the control groups were able to achieve copulation while only half of the estrogen treated groups were successful (Fig. 1).

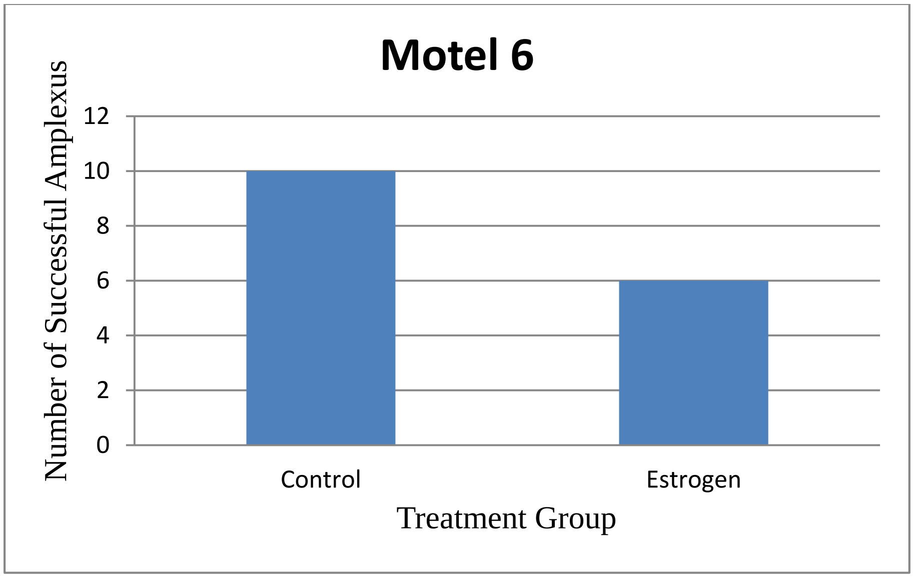

Fig. 1. Copulation Rate for Control Group and Estrogen Treated Group. Bar graph representations of a total of three "Motel 6" trials run for both Xenopus laevis groups.

Competitive "pool party"

After six trials of the pool party experiment, I found that both groups were sporadic in achieving copulation (Fig. 2).

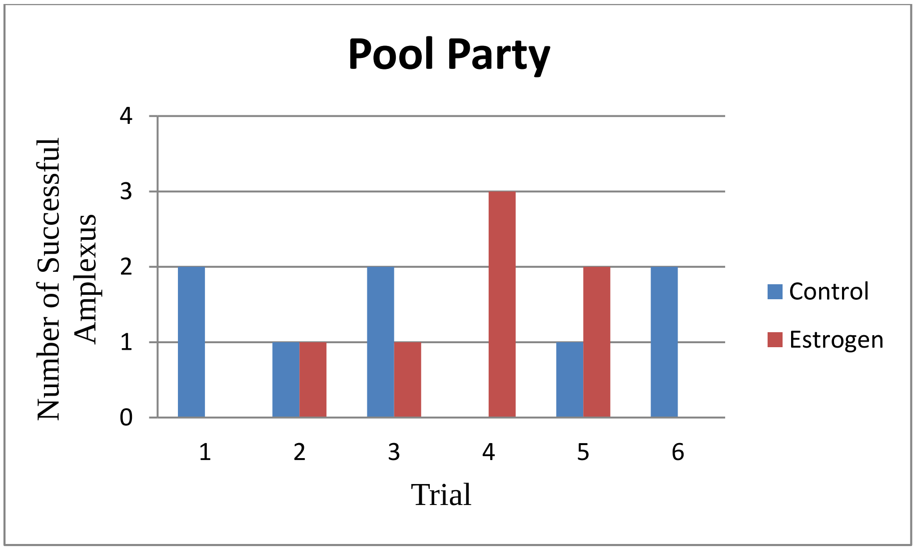

Fig. 2. Copulation Rate for Control Group and Estrogen Treated Group. Bar graph representations of a total of six "Pool Party" trials run for both Xenopus laevis groups.

Data processing

A paired T-test was used to calculate the difference in copulation success between the means of the control group and the estrogen treated group. The results allow us to see whether there is a significant difference in the number of successful amplexus events between the two groups, and if this can be attributed to estrogen. I used the R-commander software and ran the analysis of trial results separately for each breeding assay.

Analysis

Non-competitive "motel 6"

The paired T-test analysis of the non-competitive breeding assay trials with 2 degrees of freedom produced a t-value of 2. With a p-value of 0.1835, we can reject the null hypothesis that estrogen does not affect the reproductive capability of male *Xenopus laevis*.

Table 1. Summary of Paired T-test output variables for non-competitive breeding assay. From R-commander.

					95% Confidence Interval	
	T	**df**	**Sig. (2-tailed)**	**Mean Difference**	**Lower**	**Upper**
Difference	2	2	0.1835	1.333333	-1.535102	4.201768

Competitive "pool party"

The paired T-test analysis of the competitive breeding assay "Pool Party" with 5 degrees of freedom produced a t value of 0.2104. With a p-value of 0.8417, we cannot reject the null hypothesis that estrogen does not affect the reproductive competitiveness of male *Xenopus laevis*.

Table 2. Summary of Paired T-test output variables for competitive breeding assay. From R-commander.

					95% Confidence Interval	
	t	**df**	**Sig. (2-tailed)**	**Mean Difference**	**Lower**	**Upper**
Difference	0.2104	5	0.8417	0.1666667	-1.870068	2.203401

DISCUSSION

Sex hormones are vital to reproductive processes, affecting both physiology and reproductive behaviors necessary for survival of a species. EDCs in the environment have the potential to disrupt hormone balance, thereby causing abnormal reproductive behavior and development (Miyata 2000). While estrogen's ability to inhibit testosterone production and prevent successful copulation in male *Xenopus laevis* is known, I assessed how a minute trace of estrogen can lead to reproductive failure. Determining the threshold level of estrogen is necessary to understand the sensitivity of the amphibian endocrinology system and vital to addressing populations drastically affected by minute traces of endocrine disruption compounds (Cassano 2006).

Motel 6 model

Male *Xenopus laevis* were initially tested for their reproductive capabilities when exposed to estrogen. While most of the males in the control group achieved amplexus, only half of the males treated with estrogen were able to copulate. These results confirm that estrogen does in fact inhibit the frogs' ability to copulate and be reproductively successful. Estrogen's role in reproductive failure confirms that a specific threshold is necessary for this to take place. This occurs via rapid mechanisms that influence reproduction, through receptor binding in the cell membrane that produce a cascade of rapid changes within a short period of time (Steinman et al. 2010).

These Motel 6 trials consisted of estrogen treatment being added to the males' housing water every three days with a water change prior. However, in between these additions of estrogen treatment was a period of three days during which it was unknown how the concentration of hormone changed and therefore its effects on the organism altered. Metabolism is a point of concern; with the endogenous source of estrogen possibly being metabolized during this time it may have an effect on reproductive success. Moreover, these trial results did not account for estrogen having an effect on competitiveness. To account for these issues, latter experimental procedures were modified to address these questions.

Pool party model

The competitive breeding assay, "Pool Party", was used to assess if estrogen actually had an effect on the study organism's ability to compete with others for a mate. Treatments in these trials were added daily to the animal's housing water to ensure that they had what was assumed to be a constant influx of hormone. There were inconsistent amplexus results with both the control and treated groups achieving similar copulation rates. These results indicated the possibility that reproductive competitiveness is not inhibited by estrogen. However, the outcomes were not consistent enough over the course of the trials and additional trials need to be conducted before significant conclusions can be drawn.

Though estrogen treatment was added daily to experimental tanks prior to these trials, exposure to the hormone could not be accounted for when the frogs were actually competing in the shared pool setting. Therefore, there is no indication of the estrogen taking effect when the males are no longer exposed to their treatment in the competitive setting. The exogenous source of estrogen in these Pool Party trial results was not constant and thereby further alterations to the experimental procedures are necessary.

Limitations & future directions

The Golden Gate population of *Xenopus laevis* is known to be an unstable population as past behavioral study trials has yielded inconsistent results (Hayes et al. 2012). Future projects can use a more sensitive population in hopes of controlling for the study population's possible influence. Similar findings in genetically divergent zebra fish highlight the need for due consideration of the genetics of populations as they illustrate the differences in response sensitivities (Soeffker 2012). Moreover, physical attributes such as the nuptial pads of Xenopus laevis, essential in mating and as a hormone source is a factor to consider (van Wyk 2003).

Additionally, exogenous estrogen implanted into the male frogs can be a method of precision to ensure that there is a constant and effective flow of hormone within the organism's system. A similar study with testosterone implants of a higher dose successfully circulated and modulated activity in the anterior hypothalamus of male ring doves (Fusani 2003).

Broader implications & conclusion

Though my experiment verified the effectiveness of estrogen in inhibiting the ability of male *Xenopus laevis* to copulate in a noncompetitive setting, my results were unable to confirm my hypothesis of estrogen's role in limiting reproductive success in a competitive setting. There is the potential that the level of estrogen tested was below what was needed to prevent competitive behavior. Moreover, there is the possibility that the lack of treatment exposure during the duration of the experiment allowed for the estrogen treated frogs to overcome the effects of the hormone.

Testosterone treatments in male ring doves confirm the suggestion of a threshold structure needed for courtship behavior (Fusani 2003). Future experimental trials will look to address these alternative hypotheses to bring the project closer to evaluating specific hormone thresholds.

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