Naphthenic acids disrupt courtship in *Silurana tropicalis*

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Abstract

Neuroendocrine processes coordinate the behavioural, physiological, and seasonal aspects of reproduction. Some chemicals can disrupt the hypothalamus-pituitary-gonadal axis, impacting reproductive health. Naphthenic acids (NAs), the carboxylic acids in petroleum, are of emerging concern as they contaminate coastlines after oil spills and aquatic ecosystems of the Athabasca oil sands area. They are acutely toxic in fish and tadpoles and possibly endocrine disrupting at sublethal levels. I characterized courtship behaviours and disruption by NAs in the Western clawed frog, *Silurana tropicalis*. Courtship primarily consists of males producing low trills and achieving amplexus, a mating position where a male clasps a female. Adult frogs were exposed for five days to 20 mg/L NA, a dose low enough to not affect physical activity. In males, absolute calling activity was reduced. Other acoustic parameters such as dominant frequency, click rate, and trill length were not affected. Injecting human chorionic gonadotropin had a slight rescue effect. Vocalization and amplexus were both inhibited after exposure and restored after 2 weeks of recovery. However, calling behaviour did not predict competitive ability or mating success. In females, NA exposure reduced mating success, possibly through decreased attractiveness or receptivity. Receptivity can be indicated by attraction towards the sound of mating calls (phonotaxis), which is cryptic and subjective. I created an apparatus that measures phonotaxis by placing speakers inside traps with infrared lights to detect the time of entry. This novel method is widely applicable for low-visibility observations and studies of choice and preference. This work shows that an aquatic contaminant can reduce mating success in otherwise healthy frogs, and provides a detailed foundation for further investigation.

Résumé

Les aspects comportementaux, physiologiques et saisonniers de la reproduction sont coordonnés par des processus neuroendocriniens. Certains produits chimiques perturbent l'axe hypothalamus-hypophyse-gonade, avec des répercussions sur la santé reproductive. Les acides naphténiques (AN), les acides carboxyliques du pétrole, sont de plus en plus préoccupants, car ils contaminent les côtes après le déversement de pétrole ainsi que les écosystèmes aquatiques de la région des sables bitumineux de l'Athabasca. Ils sont extrêmement toxiques pour les poissons et les têtards, et peuvent perturber le système endocrinien à des niveaux sublétaux. J'ai décrit les comportements de parade nuptiale et les perturbations par les AN chez la grenouille à griffes de l'Ouest, *Silurana tropicalis*. La parade nuptiale se compose principalement de mâles produisant des trilles graves et réalisant l'amplexus, la position d'accouplement où un mâle saisit une femelle. Des grenouilles adultes ont été exposées pendant cinq jours à 20 mg / L AN, une dose suffisamment faible pour ne pas affecter l'activité physique. Chez les mâles, l'activité d'appel absolu a été réduite. D'autres paramètres acoustiques tels que la fréquence dominante, le taux de clics et la longueur du trille n'ont pas été affectés. L'injection de gonadotrophine chorionique humaine a eu un léger effet de sauvetage. La vocalisation et l'amplexus ont tous deux étés inhibés après l'exposition et rétablis après 2 semaines de récupération. Cependant, le comportement d'appel n'a pas prédit la capacité compétitive ni le succès de l'accouplement. Chez les femelles, l'exposition aux AN a réduit le succès de l'accouplement, possiblement en diminuant l'attrait ou la réceptivité. La réceptivité peut être indiquée par l'attraction vers le son des appels d'accouplement (phonotaxie), qui est cryptique et subjectif. J'ai créé un appareil qui mesure la phonotaxie en plaçant des haut-parleurs dans des pièges à lumières infrarouges pour détecter l'heure d'entrée. Cette nouvelle méthode est pertinente aux observations de faible visibilité et aux études de choix et de préférence. Ce travail montre qu'un contaminant aquatique peut réduire le succès de l'accouplement chez des grenouilles autrement saines et fournit une base détaillée pour une enquête plus approfondie.

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List of abbreviations

AVT	Arginine vasotocin
AVP	Arginine vasopressin
BCF	Bioconcentration factor
CORT	Corticosterone
DHT	Dihydrotestosterone
E2	17β-estradiol
EDC	Endocrine disrupting compound
EE2	Ethinyl-estradiol
GC-MS	Gas chromatography-mass spectrometry
hCG	Human chorionic gonadotropin
HPG	Hypothalamus-pituitary-gonadal
LC50	Median lethal dose
NAs	Naphthenic acids
OSPW	Oil sands process affected water
TMS	Tricane mesylate

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Thesis rationale and overview

Reproduction depends on complex coordination of physiological, behavioural, social, and temporal factors regulated by neuroendocrine mechanisms. Since the publication of Rachael Carson's Silent Spring in 1960s, there has been growing concern regarding exogenous chemicals that interfere with the endocrine system. In particular, chemicals disrupting the hypothlamus-pituitary-gonadal affective reproductive health. I aimed to characterize the courtship behaviours of the Western clawed frog *(Silurana tropicalis)* and examine how they are affected by naphthenic acids, a petroleum-derived aquatic pollutant and possible endocrine disruptor. Chapter 1 reviews literature on concepts relevant to the study objectives. More detailed reviews are provided later for chapter-specific topics. Chapter 2 is the main data chapter. It consists of several independent experiments focusing on male behaviours. Some preliminary results are presented earlier since they were used to develop the methods. Chapter 3 examines female behaviours, with more emphasis on methods development over experimental results. Chapter 4 summarizes the main results and discusses the significance of the findings, limitations of the study, and directions for future research.

Chapter 1: General introduction

1.1 Thesis objectives

My main goal was to examine the effect of naphthenic acids on the courtship behaviours of the Western clawed frog, *Silurana tropicalis*. This frog is a popular laboratory model for molecular and genomics research, and is easy to maintain in captivity. I first aimed to characterize and quantify the courtship behaviours of both male and female *S. tropicalis.* Naphthenic acids (NAs) are petroleum-derived aquatic contaminant of emergi\ng concern and may have endocrine disrupting effects. Since reproduction depends on hormones, l hypothesized that courtship behaviours and mating success would be inhibited through disruption of reproductive hormones.

1.2 Reproduction in anurans

Across vertebrates, reproduction is governed by neuroendocrine processes, specifically the hypothalamus-pituitary-gonadal (HPG) axis (Figure 1). The HPG axis has been extensively characterized in mammals. Studies with other taxa, particularly teleost fish, show many conserved components (Kanda, 2019). Environmental and social cues trigger the hypothalamus to release gonadotropin releasing hormone (GnRH), which then stimulates the pituitary to release gonadotropins: follicle stimulating hormone (FSH) and luteinizing hormone (LH). These gonadotropins bind to their receptors on the gonads; FSH stimulates ovarian follicle maturation and sperm production while LH initiates production of androgens which can be converted to estrogens by aromatase. The gonadotropins also negatively feedback on the hypothalamus to inhibit GnRH secretion. Sex steroids negatively feedback on both the hypothalamus and the pituitary. Courtship and reproduction are both dependent on sex steroids in anurans, and in vertebrates in general (Moore et al., 2005; Yamaguchi & Kelley, 2006).

Amphibian endocrinology is not as well understood compared to taxa such as mammals and fish. The system of gonadal sex steroids is largely recognized to regulate anuran reproduction (reviewed in Arch & Narins, 2009). In addition to sex steroids, arginine vasotocin (AVT) and the mammalian equivalent arginine vasopressin (AVP) are also involved in social and sexual behaviour in anurans and other vertebrates (Albers, 2012; Arch & Narins, 2009). In several taxa from amphibians to mammals, AVP and AVT act as neuromodulators and influence several social behaviours such as aggression, territoriality, and courtship (Goodson & Bass, 2001). In anurans, AVT enhances male courtship calls and female response to male calls (Boyd, 1994; Miranda et al, 2015; Penna et al., 1992; Wilczynski et al., 2005). Emerging evidence suggests that the activity of GnRH is regulated by inhibitory or stimulatory effects from other hormones such as gonadotropin inhibitory hormone and kisspeptins (Vu & Trudeau, 2016). Dopamine appears to play an inhibitory role. A dopamine antagonist triggered spawning in hibernating grass frogs (Sotowska-Brochocka et al., 1994), while a dopamine agonist inhibited mate calling in male green tree frogs (Creighton et al., 2013).

Sexual selection has led to a diversity of mating systems and strategies. Courtship consists of attracting mates, advertising reproductive state, indicating quality, and competing

with others (Shuster, 2009). In anurans, acoustic signals are the main mode of communication and critical for courtship. Vocalization is highly dimorphic between sexes and is a predominantly male behaviour. Males call to advertise and compete, whereas females generally only produce a release call if physically clasped, if they vocalize at all (Emerson & Boyd, 1999). Receptive females may display phonotaxis (movement towards acoustic stimuli) and approach the sound of male calls (Wilczynski & Lynch, 2011). Amplexus is the mating behaviour of anurans, as well as some other externally fertilizing species. Males clasp females dorsally with their front legs and stay in this position for several hours or days so the males can externally fertilize eggs as they are released. Anuran mating can be loosely divided into two temporal patterns with different strategies: explosive and prolonged (Wells, 1977, 2007). Explosive breeding species such as the wood frog *(Lithobates sylvaticus)* generally breed within a few days each year (Banta, 1914). Explosive breeding assemblies often contain hundreds of adults. The high density causes scramble competition with males indiscriminately clasping any moving object and physically fighting off other males. Females do not select and approach specific males as they would be quickly intercepted. This prevents males from calling to attract females, although the collective chorus signals the location of breeding (Bee, 2007). Tropical species experiencing more constant climates generally have a prolonged breeding season lasting months. In this low density environment, competition is indirect via vocalization. Males call to attract females, maintain territories, and compete with other males. Production and reception of acoustic signals are both modulated by hormones (Arch & Narins, 2009; Moore et al., 2005).

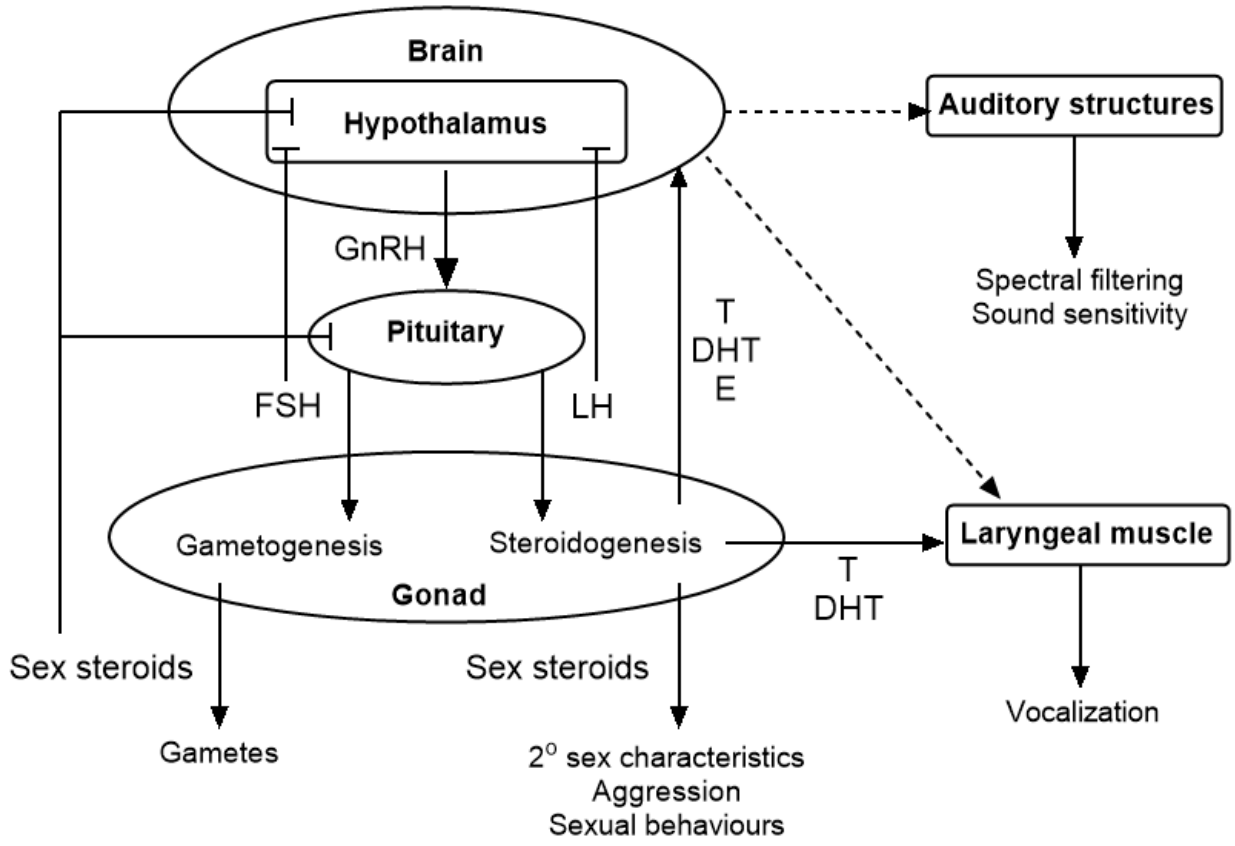

Figure 1. Neuroendocrine regulation of reproduction by the hypothalamus-pituitary-gonadal axis in vertebrates. Hypothalamic GnRH stimulates gonadotrophs in the pituitary to release the gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH). In the gonads, LH stimulates production of sex steroids while FSH stimulates production of gametes. Sex steroids are also involved in gametogenesis. Sex steroids negatively feedback on the pituitary and hypothalamus, indicated by capped arrows. The gonadotropins negatively feedback on the hypothalamus. In male anurans, androgens e.g., testosterone (T) and dihydrotestosterone (DHT), regulate vocal behaviour through androgen receptors on laryngeal muscles. Motor nuclei in the brain related to vocalization may be androgen-concentrating. In females, both androgens and estrogens (E) may modulate audio reception. Dashed lines indicate indirect action of steroids and multiple neural pathways.

1.3 Disruption of reproductive hormones

Chemical contaminants in the environment have been of increasing concern for their potential to disrupt the activity of hormones, and it has been known for decades that exogenous compounds can affect endogenous ones. These so-called endocrine disrupting compounds (EDCs) are broadly defined by the US Environmental Protection Agency as *"exogenous agents that interfere with the transport, metabolism, binding, action or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the*

regulation of developmental processes" (Kavlock & Ankley, 1996). This diverse range of chemicals includes many pesticides, pharmaceuticals, and plastics. Neuroendocrinology studies how the nervous and endocrine systems interact to regulate processes such as development, metabolism, behaviour, and reproduction (Waye & Trudeau, 2011). Neuroendocrine disruption extends the concept of endocrine disruption to encompass the exogenous chemicals, their effects on physiological targets, and indirect downstream effects (Waye & Trudeau, 2011).

Compounds with (anti-)estrogenic and (anti-)androgenic modes of action disrupt the HPG axis, impacting reproduction in wildlife as well as humans (Cevasco et al., 2008; Urbatzka et al., 2007; Zala & Penn, 2004). Flutamide, an antiandrogenic pharmaceutical, suppressed the typical breeding cycles of male sticklebacks *(Gasterosteus aculeatus)*. Despite temperature and photoperiod cues for spawning, exposed males built fewer nests and displayed fewer zig zags, a courtship behaviour, in the presence of females (Sebire et al., 2008). Aquatic exposures to flutamide and vinclozolin, an anti-androgenic fungicide, both inhibited vocalization in male Southern clawed frogs *(Xenopus laevis)* (Behrends et al., 2010; Hoffmann & Kloas, 2010).

The natural endogenous estrogen 17β-estradiol (E2) reduced courtship behaviours and male sex characteristics such as breeding tubercles in goldfish *(Carassius auratus)* (Bjerselius et al., 2001). Ethinyl estradiol (EE2), a synthetic estrogen used in contraceptives, inhibited courtship, nest building, and aggression towards conspecific competitors in male sticklebacks (Bell, 2001). Similar effects have been demonstrated in male zebrafish *(Danio rerio)*, with EE2 reducing courtship, aggression, and social dominance (Colman et al., 2009). In male Japanese quails *(Coturnix japonica)*, *in ovo* exposure to EE2 led to inhibited sexual behaviours as adults (Halldin et al., 1999). The estrogenic flame retardant TDCPP is detectable in sewage effluent and surface water. After exposing zebrafish to low doses from fertilization to sexual maturity, TDCPP was detected in the gonads. Sex steroid levels were altered in females, egg quality was decreased, and offspring developed more abnormalities than controls (Wang et al., 2013). Chemicals with other modes of action can also disrupt reproduction. Fluoxetine, an antidepressant that acts as a selective serotonin reuptake inhibitor, can also disrupt reproduction as serotonin acts centrally on the hypothalamus and pituitary (McDonald, 2017), as well as peripherally on steroid production in the gonads (Mennigen et al., 2010). Fluoxetine

altered mating strategy in guppies *(Poecilia reticulata)*; exposed males increased coercive copulation attempts instead of performing courtship displays (Fursdon et al., 2019). Male starlings *(Sturnus vulgaris)* were more aggressive and sang less to fluoxetine-treated females (Whitlock et al., 2018).

1.4 African clawed frogs as laboratory models

For this research, I used the Western clawed frog as a model organism. African clawed frogs include four extant genera, with *Xenopus* and *Silurana* as sister groups in a clade (Evans, 2008). Western *(Silurana tropicalis)* and Southern (*Xenopus laevis)* clawed frogs are both laboratory model species, and *S. tropicalis* are still often referred to as *X. tropicalis*. Adult *X. laevis* are an established model in neurobiology and for investigating *in vitro* and *in vivo* endocrine disruption (Hayes et al., 2006; Huang et al., 2005). In particular, male sexual behaviours have been shown to be sensitive endpoints. Amplexus, the main spawning behaviour, occurs when the male clasps the female with his forearms. This behaviour is androgen-sensitive and can be stimulated with gonadotropins such as human chorionic gonadotropin (hCG) (Wetzel & Kelley, 1983). Vocalizations in *X. laevis* have been characterized in detail and are also an established endpoint. Low trills composed of repeating clicks are produced by contracting laryngeal muscles to distort the larynx. Like clasps, they occur in the presence of receptive females and are enhanced by gonadotropins (Tobias & Kelley, 1987). Castrated males do not clasp females to enter amplexus even with hCG injection, showing that reproductive behaviours depends on product secreted from the gonads. Androgen implants are able to rescue clasping behaviour in castrated males and likely act as the trigger for courtship (Kelley & Pfaff, 1976). The vocal cord muscles are abundant in androgen receptors, providing further support for androgens as the trigger (Tobias and Kelley 1987). Females spent more time near a speaker playing male advertisement calls over a speaker playing white noise when injected with hCG to induce sexual receptivity (Picker, 1983).

The closely related *S. tropicalis* is also a popular laboratory model. With a fully sequenced diploid genome, they were introduced as a model for genetics and genomics research (Grainger, 2012). They are easy to maintain in captivity as a fully aquatic species.

Spawning can be artificially induced year-round and results in large numbers of embryos. As a result, much of the literature on this species uses tadpoles to assess toxicity of chemicals and disruption to metamorphosis.

The courtship behaviours and general ecology of *S. tropicalis* has been very poorly studied, especially compared to *X. laevis*. Male *S. tropicalis* vocalizations have been briefly described in a few studies. They display trill-like calls consisting of consecutive clicks. Four types of calls have been identified and defined by duration and click rate (Table 1). All calls are approximately 500-600 Hz in pitch. Calling behaviour can be induced by the presence of a female, or by hormones such as AVT and gonadotropins (Dyson and Passmore 1988; Tobias, Evans, and Kelley 2011). As male vocalizations are a hormone-dependent and clearly observable behaviour, it was the main endpoint I examined in this thesis. Since *S. tropicalis* are convenient to work with in terms of husbandry and molecular work, I also focused on characterizing their courtship behaviours in detail to further establish them as a model species.

Table 1. The four types of calls displayed by male *S. tropicalis*, as defined by (Miranda et al., 2015)

Call type	Click rate (s^{-1})	Call length
Long fast trill	>20	>1 s
Short fast trill	>20	<1 s
Slow trill	5-20	>5 clicks
Click	<5	<5 clicks

1.5 Naphthenic acids

I used naphthenic acids (NAs) as a model contaminant for the possible disruption of sexual behaviours in *S. tropicalis*. Naphthenic acids are a diverse group of compounds that encompass all carboxylic acids found in crude oil, making up 0-3% of the weight depending on the source (Derungs, 1956). They are highly complex, heterogeneous mixtures, and chemical structures can only be partially characterized. Attempts at chemical characterization face the challenge of poor resolution, but suggest the presence of hundreds, potentially thousands of

different components (Gutierrez-Villagomez et al., 2017; Holowenko, MacKinnon, & Fedorak, 2002; Rogers et al., 2002). NAs were originally recovered from petroleum to reduce corrosion of oil refinery equipment. Consequently, they have been detected in coastal sediments after major oil spills in South Korea (Wan et al., 2014) and the USA (McNutt et al., 2012). NAs have also found several commercial applications such as paint and ink dryers, wood preservatives, emulsifiers and surfactants, and hydraulic fluids (Derungs, 1956).

NAs are some of the main toxic pollutants resulting from oil sands development (Headley & McMartin, 2004; Leung et al., 2003). Alberta, Canada has the third largest oil sand reserves in the world, after Venezuela and Saudi Arabia (Alberta Energy, 2018). Among Alberta's deposits, 4800 km^2 are shallow enough to be mined and the site in Athabasca is the largest and most extensively developed. These unconventional sources of oil contain bitumen, a thick, tar-like material, bound to sand and clay. Bitumen extraction requires water and solvents, generating OSPW (oil sands-process affected water) as waste. The OSPW is what remains after 90% of bitumen is removed and contains high concentrations of NAs, with levels exceeding 100 mg/L (Headley & McMartin, 2004). Large volumes of OSPW are currently held in artificial tailings ponds. By 2017, the total area of the ponds had expanded to 220 km^2. Despite a zero-discharge policy, concentrations of 1-2 mg/L NA are detected in surface water of the Athabasca region (Headley & McMartin, 2004; Leung et al., 2003).

The toxicity of NAs has largely been studied in fish models including zebrafish *(Danio rerio)* (Scarlett et al., 2013), fathead minnows *(Pimephales promelas)* (Kavanagh et al., 2012) and walleye *(Sander vitreus)* (Marentette et al., 2015). These studies have estimated several LC50s (median lethal concentrations) ranging from 5 to >50 mg/L depending on the source of NAs. At sublethal levels, some NAs act as endocrine disruptors, especially as androgen antagonists (Thomas et al., 2009). Goldfish *(Carassius auratus)* caged in OSPW containing large amounts of NAs showed elevated plasma cortisol, indicating an endocrine stress response. Plasma levels of testosterone (T) were also reduced in both male and female goldfish (Lister et al., 2008). In fathead minnows, a 21-day exposure to an NA extract impaired reproduction. The NAs decreased fecundity in females and inhibited the development of secondary sex characteristics in males. These changes were likely the result of lowered plasma concentrations

of sex steroids, especially androgens, which regulate reproductive behaviours and spermatogenesis (Kavanagh et al., 2012).

In addition to anti-androgenicity, some NAs may be slightly estrogenic, although evidence is weak. This activity may be partly attributed to structural similarities to estradiol and estrone as gas chromatography-mass spectrometry (GC-MS) data suggest the presence of steroid-like aromatic NAs (Reinardy et al., 2013; Rowland et al., 2011). Potential estrogen-like activity has been demonstrated in larval zebrafish, with NAs upregulating gene expression for estrogen receptor-alpha and vitellogenin (Wang, Cao, Huang, & Tang, 2015).

Amphibian studies of NAs have largely examined toxicity in larvae. Tadpoles of Northern leopard frogs *(Lithobates pipiens)* and *S. tropicalis* experience 100% mortality after 24 h in 6 mg/L NA. The 72-h LC50 was estimated at 4.1 mg/L NA for *L. pipiens*, and 2.95 mg/L NA for *S. tropicalis*. Exposed tadpoles displayed irregular convulsive swimming and physical abnormalities, such as bent tails (Melvin & Trudeau, 2012a). After chronic exposure to low, environmental doses, *L. pipiens* tadpoles showed reduced glycogen stores and increased triglycerides, indicating disrupted liver function (Melvin et al., 2013). Similar effects are demonstrated in tadpoles of wood frogs *(Lithobates sylvaticus).* They experience reduced growth, an LC50 of ~3-4 mg/L, and 100% mortality at 6 mg/L (Melvin & Trudeau, 2012b). Metamorphosis was delayed or entirely inhibited in wood frog tadpoles reared in young tailings-affected wetlands, possibly due to compromised thyroid hormone ratios (Hersikorn & Smits, 2011).

Increasingly, NAs are becoming recognized for their presence in aquatic environments, and acute toxicity and developmental and reproductive disruption in several species. Given these emerging concerns, I examined how NAs may disrupt courtship in *S. tropicalis*.

Chapter 2: Male sexual behaviours are disrupted by NA exposure

Abstract

Vocalization is a signature behaviour of courtship in frogs. With high sexual dimorphism, males primarily produce advertisement calls to attract females. Sexual behaviour and spawning in

Western clawed frogs *(Silurana tropicalis)* can be hormonally induced with injections of human chorionic gonadotropin (hCG). Analysis of underwater recordings indicate that males produce low trills with a pitch of ~600 Hz and ~37 clicks/s. Range finding exposures showed that a 5-day exposure to 20 mg/L of a commercial NA extract is sub-sublethal; all frogs were still swimming and eating normally. Therefore, this dose and duration was used for all other exposures. Males were exposed, injected with hCG to induce calling, and recorded, showing that NAs consistently reduce total vocal output. The dominant pitch, clicking rate, and trill length of the calls was not affected. Increasing the hCG dose for exposed frogs slightly but non-significantly restored calling behaviour. To examine mate competition, a sexually receptive female was allowed to freely interact with two males, one control and one NA-exposed. The control males mated with the female in all trials. However, this effect disappeared after two weeks of recovery in clean water, after which control and previously exposed males were equally successful. Suppression of vocal output was also partially reversed after recovery in clean water. Surprisingly, males did not appear to engage in vocal competition as they did not consistently adjust their vocal behaviour in response to speaker playbacks or calls from a live male. This study characterized in detail the virtually unexamined courtship behaviours of a popular model animal. The results show that NAs reduce vocalization and mating success. However, these effects are not necessarily permanent, highlighting the need for ecosystem restoration efforts.

2.1 Introduction

Acoustic signals are the main mode of communication in anurans and key to successful courtship. Males call to both attract females and compete with males. Male túngara frogs *(Physalaemus pustulosus)* were more likely to call when provided with supplemental food. Calling frogs also had higher plasma T than silent frogs (Marler & Ryan, 1996). In male reed frogs *(Hyperolius marmoratus)* and gray treefrogs *(Hyla versicolor),* oxygen consumption increased linearly with calling rate (Grafe, 1996; Taigen & Wells, 1985). In *H. versicolor*, oxygen consumption was higher during peak vocalization than it was during forced intense exercise (Taigen & Wells, 1985). As calling is energetically demanding, it can be an honest signal of the male's physical condition and fitness.

Amplexus, the mating position of anurans, occurs when a male clasps a female to externally fertilize the eggs. The important role of androgens in male vocalization and reproduction has been well-established in frogs species. In *X. laevis*, amplexus and vocalization can both be induced by human chorionic gonadotropin (hCG), an analog of pituitary luteinizing hormone which triggers gonadal steroid production. In castrated adult males, hCG no longer promotes calling. However, exogenous T and its derivative DHT both restore calling, and laryngeal muscles are dense in androgen receptors, suggesting that the behaviour is dependent on testicular androgens (Wetzel & Kelley, 1983). Androgens may also act on central pathways of vocalization in the brain. Androgen-concentrating cells have been found in the brain along neural pathways involved in vocalization, although these findings are limited to *X. laevis* (Kelley, 1980, 1981; Kelley et al., 1975).

Calling was also inhibited by castration and restored with exogenous androgens in other species such as the green tree frog *(Hyla cinerea)* (Burmeister & Wilczynski, 2001). Calling and clasping were induced by injecting homogenized whole pituitaries in male leopard frogs (*Lithobates pipiens)* led to a sustained increase in plasma T and DHT, along with increased calling and clasping behaviour (Wada et al., 1976; Wada & Gorbman, 1977). However, these behaviours were abolished by castration and restored with T and testicular implants, showing that pituitary hormones act on the gonads (Palka & Gorbman, 1973; Wada & Gorbman, 1977).

In addition to courtship, vocalization is used in male-male interactions for social dominance and competition. Males respond to competitors by increasing their own calling to out signal them, or become vocally suppressed by more dominant ones. When male *X. laevis* with equal vocal output are placed together, both become vocally suppressed and form a hierarchy. The dominant male is the one who calls more than the subordinate, and can further reduce the vocal output of the subordinate (Tobias et al. 2010). Acoustic signals are especially important for clawed frogs as they live in turbid lakes with low visibility (AmphibiaWeb, 2015). Given the role of hormones in courtship and the potential endocrine disrupting effects of NAs, I examined the effects of NA exposure on vocalization and mating success in *S. tropicalis*.

2.2 Methods

Animals

All frogs were bred and raised in captivity at the University of Ottawa Aquatics Facility. Adults were fed Nasco frog brittle 3 times a week while housed in 27 L aquaria with flow-through filters in groups of up to 40. The frogs were kept in a 26^{o}C room with a 12:12 h light/dark cycle. Sexually mature males (age 1-4 years) were identified by the presence of black stripes on the forearms (nuptial pads), a secondary sex characteristic.

Exposure to NAs

Treatment solutions were produced by diluting commercially available NAs (Sigma-Aldrich, Cat# 70340, Lot# BCBK0736V) in filtered, dechlorinated water from the University of Ottawa Aquatics facility. Frogs were individually exposed in static glass tanks containing 3L of the treatment solution at 26±1 °C. Solutions were fully replaced on the 3rd day. Frogs were not fed during exposures to reduce toxicity and stress from buildup of nitrogenous waste, and to avoid potential confounding behavioural effects. In many frog species, males do not feed at breeding sites as there may be a trade-off between sexual selection (mate attraction) and natural selection (foraging) (Woolbright & Stewart, 1987). For consistency, all experiments used the same batch of a commercial NA extract which has been chemically characterized in detail (Gutierrez-Villagomez et al., 2017; Gutierrez-Villagomez et al., 2020).

The toxicity of this particular NA extract in *S. tropicalis* embryos was previously examined, reporting a 96 h LC50 of 11.7 mg/L (Gutierrez-Villagomez et al., 2019). In contrast, my range finding studies showed that adult *S. tropicalis* can tolerate very high doses. Adults survived 2 days at 40 mg/L after having already been exposed to 20 mg/L for 4 days. They do not appear to experience obvious toxicity after a 5-day exposure to 20 mg/L as exposed frogs all behaved similarly to controls: actively swimming, able to maintain equilibrium, and responsive to visual and physical stimuli. To confirm that this dose does not affect the frog's physical condition, swimming activity was measured by gently prodding the hind leg with a pipette tip to generate a swimming burst. A swimming burst is defined by the frog starting in a static position, fully extending the hind legs to propel forwards, and coming to a stop without

being stopped by the sides of the tank. Swimming trials were filmed from bird's eye view with a 1cm grid placed under the tank. Blind observers analyzed the number of grids crossed using BORIS, a video analysis software. Swimming speed was not different between control and NA ($n=11$, $t=0.8390$, $p=0.41$) (Figure 15). Although animals may be exposed to lower doses and for longer durations in nature, short higher-dose exposures are relevant in the case of a spill for example. Based on an early pilot study with lower doses, 4 mg/L NA caused some disrupting effects on calling, although the difference is not significant and the data are not robust due to problems with methodology (Figure 16). Therefore, "NA exposure" will refer to 5 days at 20 mg/L for the remainder of this thesis.

Vocalization assays

Experiments were conducted in a 28 °C room on a 12:12 h light/dark cycle. Water temperature was maintained at 26 °C. With a lack of seasonal environmental signals under laboratory conditions, vocalization is induced following a standard breeding protocol. Frogs were injected with a priming dose of 25 IU hCG (Millipore, Cat# 230734), followed by a boosting dose of 100 IU after 1 day. Injections were prepared by suspending the hCG in 0.7% saline for a volume of 50 µL per injection. After the boosting injection, the frogs were monitored to screen out non-calling individuals. After 5 days of recovery, individuals were exposed to NAs for 5 days. At the beginning of the 6th day, calling was induced with hCG and recorded. A randomized block design was used, i.e. at least 1 frog from each treatment was recorded simultaneously to counterbalance effects of time and potential uncontrollable variables (e.g., microclimate fluctuations, unexpected disturbances from surrounding rooms). Tanks were visually and acoustically isolated with pyramid foam. A hydrophone (Aquarian Hydrophones, H2A-XLR) was placed in each tank and used in combination with an external interface (UMC404HD) plugged into a computer. Calls were recorded with multichannel recording software (MixPad, NCH Software) and exported to audio editing software (Audacity, Version 2.4.2) to visualize sound waveforms and spectrograms. Audio files were analyzed by several blind observers, with a total of 36 files analyzed by 2 different people. The inter-observer repeatability, i.e., the correlation between the values obtained by two observers for the same file was very high ($n=36$, $r>0.99$) (Figure 17). Absolute calling duration was measured by adding the duration of each trill.

Although there was no quantitative definition of a trill or inter-trill intervals, individual trills are visually and acoustically distinguishable with a clear start and end. Spectral plots were generated by Fast Fourier Transform on pooled calls from a frog. The frequency of the highest peak was recorded, obtaining one dominant frequency per frog. Click rate was manually counted over time and averaged over 5 randomly selected trills from each frog. The four types of *S. tropicalis* calls were observed (Figure 2) and consistent with previous descriptions (Miranda et al., 2015). However slow trills were very rare and not recorded for most frogs. Clicks are difficult to distinguish from background noise. Therefore, only fast trills were considered for the analyses.

This general experimental plan was repeated three times with slight modifications. In Feb 2018, 4-year-old frogs were injected with hCG and screened to ensure reliable calling 5 days before the experiment. In May 2018, 1-year-old frogs were also screened 5 days before the experiment. In May 2019, 1-year-old frogs were not screened since all individuals called in the previous experiments.

Immediately after recording vocalizations, frogs were anesthetized in buffered 1g/L tricane mesylate (TMS). Testes were dissected and blood was taken by cardiac puncture. The order of sample collection was alternated between treatments to avoid confounding effects of time on steroid levels. Blood was centrifuged in heparinized capillary tubes to separate fractions. Plasma and testes were frozen in dry ice and stored at -80 °C for later analysis.

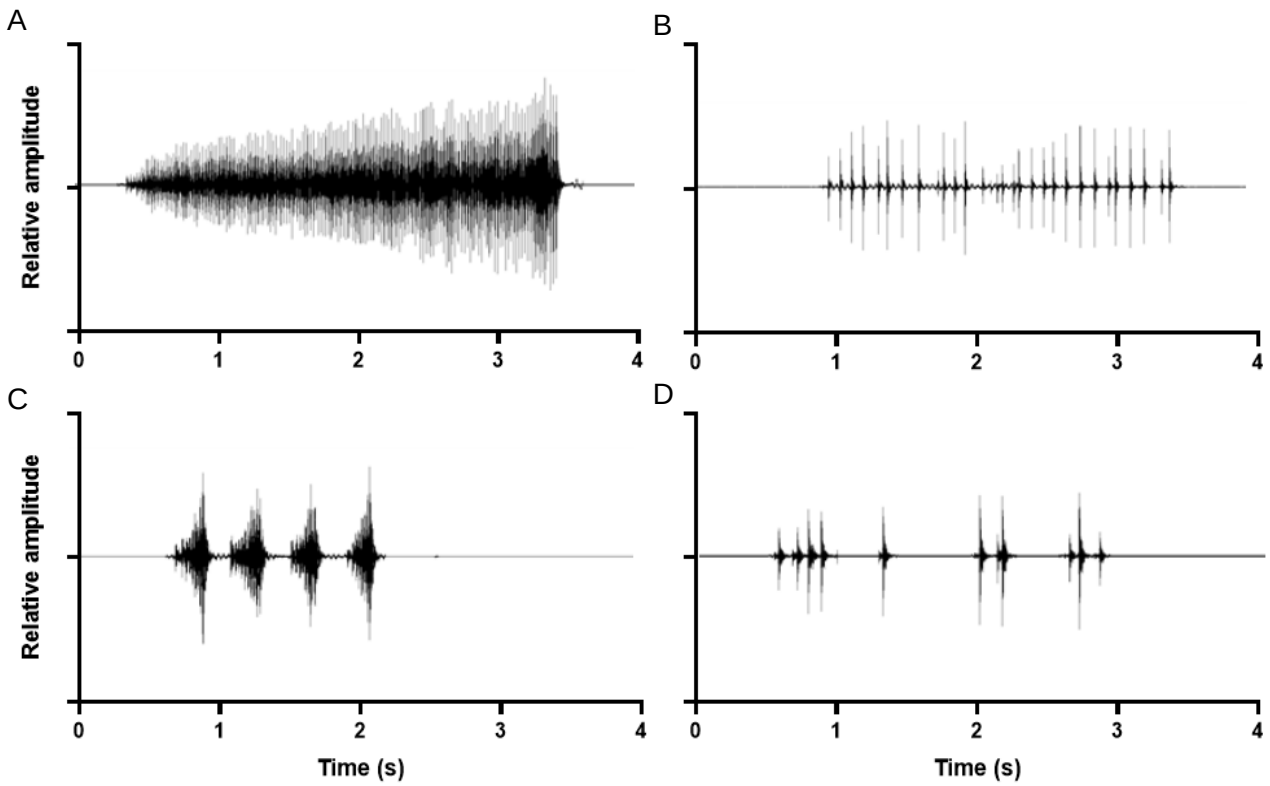

Figure 2. Examples of calls displayed by male Western clawed frogs. A) Advertisement call, B) Slow trill, C) Short fast trills, D: Clicks. Recordings obtained from control frogs in pilot studies. Waveforms visualized as relative amplitudes without units.

Rescue of NA-disrupted vocalization with androgens and hCG

In several anurans, androgens induce or increase vocalization and rescue the behaviour after it is abolished via castration (Burmeister & Wilczynski, 2001; Wada et al., 1976; Wada & Gorbman, 1977; Wetzel & Kelley, 1983; Yamaguchi & Kelley, 2006). Exposure to anti-androgenic compounds also inhibits calling behaviour in *X. laevis* (Behrends et al., 2010; Hoffmann & Kloas, 2010). Since the NA-exposed *S. tropicalis* displayed reduced vocal output, the NAs may have reduced the frogs' androgen production. Therefore, I attempted to rescue vocal behaviour in NA-exposed frogs using the androgens T and DHT. Several methods of administering the steroids were tested, including dissolving in ethanol and mixing into the tank water, and injecting the dissolved hormone intraperitoneally in the frog. The androgens alone never induced any calling even after several days of continuous administration. The frogs either did not take up the androgens, or not enough to affect vocalization. Injecting hCG induced calling in

frogs treated with androgens but the amount of calling was not different from those who received only hCG (n=4, p=0.8). It is also possible that the NAs have wider effects beyond the hypothesized effect on androgen production. For example, the larynx may have lower androgen sensitivity via reduced receptors, or the gonads may be less responsive to gonadotropins. Since the mechanism of disruption by NAs is unknown, I instead used higher doses of hCG to rescue. Under normal conditions, the effects of hCG increase with dose. As hCG is an analogue of LH, the hCG rescue can also assess response to gonadotropins. A vocalization assay was conducted with some NA frogs receiving higher boosting doses of hCG: 300 or 600 IU.

Competition and recovery

To examine how males respond to the calls of a competitor, individuals were injected with hCG (25 IU priming and 100 IU boosting after 24 h) and placed in a tank with 3L water. A waterproofed speaker was secured to the side of the tank underwater. The frog was left for ~1-2 h to start calling, then recorded while the speaker played white noise for 30 min followed by another male's calls for 30 min. The playbacks consisted of calls from previously recorded control males. A hydrophone was used to monitor the speaker playbacks and adjust the volume so that it was similar to that of live frogs.

To test vocal and mating competition between males, pairs were assigned to either control or the NA treatment. Although body weight does not seem to be associated with vocalization, pairs were nevertheless weight matched (<1g difference) to control for potential differences in physical competitive ability. Males were exposed and recorded in isolation. They were placed in opposite ends of a larger tank (91.5 x 31.5 cm) separated by mesh dividers. The dividers enclosed each frog in a 15 x 31.5 cm compartment (Figure 3). Each compartment had a hydrophone and the difference in intensity between the two signals identifies the caller. The side that the control and exposed frogs were placed in was randomized. After 1 h, the dividers were removed and a receptive female was added. The trio was allowed to freely interact undisturbed until the female was in amplexus. The frogs from the competition experiment were

maintained under control conditions for 14 days, then retested to determine if the inhibitory effects of NAs are reversible.

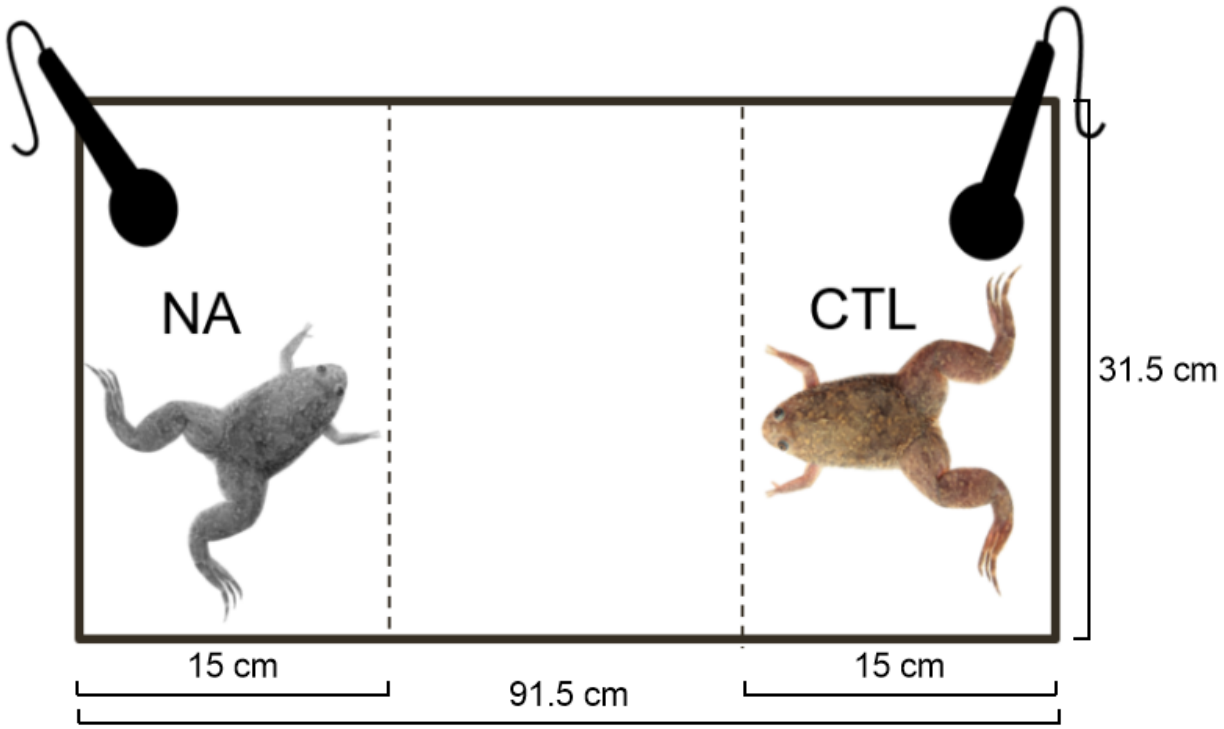

Figure 3. Setup for vocal competition recording. Mesh dividers (dashed lines) spatially isolated a pair of frogs while without blocking sound or water movements. The frogs were weight-matched and exposed to NA or control conditions. Two hydrophones on opposite sides recorded both frogs with differences in intensity due to distance. The discrepancy between the two recordings identifies the caller.

Statistical analysis

Analyses were conducted using Graph Pad Prism (Version 8.0) and R (Version 3.6.0). All p-values are two-tailed with α=0.05. Normality of residuals were tested using the Shapiro-Wilk test and visualized with QQ plots. Homogeneity of variances were tested using Levene's test and visualized with residual plots respectively. Calling duration between groups was compared with Kruskal-Wallis ANOVA or Mann-Whitney-U test on ranks as the data were not parametric. The exception is the data from the May 2018 assay which were parametric and analyzed with a t-test. The latency data from the rescue experiment were analyzed with log-rank comparison of survival curves. Frogs who never called were censored and assigned a latency of 5400 s (the length of the experiment). The mate competition data were analyzed using a binomial

probability test, comparing results to 0.5 probability of success expected by random chance. To compare vocal output between control and NA frogs, all data of individual recordings after exposure were pooled: the vocalization assays, the CTL+100 IU hCG and NA+100 IU hCG of the rescue experiment, and unpaired data from the competition experiment after exposure. The data were normalized by experiment with the lowest value as 0 and the highest value as 1, and analyzed with Mann-Whitney-U test on ranks.

Vocal competition data were log(x+1) transformed and analyzed with two-way repeated-measures ANOVA with subject and replicate (or pair) as random effects and phase (alone or competing) and treatment as fixed effects. Separate analyses were conducted for after exposure and after recovery. Individual calling data were log(x+1) transformed and analyzed with two-way repeated-measures ANOVA with subject and replicate as random effect and time and treatment as fixed effects. Models were fitted using the lme4 package in R (scripts in appendix).

To assess recovery of vocalization, the calling data from the alone phase of the vocal competition experiments were analyzed, before and after the two weeks of recovery. Only individual calling data was used for consistency with previous vocalization assays (1 h of recording after 5 days of exposure), and since NA-exposed frogs were no longer in the NA solution when paired.

2.3 Results

Description of behaviours

The four types of male *S. tropicalis* calls previously reported (Miranda et al., 2015) were observed (Figure 2) and further characterized. Slow trills were very uncommon and were not produced by most frogs. Clicks resembled background noise, and accurate durations were also difficult to obtain as they usually lasted <0.1 s. Males generally start vocalizing around 30 min after the boosting injection of hCG, and some continued calling over 24 h later. The frogs produce several frequencies with an average of 584 Hz (n=80, range 324-808). Pitch was not affected by NAs in any of the experiments (Figure 4A). The pooled averages were 581.4 Hz and 586.1 Hz for control and NA (n=40, t=0.21, p=0.83). The trills consist of rapid clicking with an

average rate of 37 clicks/s (range 31-44). The pooled averages were 37.9 and 36.9 for control and NA (n=25 and 33, t=1.8, p=0.08) (Figure 4B). The average trill length for each frog was calculated as total duration divided by total number of calls and ranged from 0.5-8.3 s/call. They were not different between control and NA (n=37 and 40, median=1.5 and 1.6 s/call, U=726, p=0.89) (Figure 4C).

In general, the frogs were observed to be inactive. Males were often stationary as they call. Receptive males oriented towards frogs who were swimming close by, then chased and attempted to clasp the other frog, whether they are other male or female. If the clasped frog is a male or an unreceptive female, the clasping male will release. If a receptive female is clasped, the pair will remain in amplexus. Males continued to call while in amplexus. Several frog species have a release call produced by males and unreceptive females when clasped, however no such vocalizations were recorded from *S. tropicalis*. It is unclear how females communicate non-receptivity to males. When frogs of both sexes were handled, such as during injections, they sometimes produced a low and very quiet rasping sound. This may represent a release call not detected by the hydrophone settings used, or they may have other signals for unreceptivity.

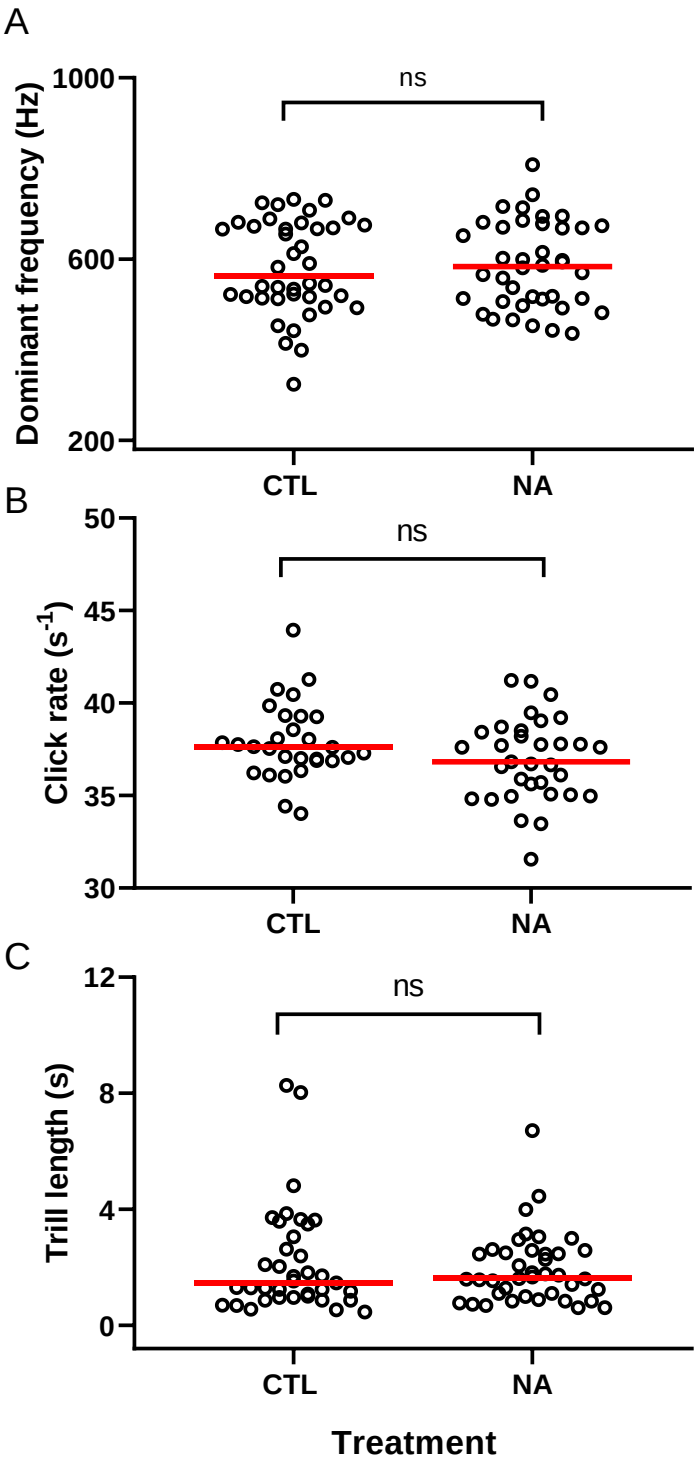

Figure 4. Effect of NA exposure on acoustic properties of vocalizations: A) dominant frequency, B) average click rate, or C) average trill length. Individual data presented and pooled across all experiments with red lines at medians. No significant effects were found ($p>0.05$)

Disruption and rescue of vocalization

In all three experiments, vocal output was clearly lower in the NA-exposed frogs (Figure 5). Median calling durations were 119.7 and 0 s in Feb 2018 (n=7, U=1, p=0.001), 664.2 and 307.6 s in May 2018 (n=12, t=2.8, p=0.009), and 65.27 and 0 s in May 2019 (n=8, U=7, p=0.006). Pooling normalized individual vocalization data from all experiments confirms that NAs reduce vocal output (U=115, p<0.0001). In total, 43/44 (98%) control frogs called, while 27/44 (61%) NA-exposed frogs called (Fisher's exact p<0.0001) (Figure 6).

In the hCG rescue experiment, the control, NA, NA + 3x hCG, and NA + 6x hCG groups had median calling durations of 220.9, 6.1, 19.9, and 7.6 s respectively, which overall was different between groups (KW=8.7, p=0.03). Dunn's multiple comparisons show that calling duration was lower in the NA frogs than control (p=0.04), consistent with previous assays. Durations were not significantly different between control and two NA rescue groups (p=0.17 and 0.2), although the three NA groups were also not different from each other (p>0.99). Controls start calling after around 30 min (median latency=1662.5 s), much earlier than the NA, NA+3x hCG, and NA+6x hCG groups (median latency=4732.66, 4279.47, and 3105.84 s respectively). Log-rank test shows an overall difference in latency between groups (X^2=34.3, p<0.0001). Although there appears to be a dose-dependent rescue effect, the difference between the NA groups is not significant (X^2=3, p=0.2) (Figure 7).

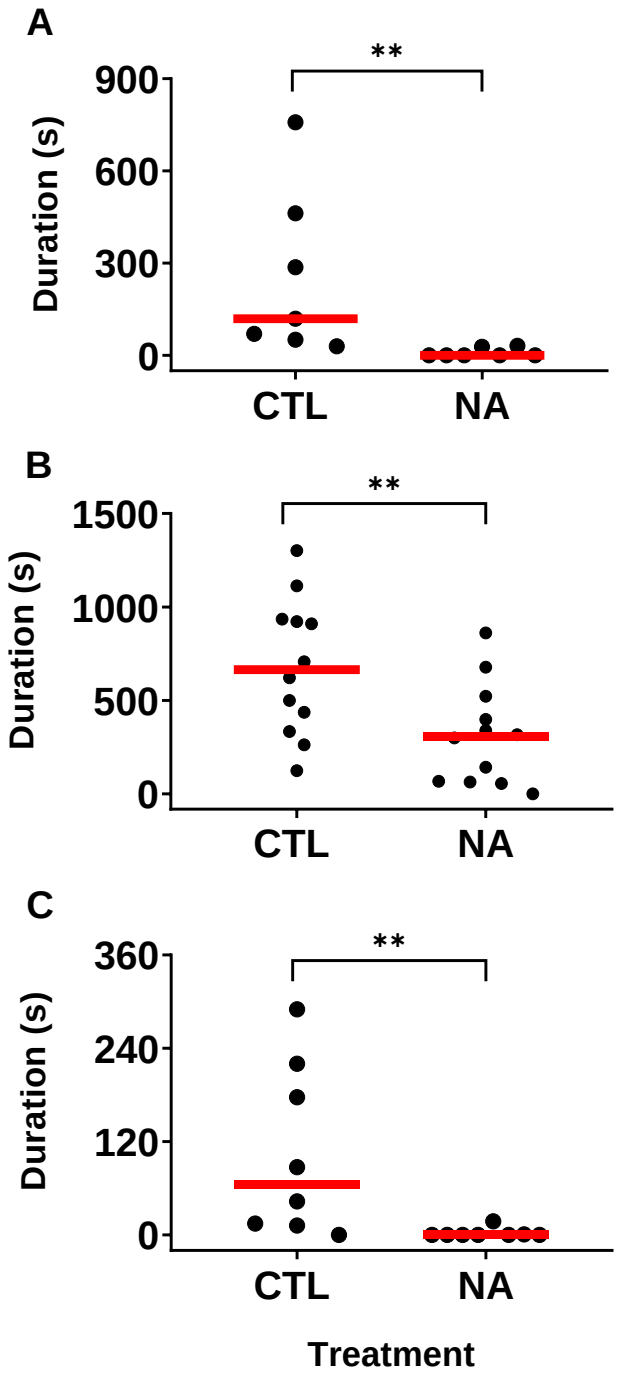

Figure 5. Effect of NA exposure on total calling duration in A) Feb 2018 B) May 2018 and C) May 2019. Individual data presented with red lines at medians. Statistical significance indicated by asterisks. **p<0.01

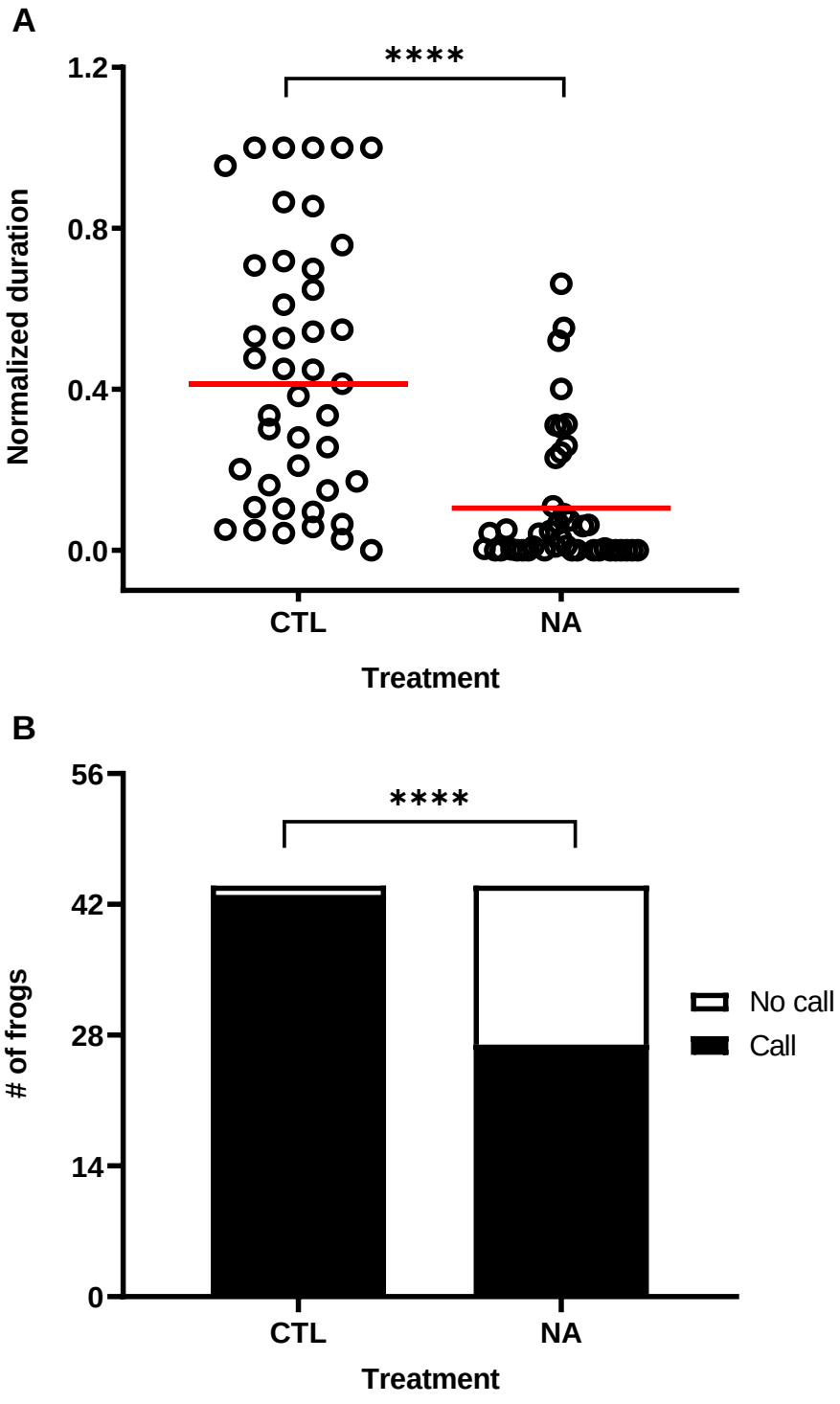

Figure 6. Effect of NA exposure on total calling duration, pooled across all experiments. A) Individual data normalized proportionately from 0-1 and with red lines at medians and B) count data of calling and non-calling frogs. Statistical significance indicated by asterisks. ****p<0.0001

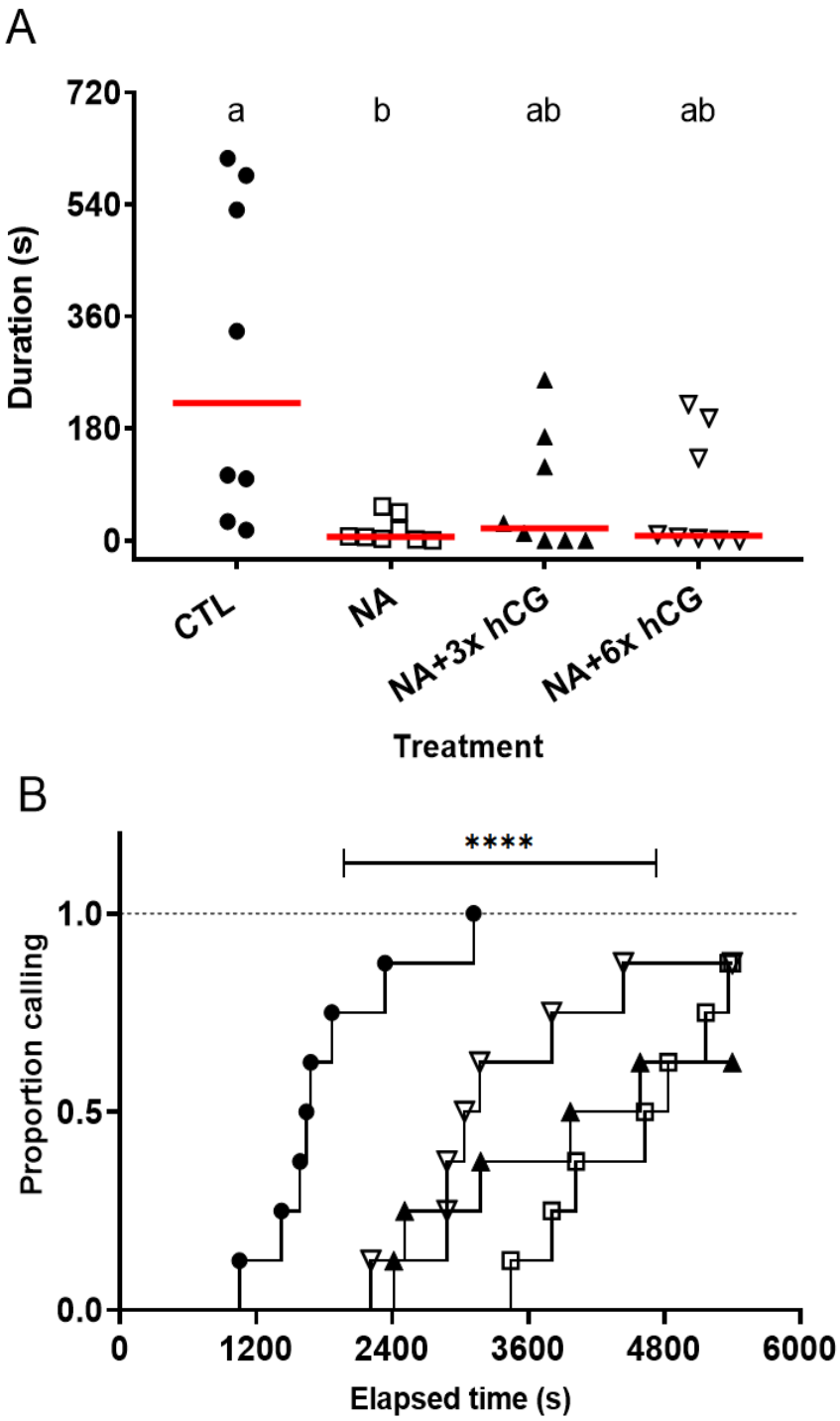

Figure 7. Effect of NA exposure and hCG rescue on calling duration and latency to call. A) Individual total calling duration data presented with red lines at medians. Different letters represent statistically significant difference ($p<0.05$). B) Individual latency data presented as survival curves. Statistically significant difference indicated by asterisks. ****$p<0.0001$

Inter-male vocal and mating competition

Exposing vocalizing males to speaker playbacks of other males did not show evidence of vocal competition, or the vocal suppression typically seen in *X. laevis* (Tobias et al., 2004, 2010). Once males had started calling, they were individually recorded with white noise and male call playbacks. There was no trend in how they respond to the "competitor"; some increased, decreased, or did not change their vocal output (n=10, paired t=0.6, p=0.6) (Figure 8). Since olfactory cues or physical movements detected in the water may also play a role in competition, live frogs were used instead of playbacks for the remaining experiments.

Pairs of control and NA-exposed males were recorded calling during two phases: first alone, then as a pair. There was no significant random effect of frog or pair ID; full and reduced models were not significantly different ($0.36<p<0.99$). Statistics reported hereon are from reduced models with only the fixed effects of treatment (control or NA), phase (alone or paired) and their interaction. Excluding random effects did not affect the significance of any results.

There was a significant effect of treatment ($F_{1,36}=11.8$, $p=0.0006$) and pairing ($F_{1,36}=10.6$, $p=0.002$), but no interaction was evident ($F_{1,36}=1.3$, $p=0.25$) (Figure 9A). Tukey's post-hoc indicated that during the alone phase, controls called more than the exposed frogs ($p=0.007$). Within individuals, the exposed frogs called more during the paired phase than alone ($p=0.02$) and this effect of phase was not found in the control frogs. During the paired phase, control males generally called more among the pair, although the difference in calling duration between control and exposed males are not significant (Figure 9B).

After two weeks of recovery in clean water, the same frogs were retested. Now, there was no effect of treatment, phase, or treatment x phase interaction ($p=0.8$, $p=0.07$, $p=0.3$) (Figure 9C). Tukey's post-hoc indicated that within individuals, the previously NA-exposed males called more during the paired phase than the alone phase ($p=0.03$). During the paired phase, there is no trend regarding which frog vocalized more (Figure 9D). Data were also analyzed by mating outcome (yes/no) instead of treatment. In this model, there was no effect of mating outcome, pairing, or interaction ($p=0.9$, 0.2, 0.4).

After audio recording for the vocal competition experiments, mating competition was tested as the two males were allowed to freely interact with a sexually receptive female. In the

first set of trials following the NA exposure, the exposed males failed to amplex the females in 10/10 trials (z=2.85, p=0.001). In the second set of trials after two weeks of recovery in clean water, control and NA-exposed males both amplexed in 5/10 trials (z=0, p=0.25) (Figure 10).

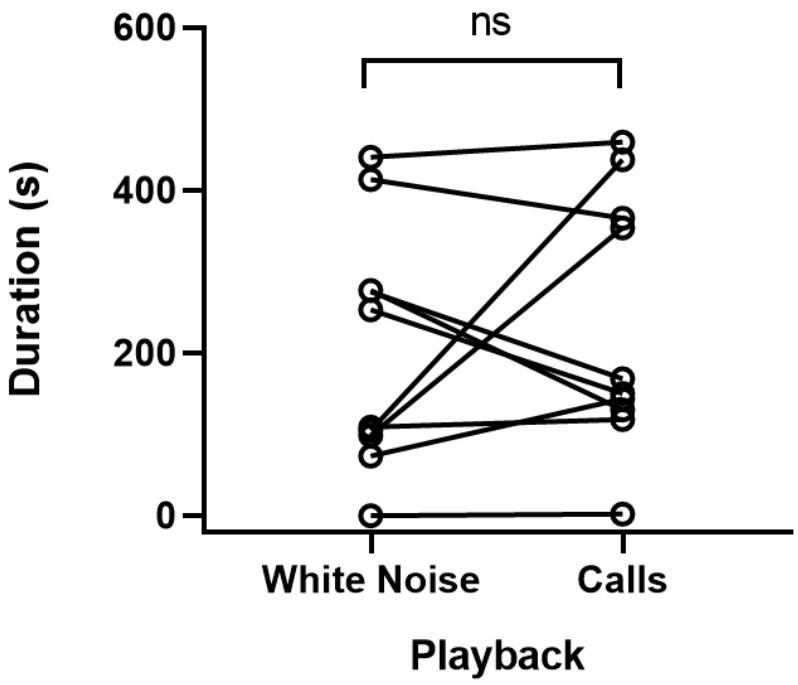

Figure 8. Total calling duration of males exposed to playbacks of white noise, followed by playbacks of previously recorded male vocalizations. Individual data presented with lines connecting the same frog. Difference in calling duration was not significantly difference during the two playbacks.

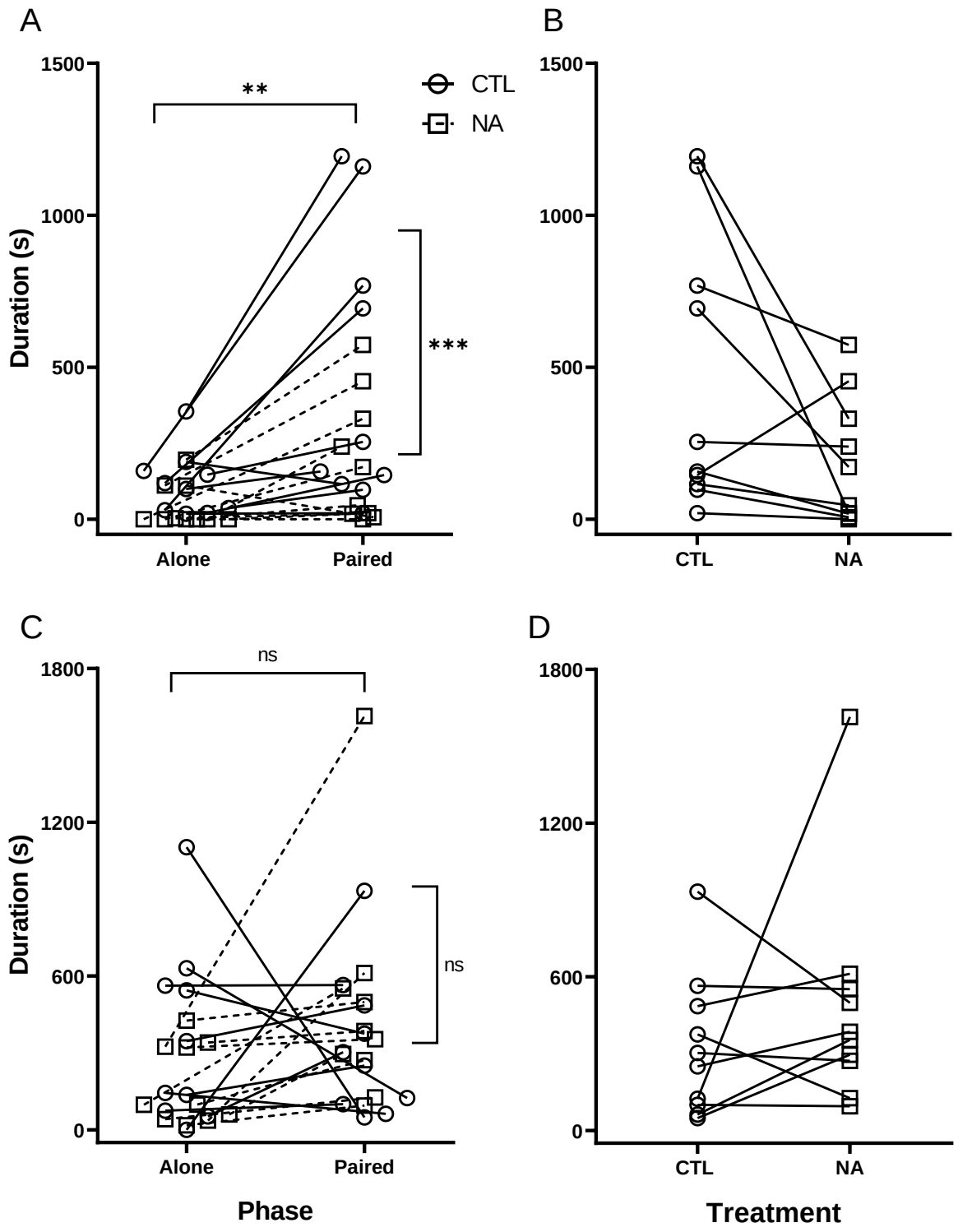

Figure 9. Effect of NA exposure and phase (alone or paired) on total calling duration. A) Lines connect individuals across phases. B) Same data as paired phase of A with lines connecting pairs. C and D) All frogs were retested after two weeks following the same procedures. Analyses performed on log(x+1) transformed data, raw data presented. Asterisks indicate statistical significance. **p<0.01, ***p<0.001

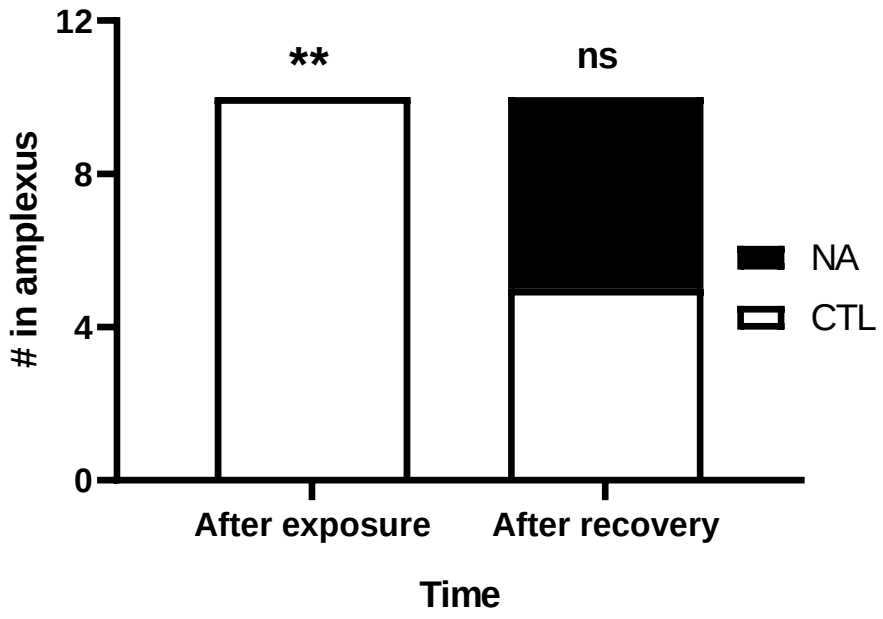

Figure 10. Mating outcome of pairs of a control and exposed male with one female. After exposure and after recovery. Count data presented. Separate analyses performed for after exposure and after recovery. Asterisks indicate statistical significance. **p<0.01

Disruption and recovery of vocalization

Recovery of vocalization in NA-exposed frogs after two weeks in clean water was assessed by comparing individual calling data from the alone phase of the vocal competition experiments. The fixed effects used in the model were treatment, time (after exposure and after recovery), and their interaction. There were significant effects of time, treatment, and interaction ($F_{1,36}$=11.63, p=0.0016; $F_{1,36}$=8.3, p=0.007; and $F_{1,36}$=4.9, p=0.03 respectively) (Figure 11). Tukey's post-hoc shows that controls had higher vocal output than exposed frogs after exposure, consistent with all previous vocalization assays (p=0.005). After recovery, the NA-exposed frogs called more than they did before (p=0.005), and were no longer different from controls (p=0.9). The random effect of individual was not significant (p=1) and the results did not change when it was excluded from the model.

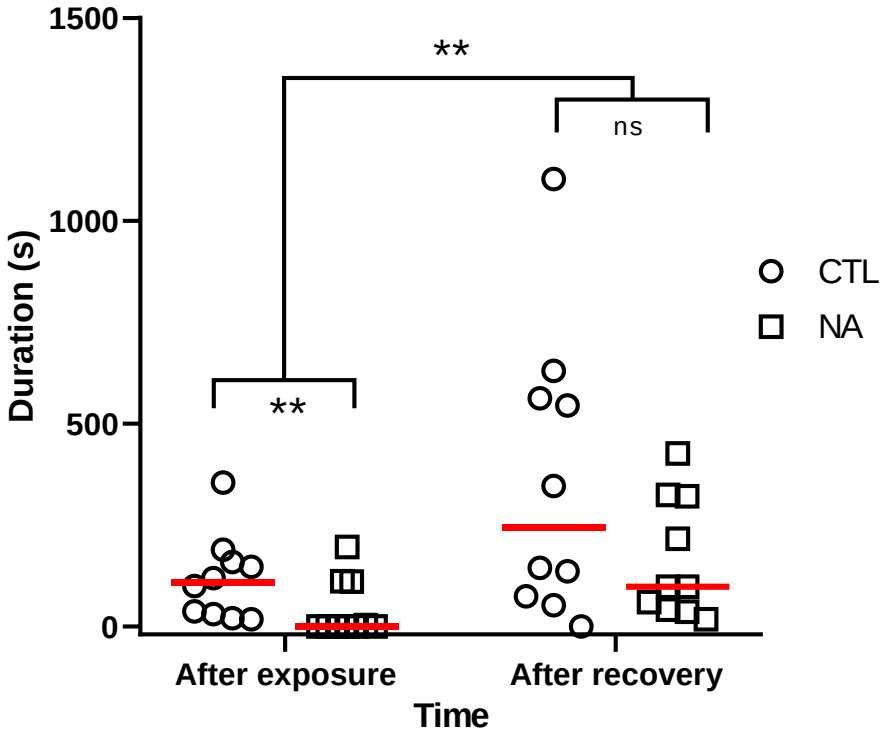

Figure 11. Effect of NA exposure and two weeks of recovery in clean water on individual calling duration. Analyses performed on log(x+1) transformed data. Raw untransformed data presented with red lines at medians. Statistical significance indicated by asterisks. **p<0.01

2.4 Discussion

This study reports for the first time the effects of NAs on behaviours in adult frogs. Adult *S. tropicalis* were unexpectedly resistant to overt toxicity of NAs, considering the sensitivity of larvae. Adults were still alive and mobile after 4 days at 20 mg/L followed by 2 days at 40 mg/L, whereas larval *S. tropicalis* experienced an LC50 of 11.7 mg/L NA after 96 h of exposure to the same NA product (Gutierrez-Villagomez et al., 2019). Toxicity assays have also been conducted with *S. tropicalis* embryos using a slightly different NA extract (Sigma-Aldrich, Cat# 70340, Lot# BCBC9959V), with varying results. One reported a 96 h LC50 of 10.4 mg/L NA (Gutierrez-Villagomez et al., 2019). Another study reported a 72 h LC50 of 2.95 mg/L NA, and had 100% mortality after 24 h at 6 mg/L (Melvin & Trudeau, 2012a). This NA product has the same catalogue number as the one used in this thesis but a different lot number. This highlights the

complexity of NAs and difficulties in comparing studies; results can vary between studies using the same product and species, and more so between different NA products and species.

Only one study has previously exposed adult frogs to NAs. Female *L. pipiens* did not experience any mortality or acute toxicity after 28 days at 40 mg/L NA, and continued to feed normally. At 60 mg/L NA, they developed systemic edema but there was still no mortality (Smits et al., 2012). As this study used another NA extract (purchased from Merichem) as well as a different species, the results are difficult to compare to the *S. tropicalis* data. An adult LC50 was not established for *S. tropicalis* due to limited sample sizes and difficulties of handling such high concentrations.

The vocalizations observed were consistent with a previous study (Miranda et al., 2015) which reported four different types of calls with an average dominant frequency of ~600 Hz. The vocalization assays represent the first on the behavioural effects of NAs. The results varied in scale and magnitude between experiments due to differences in age, cohort, and methods. However, the NA-exposed frogs consistently call for lower durations than controls. This result is consistent with the effects of various other EDCs in *X. laevis*. Vinclozolin, a commonly used fungicide with anti-androgenic effects, affected male vocalization. Males exposed for 96 h experienced a decrease in advertisement calls and an increase in calls indicating sexual non-receptivity (Hoffmann & Kloas, 2010). Exposure to flutamide, a model anti-androgen, also reduced advertisement calls (Behrends et al., 2010). Opposite effects were produced with the potent androgen agonist methyl-dihydrotestosterone (Hoffmann & Kloas, 2012a).

Although the role of androgens in inducing male vocal behaviour is well established in several anuran species, administering T or DHT did not affect calling in *S. tropicalis*. A study with *L. pipiens* also failed to restore calling in castrated males using daily intraperitoneal injections of T. However, calling was restored when intercranial T implants were placed directly into the brain (Wada & Gorbman, 1977). Androgens modulate central pattern generators of vocalization in teleosts and amphibians (Bass & Remage-Healey, 2008), and androgen receptors are known to be expressed in the vocal nuclei of *X. laevis* (Kelley, 1986; Pérez et al., 1996).

Direct administration of T or DHT into the brain may have been more effective, but also more invasive and riskier. As experimental data and several years of colony maintenance shows

a dose-dependent effect of hCG (Miranda et al., 2015), the increased dose was expected to rescue the suppressed vocalization. Total calling duration was reduced in the NA-exposed frogs, as previously shown. The three groups of NA-exposed frogs had very similar median calling durations. Control frogs began vocalizing after approximately 30 min, consistent with previous experiments. Increasing hCG appears to partly restore latency to control levels, although the difference is not significant. Overall, increasing hCG dose only slightly and non-significantly increased the vocal output that was suppressed caused by NA exposure. The effect of NAs may be severe enough that rescue was minimal, or NAs could be affecting hormone receptor abundance and sensitivity instead of production. In *X. laevis*, androgen receptors are found in laryngeal muscles and in parts of the brain responsible for vocalization (Kelley, 1980), and similar receptors may be disrupted by NAs in *S. tropicalis*.

As courtship involves both attracting mates and competing, male calling behaviour was examined in the context of male-male competition. After NA exposure, pairs of a control and exposed male were recorded alone, then in a pair. Frogs in both treatments called more when paired. However, time was a confounding factor because the alone phase always took place before the paired phase. Frogs generally start with few, infrequent calls before reaching peak vocal output. The increase in calling is likely an effect of time, not a response to a competitor, and it is therefore difficult to conclude if vocal competition is occurring at all. Vocal competition has been studied in *X. laevis* using a similar experiment design; males injected with hCG were recorded alone, paired, and alone again. In this case, the calling followed a very clear trend. Duration decreased for both frogs when they were together and a hierarchy was formed with the subordinate calling less than the dominant frog. Both frogs then returned to original calling levels after being separated. This experiment was conducted ~6 h after hCG injection, so the frogs were likely at their peak calling levels (Tobias et al., 2004). The playback of recorded advertisement calls also reduced the calling of live males, showing that acoustics alone without tactile, visual, or olfactory signals can cause vocal suppression (Tobias et al. 2010). This trend was not seen in the playback experiment with *S. tropicalis*.

After two weeks of recovery, calling duration was no longer different between control and previously NA-exposed frogs. Similarly, burbot fish exposed to the anti-anxiety drug

oxazepam showed decreased sheltering behaviour, and the effect was reversed after 5-7 days in clean water (Sundin et al., 2019). Furthermore, there was no longer a significant difference between calling output when *S. tropicalis* males were alone or paired with another. Since the hCG injections two weeks prior may have had priming effects, these frogs may have started calling sooner so that the recording window did not capture the initial increase.

Mate competition experiments were conducted immediately after vocal competition recordings by allowing the control and exposed male to freely interacted with a sexually receptive female. Following NA exposure, the control males successfully amplexed the female in all trials. By the time the male recordings were complete and a female was added, most females had already started laying eggs (~4hr post-injection) and were therefore highly receptive. Control males would immediately respond to her presence. When the female swam within one body length, the male would orient towards, chase after, and attempt clasp the female. Within a few minutes of adding her to the tank, the female would be in amplexus with one of the males. The NA-exposed males did not display these behaviours towards the female or make attempts to clasp. Even when the female swam close by or made contact, they would not orient towards or approach her. Although 10/10 controls were successful in mating, they did not necessarily do so by outcompeting the other male, as the exposed males were not observed competing or attempting to mate.

After recovery, mate competition was clearly observed as both males attempted to clasp the female. They physically competed when both attempted to clasp and dislodge the other by kicking. Some amplexus attempts were not successful as the female also dislodged males, providing further support for the role of female choice and receptivity in courtship. As these observations were qualitative, future work may examine and quantify mate competition behaviours in detail. A similar mating trial was conducted with *X. laevis* has been conducted previously. Four females, four control males, and four males exposed to atrazine, an anti-androgenic herbicide, were left to freely interact overnight (Hayes et al., 2010). Amplexus was achieved almost exclusively by control *X. laevis* males, but the frogs were not observed. It is unclear if the controls more successfully attracted the females and outcompeted the exposed males, or if the exposed males made fewer attempts to mate.

Interestingly, male *S. tropicalis'* vocal output was not a reliable predictor of competitive ability or mating success. Analyzing call duration by mating outcome did not show a relationship between vocal output and mating success. Within pairs, the winner did not necessarily call more than the frog he outcompeted (Figure 9B and 9D). These results were unexpected; based on their prolonged and low-density breeding system, male *S. tropicalis* mating success should depend on vocally attracting the female and outcompeting males. Advertisement calls are particularly important in low-visibility conditions such as turbid ponds at night. However, in the lab setting, the males were able to quickly locate and approach females, making attractive calling less important. This setup essentially led to scramble competition typical of explosive breeding, with males physically fighting for the female. The males were also able to approach the female immediately after she was introduced, so she may not have had enough time to evaluate the calls and make a choice. Reduced vocal output may have more significant effects in natural conditions, where breeding season lasts several months with a small proportion of females receptive at a given time. Individuals are more dispersed and rely on acoustic signals and phonotaxis to locate mates, rather than males physically fighting as seen in the experiment. To elucidate the males' ability to vocally attract females and ecological consequences of reduced vocal output, males could be physically but not acoustically isolated so that the females must approach her preferred mate based on song quality. Such an experiment would be relatively easy to conduct in both lab and field conditions by placing and recording individual males inside the traps later described in Chapter 3.

Since most females had already started laying eggs by the time the mating trials began, it is possible that they were no longer selective and accepted a male regardless of his relative attractiveness. As female túngara frogs *(Physalaemus pustulosus)* near the time of egg-laying, they approach playbacks with lower latency and become more likely to approach an unattractive call, becoming more receptive and more permissive (Lynch et al., 2005). The *S. tropicalis* females could have been injected with a lower dose and placed with the males before they start ovulating. This way, she can evaluate potential mates when she is less receptive and consequently, more selective.

Although the exposed frogs have lower absolute calling activity, the call characteristics (pitch, click rate, and trill length) do not appear to be affected by NA exposure (Figure 4)Figure 4. Effect of NA exposure on acoustic properties of vocalizations: A) dominant frequency, B) average click rate, or C) average trill length. Individual data presented and pooled across all experiments with red lines at medians. No significant effects were found ($p>0.05$). As these acoustic parameters depend on larynx morphology and muscle mechanics, NAs may be acting on the neuroendocrine triggers of vocalization rather than physically damaging the anatomy. Given the high energetic demands of vocalization, it is possible that the stress of exposure is simply diverting energy away from courtship. This may also explain why exposed males who vocalized still do not show interest in or attempt to mate with females. Body condition was assumed to be the same in controls and exposed frogs since they were weight-matched, had similar swimming speed, and qualitatively appeared to have the same body size and activity.

In this chapter, I described the acoustic properties of male *S. tropicalis* vocalizations. I found that exposure to NAs reduces vocal output and clasping behaviour, and that the effects of NAs may be reversible through recovery in clean water. Future research may examine the mechanisms of disruption, male-male competition, and role of female behaviours in courtship.

Chapter 3: Development of methods to quantify female *S. tropicalis* behaviours

3.1 Introduction

The study of sexual selection initially focused on male competitive strategies and female choosiness. First recognized by Darwin, sexual selection was described as *"special weapons, confined to the male sex''* and females who *"standing by as spectators, at last choose the most attractive partner"* (Shuster, 2009). Anuran vocalization exhibits extreme sexual dimorphism and is largely used by males for competition and courtship. As such, the main courtship behaviour of female anurans is phonotaxis, or movement towards an acoustic signal (Zelick et al., 1999).

Binary choice experiments are a commonly used tool to test preference, and two-speaker phonotaxis tests have been extensively used to study the effects of varying acoustic parameters on female response. Túngara frogs *(Physalaemus pustulosus)* are the most well-

established model system for sexual selection via female choice. Male calls consist of two components: whine and chuck. Females have a strong preference for complex calls, i.e., calls with both components, consistent over 19 years of testing (Ryan et al., 2019). More general parameters have also been tested, with consistent preferences (Dyson & Passmore, 1988). As calling is very energetically demanding, vocal behaviour can act as honest indicators regarding the male's physical fitness. Female *P. pustulosus* prefer larger males, are able to distinguish calls of small and large males. Lower pitches are preferred, as call frequency negatively correlates with size (Ryan, 1980). Intense and robust callers, i.e., high vocal output, are also preferred. Female grey tree frogs preferred calls of longer duration (Gerhardt et al., 2000). Female midwife toads *(Alytes obstetricans* and *Alytes cisternasii)* almost exclusively approached the higher call rate speaker (Bosch & Márquez, 2010). Comparisons of half-siblings show that larvae of long callers grow more and reach metamorphosis faster than larvae of short callers, suggesting genetic benefits to females (Welch et al., 1998). Calls are an honest signal for resources in male Emei music frogs *(Babina dauchina),* who build burrows for tadpoles. Resonating through a burrow changes the pitch of calls, and females strongly prefer calls produced inside over those produced outside, even from the same male (Cui et al., 2012). Since NA exposure in *S. tropicalis* decreased total calling duration and due to fewer individual calls overall (Figure 6, Chapter 2), their vocalization may be less likely to induce phonotaxis.

Playback experiments have also demonstrated hormonal control of phonotaxis toward conspecific calls. Female bullfrogs *(Rana catesbeiana)* approached speakers playing male advertisement calls, and with a shorter latency when injected with AVT (Boyd, 1994). Phonotaxis also depends on sex steroids. Female grey tree frogs *(Hyla versicolor)* displayed stronger phonotaxis responses when injected with progesterone and prostaglandin. Prostaglandin levels also correlated with E2 levels (Gordon & Gerhardt, 2009). Phonotaxis was induced in female *P. pustulosus* with injections of hCG, E2, and co-injection of E2 and progesterone. Co-injection of hCG and fadrozole, an aromatase inhibitor, failed to induce phonotaxis, suggesting that the behaviour depends on estrogens (Chakraborty & Burmeister, 2009). In *P. putulosus*, seasonal fluctuations in circulating E2 and progesterone also correlated

with reproductive state and peaked when females were gravid and most likely to display phonotaxis (Lynch & Wilczynski, 2005).

While the study of sexual selection generally focuses on competing males and choosy females, females also communicate to and attract males, and male mate choice is now widely recognized as an important component of courtship (Edward & Chapman, 2011). Female anurans generally produce only release calls in response to amplexus attempts when they are not sexually receptive (Emerson & Boyd, 1999). However, in a few species, females vocalize to attract males (Leary et al., 2004). Female *X. laevis* produce a receptive call, rapping, in response to male advertisement calls. Rapping consists of a series of rapid clicks, and can induce more advertisement calling from males, forming a duet (Tobias, Viswanathan, & Kelley, 1998). Female torrent frogs *(Odorrana tormota)* produce ultrasonic calls, attracting and evoking response calls in males (Shen et al., 2008). Female leaf-litter frogs *(Eleutherodactylus podiciferus)* produce a call as they approach or are approached by an advertising male (Schlaepfer & Figeroa-Sandí, 1998).

In the natural habitat of *S. tropicalis*, tadpoles can be continuously observed for several months throughout the rainy season, indicating asynchronous and therefore low-density breeding. Visibility is poor as *S. tropicalis* inhabit turbid ponds and are more active at night (AmphibiaWeb, 2015). Given these factors, males likely compete indirectly for females through vocalizations and females likely select and approach males via phonotaxis, although the natural breeding behaviours of *S. tropicalis* have not been studied.

Phonotaxis has not been studied in *S. tropicalis*, and only a few studies have been conducted using *X. laevis*. After hCG injection, female *X. laevis* approached a speaker with advertisement call playbacks by swimming in zigzags and in increasingly tight circles. Around 30% of females were phonotactic, defined as orienting towards and reaching 6 cm from the speaker (Picker, 1983). They were also able to consistently approach the conspecific call over the call of another species of clawed frog, *Xenopus muelleri* (Picker, 1983).

In this chapter, I characterized female behaviours and the effects of NAs. I also developed and validated a method to objectively measure phonotaxis in females, a cryptic and subjective behaviour. Since these frogs breed at night under natural conditions and females

only displayed phonotaxis in complete darkness, I developed an apparatus to track movement using infrared sensors.

3.2 General methods

Females were maintained under the same condition as males in sex-segregated tanks. As they are larger than males, they were fed ~50% more. They also received higher doses of hCG than males and several priming and boosting doses were tested. While females do not have clear secondary sex characteristics to indicate maturity, adults can be roughly identified by a more pronounced cloaca and bulges around the hips indicating eggs. However, one-year-old frogs who appeared mature were dissected and found to have partially immature ovaries (Figure 12). Fully developed eggs are large, spherical, and have distinct black and white halves. Immature eggs are smaller without clearly separated black and white. Therefore, the females used for the remainder of these studies were at least two years old. Exposures and injections followed the same protocols as males. Data were collected as discrete quantities analyzed as contingency tables using Fisher's exact test.

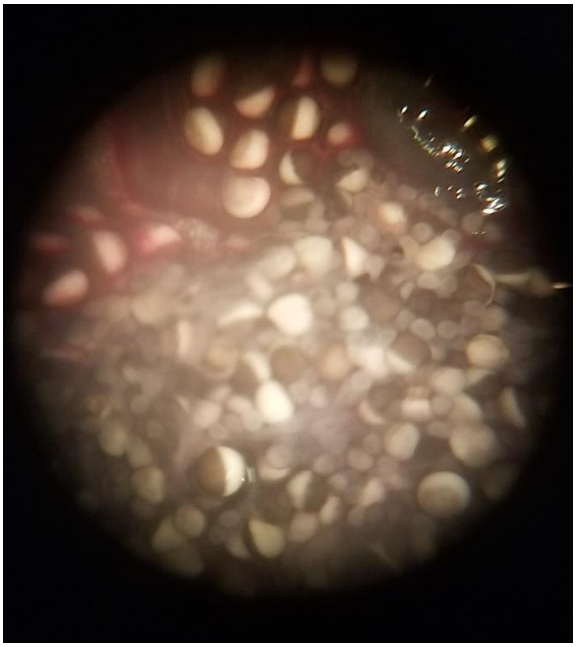

Figure 12. Partially mature ovaries of a one-year-old female. Mature eggs are larger and half black, half white. Immature eggs are smaller and grey, without distinct black and white sections.

3.3 Description of behaviours

After hCG injection, the female's cloaca everts and becomes red and swollen. This is also used as an indicator of sexual receptivity in wild-caught *X. laevis* (Tobias et al., 1998). Within a few hours, they ovulate and begin slowly laying a few eggs at a time regardless of the presence of males. At this point, gently squeezing the abdomen easily released eggs. The female would then lay an egg mass and no longer be receptive. Males may still attempt to clasp but do not achieve amplexus.

Recordings of females alone did not show any vocalization, with or without hCG injection. When receptive females were with hCG-injected males, a slow loud click with an inter-click interval of 1-3 seconds with an was observed. This click began shortly after the couple entered amplexus. This sound was never observed in males alone or males with unreceptive females. Therefore, it is most likely a female vocalization that indicates receptivity. The call was not observed in single females before amplexus so is unlikely for attracting males. It is not possible to confirm that the sound was produced by the female since a male was also present. However, the males continue to vocalize during amplexus with their typical trills sometimes overlaying the slow clicks, further supporting that the clicks are produced by the female (Figure 13). The female could be confirmed to be the caller with follow-up studies in which she is artificially clasped, or if she produces the sound while in amplexus with a male who has been experimentally altered to be unable to call (e.g., laryngectomy, freezing laryngeal muscles, etc).

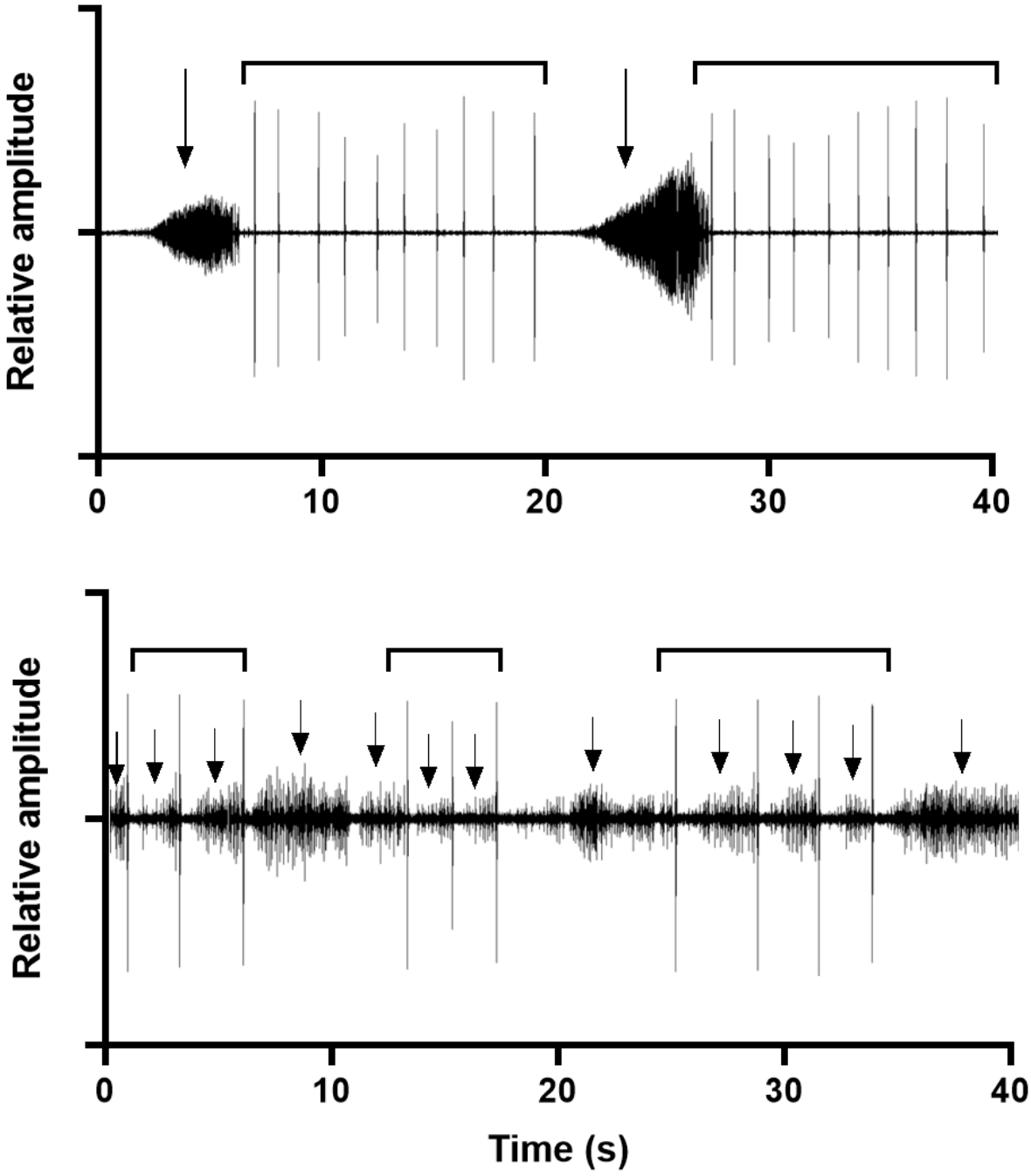

Figure 13. Vocalizations of a male and female in amplexus. Each waveform represents a different couple. Males produce trills, consistent with previously described advertisement calls indicated by arrows. Slow, repetitive clicks (indicated by brackets) were observed simultaneously. As this click was only observed in couples after achieving amplexus and had not been previously observed in recordings of males or females alone, it is likely produced by the female and may indicate receptivity.

3.4 Female mating success

Pairs of females were weight-matched (<1 g difference within each pair), identified with toe clips, and randomly assigned to control or NA exposure. After a 50 IU hCG priming injection and 200 IU boosting injection, females were rinsed and placed in 3 L dechlorinated water for 30 min to reduce surface NAs. Mature males (i.e., previously successful breeders) were injected with 25 IU and 100 IU hCG priming and boosting dose, then placed in 3L water with one control female and one exposed female. The frogs were left undisturbed in the dark for 2 h. At this point, all males were in amplexus and the females were identified. In 9/11 trios, the male was in amplexus with the control female and in 2/11 trios, with the exposed female (binomial test, z=-1.8, p=0.03). As the frogs were not directly observed during courtship, it is unknown how or why the males achieved amplexus with one female over the other. The male may have attempted to clasp both and achieved amplexus with the more receptive female, or preferentially attempted to mate with the more attractive female. Due to the lack of visual cues, no difference in size, and no evidence of mate-attracting calls from females, any male preference would have been likely dependent on olfactory signals. The amphibian chemosignalling literature focuses on urodeles (newts and salamanders), however anurans are also known to produce pheromones. Exposure to endosulfan, an insecticide that acts as a competitive inhibitor of EE2, disrupted pheromonal communication in red-spotted newts *(Notophthalmus viridescens).* Males preferentially approached water flow containing control females and mated with controls over exposed females. The control females also triggered more electrophysiological olfactory responses in males (Park et al., 2001).

It is possible that despite the 30 min wash in clean water, females were still purging NAs which repelled the male. Avoidance behaviour of contaminated water has been measured in several aquatic animals (Rosa et al., 2008). Rainbow trout *(Oncorhynchus mykiss)* showed a bioconcentration factor (BCF) of ~2; their tissues reached and plateaued at 5.6 mg/kg NA when exposed to 3 mg/L NA. After 1 day in clean water, NA concentration was down to 0.3 mg/kg. After 2 days, it was 0.1 mg/kg which is the detection limit. Among fish wild-caught near the Athabasca oil sands, NAs were detectable in the tissues of 4/23 (Young et al., 2008). Yellow perch *(Perca flavescens)* were kept in ponds of OSPW with <1-~13 mg/L for 4 months, but NA

accumulation was not consistent with water concentrations (Van den Heuvel et al., 2014). Due to apparent species differences, variability in quantification methods, and heterogeneity of NA mixtures, it is difficult to estimate depuration of this particular NA extract in *S. tropicalis*. Furthermore, gills are the main site of solute uptake in fish (Arnot & Gobas, 2006). A single study has been conducted with adult frogs*, L. pipiens*. Tissues contained 4.9 mg/kg NA when exposed to 20 mg/L and 9.2 mg/kg when exposed to 40 mg/L for a BCF of ~0.25 (Smits et al., 2012). However, (Smits et al., 2012) used a different commercial NA extract purchased from Merichem. To simply measure rate of purging in *S. tropicalis* for the specific NA extract used (Sigma-Aldrich, Lot # BCBK0736V) without sacrificing animals, water samples could be regularly taken after moving an exposed frog to clean water.

Finally, the exposed females may have been less receptive, or responded to the hCG with higher latency. This is likely as NAs greatly increased latency to call in males, so the females may have not become receptive until after the control female was already in amplexus. Males have been observed attempting to clasp saline-injected females and quickly releasing, perhaps due to a currently unidentified release call indicating unreceptivity. Quantification of male clasping can reveal if the exposed females were unreceptive or unattractive, i.e. if males attempted but failed to achieve amplexus, or if males did not attempt to clasp. I then aimed to measure phonotaxis as an indicator of female receptivity.

3.5 Measuring phonotaxis

Binary choice tests are commonly used to study phonotaxis, as well as responses to many other stimuli; the test subject starts in the middle of a tank (neutral zone) and the time spent in proximity to two stimuli (choice zones) on opposite sides is measured. However, this method did not work well for *S. tropicalis*. The binary choice test is well-validated for guppies *(Poecilia reticulata)* (Jeswiet & Godin, 2011) but in general, these frogs are quite inactive and spend most of their time hovering in one location. This limited the amount of spatial and locomotor data that could be collected. The frogs appeared to be fearful of the test tank and sensitive to light, even dim red light. Most frogs immediately swam to an end of the tank and stayed near corners and sides. Since the choices zones were on opposite ends, latency to

approach was not a reliable indicator of attraction to sound. Placing the speaker in the middle of the tank led to the opposite problem: the frogs appeared to not be phonotactic. This also made it difficult to measure the lack of receptivity, since the frogs avoided the middle of the tank (the neutral zone), and would be in a choice zone by default. Our space limitations also reduced the sensitivity of the test as it was more likely for the frog to be in any part of the tank by chance. To visualize night-time phonotaxis in Australian tree frogs *(Litoria chloris),* females were fitted with "backpacks" holding LED lights (Aihara et al., 2017). Due to complications of waterproofing electronics, I attempted to secure small luminescent and reflective sheets to the frogs back. However, the frogs appeared distressed and scraped off the materials with their hind legs.

The design of playback experiments has also raised concerns regarding the response variables measured (McGregor, 2000). Preference is inferred from time spent in proximity to the stimulus (in choice zones or neutral zones), or latency to approach. However, spending time in one place may simply result from avoiding other places, especially if the stimulus is aversive or induces neophobia. The test may also fail to detect a difference if the animal cannot perceive a difference, if they respond consistently to any stimulus, or if the test is not sensitive enough.

Through addressing these issues, I developed a test to objectively measure a cryptic behaviour and run in complete darkness. Several different setups were tested, and the final design is shown below. Speakers are placed inside traps (23 x 23 cm) with infrared sensors. The opening is funnel shaped, measuring 6 cm at one end and 2.5 cm at the other, allowing the frog to enter but not exit. It also prevents false positives as without the funnel, the frogs would swim in and out of the trap randomly. Entry into a trap with playbacks is considered phonotaxis. Playbacks are made by selecting five advertisement calls from one male. White noise tracks are made from the background sound recorded between calls. Each playback uses calls from different males to prevent pseudo-replication. Sides of playbacks and the physical speakers were both alternated. The test is conducted by placing two traps with speakers in a soundproof chamber (60 x 30 cm) made by stacking two plastic tubs with a layer of acoustic pyramid foam in between (Figure 14A).

The sensor consists of two LED lights: a transmitter and a receiver (QED123 and QSD123, ON Semiconductor). A beam of infrared light (880 nmλ) is emitted from the transmitter to the detector. A plastic frame keeps the transmitter and detector aligned 16 cm apart and running across the opening of the trap. Aquarium-grade silicone sealant waterproofs the lights and connections. The detector is connected to a data acquisition box (USB-6000, National Instruments) plugged into a computer to track voltage. At rest, the input from the light is 5v. When the beam of light is broken by a frog crossing, the voltage drops and the time is recorded by a custom-made program. Although there is no direct evidence, it is assumed the frogs cannot see 880 nm light waves. Furthermore, the lights are placed in the trap such that the frog would not see until they are already inside. Entry into the trap should not result from visual attraction to the lights (Figure 14C).

The infrared signal was found to be too weak to be detected underwater due to optical deflection. The baseline signal was so low that disruption of the beam did not generate a detectable voltage drop. However, these lights were the best choice due to cost, availability, and visibility. To strengthen the signal, the detectors were placed vertically to minimize the amount of water between the detector and transmitter (Figure 14B). This setup is functional, although should be further modified to be more user-friendly, perhaps by using higher intensity lights with a higher baseline signal. Photos of the trap and sensor are shown in the Appendix.

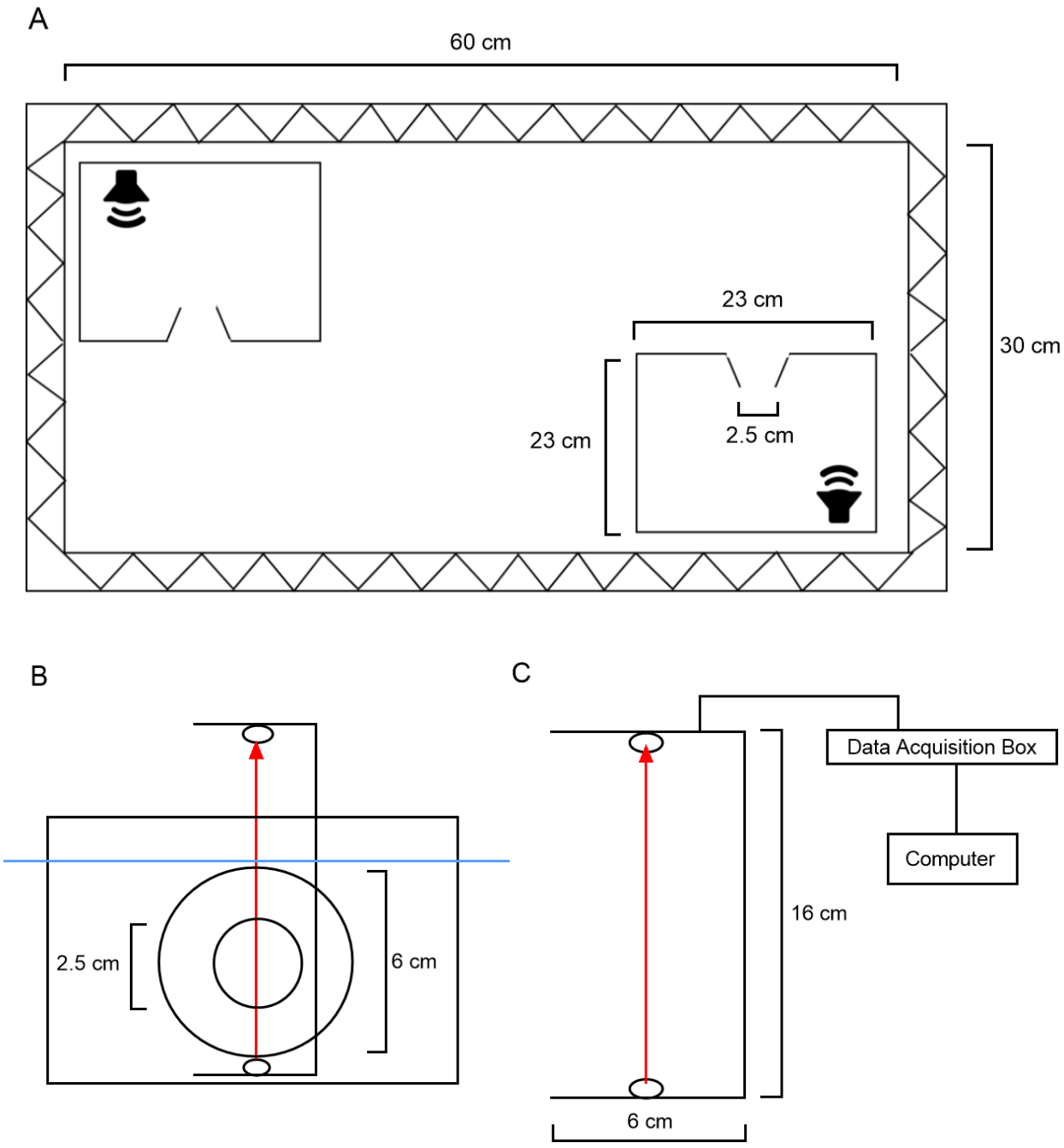

Figure 14. Phonotaxis test apparatus. A) Bird's eye view of test arena. Speakers are inside traps with funnel-shaped openings so that frogs can enter but not exit. Infrared sensors are placed at the opening. Two traps are placed in opposite corners of plastic tub soundproofed with foam. B) Side view of the sensor and trap. The trap opening tapers in and is funnel shaped (6 cm to 2.5 cm) C) Infrared sensor. Two LEDs are aligned and secured to a plastic frame with silicone sealant. A beam of infrared light (indicated by the red line) is transmitted by one LED across the entrance of the trap and received by the

other LED. The apparatus is placed vertically inside the trap to minimize light signal attenuation by water. A data acquisition box and custom-made computer program records the time that the beam is broken by the frog crossing. The test arena was filled with water just deep enough to submerge the opening of the trap (indicated by the blue line). Photos shown in Appendix (Figure 18).

3.6 Testing the apparatus

I had planned to test each female for ~10-15 minutes a few hours after hCG injection, as previously done with *X. laevis* (Hoffmann & Kloas, 2012a). This would allow several replicates to be tested consecutively, and repeated measures within individuals. However, the time to respond to the hCG was extremely variable and it was not possible to find a window to test all the frogs. Some frogs would have laid eggs already and no longer be receptive, while others would not be receptive yet. Due to these challenges, I decided to instead test each female one time for a longer duration. Immediately after hCG injection, females were placed in the middle of the test arena inside a cylinder. Playbacks started after ~5 min and ran continuously for 4 h, regardless of if or when the frog approaches a speaker since simply hearing calls may affect hormone levels. In female *P. pustulosus*, exposure to playbacks of male calls for 10 nights increased plasma E2 levels relative to females who were exposed to playbacks of random tones (Lynch & Wilczynski, 2006). For most of the trials, the *S. tropicalis* females were primed with 50 IU and boosted with 200 IU hCG. In some later trials, some females laid eggs overnight from the priming dose alone. These females tended to be younger, or previously injected within the past few months. With little ovarian tissue left, they did not respond well to the boosting injection. They did not display phonotaxis or allow males to clasp them. These trials were discarded and the hCG doses were then changed to 25 IU for priming and 200 IU for boosting.

The apparatus was first piloted using 4-5-year-old females that were regularly used for breeding and colony maintenance. In general, frogs did not enter empty traps, or traps with white noise playbacks. Saline-injected females and females who finished laying eggs did not exhibit phonotaxis. Some trials had two call playbacks and some had one call and one white noise. In 9 of the trials, there was one calling track and 8/9 frogs displayed phonotaxis regardless of side. One of the frogs did not enter either trap. In the other 20 trials, both speakers played calls. The only parameter altered between the two tracks was total vocal

output, or inter-call interval. This was done by adding/removing white noise between calls of the same track. Several ratios of vocal output were tested, up to 20:1. In the binary choice tests, the frogs almost exclusively went to the same side regardless of playback intensity. The animal facility had a lot of background noise from various systems and activity in surrounding rooms, which may have caused the side bias. Playback tests are generally conducted in sound-proofed chambers, and better acoustic isolation is required for future binary choice tests. In total, 25/32 of these experienced females exhibited phonotaxis, usually within 2-4 h of the boosting hCG injection.

To control for possible effects of past experience and hCG injections on behaviour and hCG response, I then used naïve females. They were 2.5 years old, naïve to hCG and to males, and tested with a single call playback. Of the first eight naïve females tested, none displayed phonotaxis. They did lay eggs, showing that they were sexually mature and responsive to hCG. The only difference between these females and the ones who previously demonstrated phonotaxis was lack of experience with males.

To give the females experience with courtship, a male induced to call with hCG was placed with two naïve, unreceptive (i.e., saline-injected) females for several hours the day before testing to familiarize the females with the sound of a vocalizing male. However, these females still did not display phonotaxis the day after. All naïve females were then bred with mature males (i.e., reliable breeders) in large groups, ~10-15 of each sex. All couples achieved amplexus and laid eggs, which ensures that each female had heard calls from several different males and has experienced breeding. The females were returned to their regular tanks and were fed ad libitum for ~2 months until they regenerated eggs, estimated by regaining their characteristic bulges of eggs around the hips. At this point, all females had had the same experience with hCG injection, courtship, and mating, and the same duration of gamete production. After breeding once, the females were tested and 4/5 displayed phonotaxis, suggesting that courtship and mating experience may be required for females to display phonotaxis. Overall, 29/37 experienced females exhibited phonotaxis, compared to 0/8 naïve (Fisher's exact p= 0.02).

Table 2. Results of all phonotaxis trials. Naïve females were naïve to both hCG injection and interaction with males. Females mated several times were 4-5 years old and regularly bred to obtain tadpoles. Females mated once were 2.5 years old and tested ~2 months after breeding for the first time.

Description	Displayed phonotaxis (#)	Total tested (#)	Displayed phonotaxis (proportion)
Naïve	0	8	0
Mated several times	25	32	0.78
Mated once	4	5	0.8

3.7 Discussion

Female vocalization is a rare phenomenon for anurans (Emerson & Boyd, 1999). For the first time in *S. tropicalis,* a call likely produced by females to communicate receptivity was observed. This call should be examined more closely to confirm if it is produced by the female, and to characterize its function during courtship. Release calls are commonly produced by unreceptive female anurans in response to being clasped (Emerson & Boyd, 1999). In *S. tropicalis*, no such call was recorded, but females appear to communicate non-receptivity to males as males attempted to clasp and quickly released saline-injected females. Further observations may identify a release recall, perhaps using more sensitive hydrophones.

Phonotaxis was also studied for the first time in *S. tropicalis.* Troubleshooting the binary choice test and resolving the side bias can allow studies on female preference. Indirect effects of NA exposure can be studied by evaluating the males' ability to vocally attract females. Female *X. laevis* showed a preference for calls from control males compared to calls from males exposed to EE2 for 96 h as their calls lacked accentuated clicks (Hoffmann & Kloas, 2012b). Although the pitch and click rate of *S. tropicalis* male calls were not affected by NA exposure, the absolute output was reduced. In grey treefrogs *(Hyla versicolor),* vocal output indicated energetic costs to males, and half-sibling studies found that males who produce longer calls have offspring with higher fitness (Welch et al., 1998). Females of some frog species show a preference for high calling intensity and vocal output (Bosch & Márquez, 2010; Gerhardt et al., 2000), suggesting that the overall songs of NA-exposed *S. tropicalis* males may be less attractive than those of control males.

Direct endocrine disruption of phonotaxis has only been tested in a single study with *X. laevis*, with weak and inconclusive results. A 96-h exposure to the androgen agonist methyl-dihydrotestosterone made females more or less phonotactic, depending on dose (Hoffmann & Kloas, 2012a). A future step would be to examine the effects of NA exposure on phonotaxis in *S. tropicalis.* This was not examined in this thesis due to several unexpected challenges regarding timing, age, and experience which required extensive troubleshooting. Considering that males experienced higher latency to start calling after hCG injection (Figure 7B), it is possible that phonotaxis would also be delayed or inhibited by NAs.

The lack of response to male playbacks in naïve females despite responding to hCG (i.e., ovulating after injection) suggests that mating experience influences display of phonotaxis. The role of social experience in courtship has been mainly studied in the context of songbird vocalization, as well as other species (Freeberg, 2000). In Weddell seals *(Leptonychotes weddellii),* paternity tests showed that male reproductive success is explained by mating experience (Harcourt et al., 2007). In pairs of fruit flies *(Drosophila melanogaster)*, males with mating experience outcompeted naïve males for a female. This effect was not seen in males with courtship but not mating experience (Saleem et al., 2014). Similarly, the female *S. tropicalis* who experienced courtship without mating were still not exhibiting phonotaxis.

Further validation will be required to show that the playback a female chooses predicts mating outcomes. For example, the stimulus fish that *P. reticulata* spend more time associating with in binary mate choice tests were shown to predict the mating outcome when the fish are allowed to freely interact (Jeswiet & Godin, 2011). The apparatus provides unambiguous binary data and the concept can be widely applied to choice and preference studies, such as chemotaxis towards pheromones. The infrared sensors are non-invasive and can be used in the lab or in the field for tracking movement under low visibility, such as at night or underwater.

Chapter 4: General discussion

4.1 Summary and significance

The courtship behaviours of *Silurana tropicalis* and non-lethal effects of naphthenic acids are both poorly studied. In this thesis, I have provided detailed descriptions of male vocal behaviour, and report a previously undescribed female vocalization. A 5-day exposure to 20 mg/L NA consistently inhibited vocalization and mating effort in males, although calling was not predictive of competitive ability. These effects were reversed after 2 weeks of recovery in clean water. Mating success was also reduced in females exposed to NAs, possibly through reduced attractiveness or receptivity. A novel method was created to measure phonotaxis, the main receptive behaviour. This apparatus may also be used to assess both male and female mate choice, with broader applications in measuring preference and tracking activity in the dark.

Throughout this project, the frogs were extensively observed both for experimental data collection and from general qualitative observations during handling. This large volume of direct handling and observation reveals priming effects and dose-dependent responses to hCG. The priming effects of previous injections are seen in males after up to two weeks when they were retested in the recovery experiment. Females may be affected for longer. Two months after breeding, some frogs laid eggs after a priming injection that would normally not induce a response. This information is important for the design of follow-up studies and for improving general colony maintenance practices, such as timing of breeding.

4.2 Limitations, future directions, and applications

For consistency across experiments, a single commercial extract of NAs was used for all exposures. This specific product was selected as previous work in the Trudeau lab has characterized its chemical composition in detail (Gutierrez-Villagomez et al., 2017, 2020) and toxicity in tadpoles (Gutierrez-Villagomez et al., 2019; Melvin & Trudeau, 2012a). However, NAs are highly complex and heterogeneous in composition depending on the source and extraction method. Despite advances in analytical methods, elucidation of individual components has been largely unsuccessful and hundreds or thousands of chemicals remain uncharacterized

(Clemente & Fedorak, 2005). A large body of evidence shows that NAs differ greatly in composition depending on the source (Barrow et al., 2003; Fan, 1991). This creates variation in structure-dependent uptake, toxicity, and neuroendocrine disrupting modes of action. Furthermore, the term oil sands-process affected water (OSPW) NAs is misleading as it actually refers to acid-extractable organics, which contain non-naphthenic carboxylic acids (Grewer et al., 2010). As such, commercial extracts are typically more toxic than those from OSPW and LC50s can be several times lower (Kinley et al., 2016; Lister et al., 2008). Several other factors contribute to differences among and within tailings ponds, such as water depth, oxygen availability, and activity of NA-degrading microorganisms (Han et al., 2009). Therefore, data from different studies are difficult to compare. However, the methods used to assay the effects of NAs can be used for other chemicals, perhaps along with pharmaceutical EDCs (i.e., known agonists and antagonists) as positive controls to identify modes of action. The 20 mg/L dose used was high enough that around one third of males did not call at all, skewing the data. With a slightly lower dose that reduces but not entirely inhibits calling, the NA data may have more variation to link to parameters such as physiological processes. Preliminary data suggests that vocalization may be affected at around 4 mg/L NA (Figure 16, Appendix).

The exact mechanisms of how NAs disrupt vocalization and amplexus are unknown. In the May 2019 vocalization assay, another set of males were concurrently exposed but injected with saline. Gonad tissue was analyzed for gene expression of key steps in steroid production. The unpublished data on testicular mRNA levels were obtained by Elizabeth Farmer, a Honour's and NSERC USRA student (Table 3, Appendix). Surprisingly, NA-exposed frogs showed higher expression of *lhr*, the LH receptor that hCG binds to trigger steroid production. Injecting hCG upregulated *star*, the first and rate-limiting step of steroidogenesis, and this effect was enhanced by NA exposure, perhaps due to upregulated receptors. Both hCG injection and NA exposure upregulated *cyp17a1*, a major enzyme in androgen production. Since NAs slightly downregulated *srd5a,* which converts testosterone to its more bioactive form DHT, the upregulation of *lhr, star,* and *cyp17a1* may be compensatory. The gene *cyp19* codes for aromatase, an enzyme that converts androgens to estrogens, and was not affected by either NAs or hCG. Calling duration did not correlate with any gene expression and there are many

steps between transcription and translation of the final protein product. The next step is to measure the key steroids T and DHT in the blood plasma. Given the large body of literature demonstrating androgen-dependent calling, I hypothesize that androgens will be reduced by NA exposure. However, genes related to steroid synthesis were upregulated in the testes despite reduced singing, so NAs may be affecting steroid binding to androgen receptors in addition to androgen production.

Estrogen-related disruption is also possible, although the evidence for estrogenic activities in NAs is weak and indirect. Testicular expression of *cyp19*, which codes for the enzyme aromatase that converts androgens to estrogens, was unaffected by NA exposure (

Table 3). One study found that a 30-day exposure to E2 and the xenoestrogen octylphenol increased calling and male courtship behaviours in *S. tropicalis* (Schwendiman & Propper, 2012). This is surprising since these same chemicals inhibited male behaviours in medaka *(Oryzias latipes)* and guppies *(Poecilia reticulata)* (Bayley et al., 1999; Gray et al., 1999). Furthermore, the literature consistently shows that estrogenic disruption inhibits male characteristics in a wide range of species from goldfish (Bjerselius et al., 2001) to quail (Brunström et al., 2009). However, the findings of the *S. tropicalis* study (Schwendiman & Propper, 2012) seem doubtful due to the methods. Underwater vocalizations were conducted by a live observer without using a hydrophone, and any sounds heard were counted as a call. The other behaviours recorded include arm waving, which sounds like the frogs' feeding motion rather than courtship. These issues with data collection highlight the need for clearly defined behaviours and accurate methods of analysis.

Corticosterone (CORT), the amphibian stress hormone, should be measured since the stress response reduces investment into reproduction in favour of immediate survival. CORT can be measured in plasma or sampled non-invasively in water. In *P. pustulosus*, CORT levels in plasma and whole body homogenates were positively correlated with levels of water-borne CORT after 60 min of submersion (Baugh et al., 2018). Frog vocalization is metabolically demanding and glucocorticoids function to mobilize energy reserves, but stress can also reduce androgen levels. Theoretical models suggest that increasing CORT supports the energetic demands of calling up to a point, after which energy depletion leads to CORT reducing calling

and androgen levels (Emerson & Hess, 2001). In the toads *Bufo woodhousii* and *Bufo cognatus*, calling males had higher CORT and better physical condition than non-calling satellite males, but androgen levels did not differ (Leary et al., 2004). Injections of CORT increased the use of satellite strategy independent of androgen production, so CORT may act directly on the brain and on AVT-producing neurons in the vocal pathway (Leary, Garcia, and Knapp 2006). Injecting CORT in the green treefrog *(Hyla cinerea)* also reduced advertisement calling while increasing aggressive calls and use of the satellite tactic, behaviours known to be less attractive to females. These effects were also independent of androgen levels and body condition (Leary & Crocker-Buta, 2018). In *S. tropicalis*, I found that the males with reduced calling did not have different acoustic characteristics or noticeably poorer body condition. It is also uncertain whether they use the calling/satellite strategy. Empirically, the relationship between stress, sex steroids, and courtship in anurans is highly variable, reflecting species differences in physiology, social structure, and mating systems.

Using *S. tropicalis* as a model also presented limitations. In my experience, behaviours never occurred unless hormonally induced. While this allows for controlled timing of experiments, it created challenges as well. Previous hCG injections affect future responses so the same individual cannot be tested too often. The induction of behaviours provides a limited window to complete data collection, especially for females. Females do not display phonotaxis or become receptive to amplexus without eggs to lay, and cannot be used for months as they regenerate gametes. It would also be difficult to take repeated measures with females as they would need to undergo multiple short tests within a few hours. As little is known about the behaviour and ecology of *S. tropicalis*, many of the predictions were based on literature for *X. laevis* as it is a similar African clawed frog with courtship behaviours characterized in detail. However, my findings revealed significant differences. Sexual behaviours also need to be artificially induced in *X. laevis*, most commonly with hCG. They then call continuously every night for up to a month, allowing for long-term, inter-individual data collection. The *S. tropicalis* were sometimes heard vocalizing up to a day after an experiment, but none were still calling by the second day. Vocal competition between males is easily measurable in *X. laevis*; clear dominance hierarchies form between pairs despite no difference in vocal output between

dominant and subordinate males before or after pairing (Tobias et al. 2004; 2010). The lack of evidence for vocal competition in *S. tropicalis* may reflect species differences in mating strategy and social behaviour. In *X. laevis*, playbacks of a receptive female increase the calling of both males such that calling of the subordinate is no longer less than that of the dominant (Xu et al., 2012). Competition in *S. tropicalis* should also be examined in different social contexts, such as with females. After the female was added to the pair of males in the mate competition experiments, they could be heard continuing to vocalize but were not recorded since it was not possible to identify the caller.

Non-acoustic communication, such as chemical signals, may also influence female mate choice. In some species of the dwarf African clawed frog (*Hymenochirus* sp), receptive females were attracted to water containing live males or homogenized male breeding glands (nuptial pads), but not to water containing females or gland-ablated males, showing that breeding glands produce chemosignals to attract females (Pearl et al., 2000). The male *S. tropicalis* used in my studies were selected based on the presence of nuptial pads as an indicator of sexual maturity. However, the nuptial pads were simply noted as present or absent without being quantified or closely examined. As there was qualitatively noticeable, but not recorded, variation in darkness and texture between and within males over time, nuptial pad parameters can be quantified as another endpoint. In *X. laevis*, the antiandrogens flutamide and vinclozolin reduced the size of breeding glands in the nuptial pads (Van Wyk et al., 2003). Castration and T treatments in *L. pipiens* shows that nuptial pads are androgen-dependent, and are also believed to enhance grip for males to stay in amplexus (Thomas and Licht 1993). Since males were observed being physically dislodged by the female or other males, the role of nuptial pads should be examined further in terms of mating success while under the influence of endocrine disruption.

With differences in physiology and mating system across species, other frogs should also be studied. As OSPW is a major source of NA contamination, species native to oil sands areas such as wood frogs *(Lithobates sylvaticus)* would be a relevant study species. They are an explosive breeding species with a very short breeding season, and mating strategy may influence the role of vocalization and other courtship behaviours. In early spring, *L. sylvaticus*

gather in large groups of up to a thousand adults and breed within a few days per year. This protects the offspring by providing thermal insulation and by reducing risk of predation and cannibalism (Petranka & Thomas, 1995; Waldman, 1982). Breeding takes place in ephemeral ponds with unpredictable times and locations, so frogs follow the sound of conspecific choruses (Bee, 2007). Therefore, the ability of males to produce frequent and intense calls is critical for the formation of the explosive breeding assembly and consequently, for spawning success and offspring survival. The Northern leopard frog *(Lithobates pipiens)* is also found in oil sands areas. Some of their western populations are in decline, and are listed as special concern and endangered by COSEWIC (Rogers and Peacock 2012).

Effects of early developmental exposure to NAs on behaviours as adults remains unexamined, as all frogs in the current studies were exposed acutely as sexually mature adults. Fathead minnows *(Pimephales promelas)* exposed as eggs and larvae for 21 days to bitumen-containing sediment from the Athabasca oil sands area exhibited growth delays after moving to clean water. At adulthood, growth recovered but jaw deformities in the minnows persisted (Vignet et al., 2019). As NAs are much more toxic to *S. tropicalis* tadpoles than adults, very low doses representative of low environmental levels of NAs could potentially have effects. Androgens contribute to development of sexual dimorphism, including vocalization. Gonadectomized juvenile *X. laevis* of both sexes implanted with testes of other males produce male advertisement calls as adults but only if the frogs received the implant at <6 months old, suggesting a critical period (Watson & Kelley, 1992). Male *X. laevis* exposed to atrazine from embryo to adult had lower plasma testosterone levels than controls, and were out-competed by controls when attempting to mate with females. They also displayed feminized morphology, including changes in larynx structure (Hayes et al., 2010). Unlike with adult exposures, effects of developmental disruption by NAs may be irreversible through recovery in water if anatomy is altered.

Controlled laboratory experiments do not necessarily extrapolate well to natural conditions, but comprise the majority of the literature on determinants of anuran mating success. A field study showed that male agile frogs *(Rana dalmatina)* with low-pitched calls had higher breeding success, indicated by more and larger clutches in their territory (Lesbarrères et

al., 2008). Literature on behavioural disruption by contaminants is entirely laboratory-based, although toxicity and endocrine disruption has been examined through observational field studies. Juvenile alligators from eggs collected from a pesticide-polluted lake showed disrupted steroidogenesis and lower plasma T compared to alligators from eggs collected from a control lake (Crain et al., 1997). Field sampling is used for surveys, for example, to assess potential endocrine disruption in contaminated agricultural ponds (Mandiki et al., 2014).

Field studies have also been used to evaluate the long-term toxicity of tailings ponds. A proposed method of reclaiming oil sands tailings involves aging for natural degradation and capping with clean water. To comply with environmental regulation, the resulting artificial lakes must develop into ecosystems capable of sustaining aquatic life. Levels of NAs decrease over time via natural degradation, although higher weight compounds may remain longer (Quagraine et al., 2005). The viability of the lakes is evaluated by toxicity assays with several aquatic organisms (Bauer et al., 2019). A bioassay with *L. sylvaticus* tadpoles showed that reclaimed wetlands over seven years old have significantly reduced toxicity and can sustain amphibian life (Hersikorn et al., 2010). Calling behaviour can be another endpoint to consider evaluating. Long-term bioacoustic monitoring and semi-automated analysis has been able to reveal seasonal breeding patterns of the invasive cane toad *(Rhinella marina)* in Australia (Brodie et al., 2020). As it is non-invasive and cost-effective, passive acoustic monitoring has been proposed as a method of assessing biodiversity and ecosystem health (Desjonquères et al., 2020).

I have characterized the courtship behaviours of a popular anuran model species. By developing novel behavioural assays, I showed that behaviours can be reversibly disrupted by NA exposure. With conserved endocrine systems across vertebrates and a need for more environmental research and monitoring, this work may have broader implications for the health of wildlife, ecosystems, and humans in an increasingly contaminated world.

Appendix

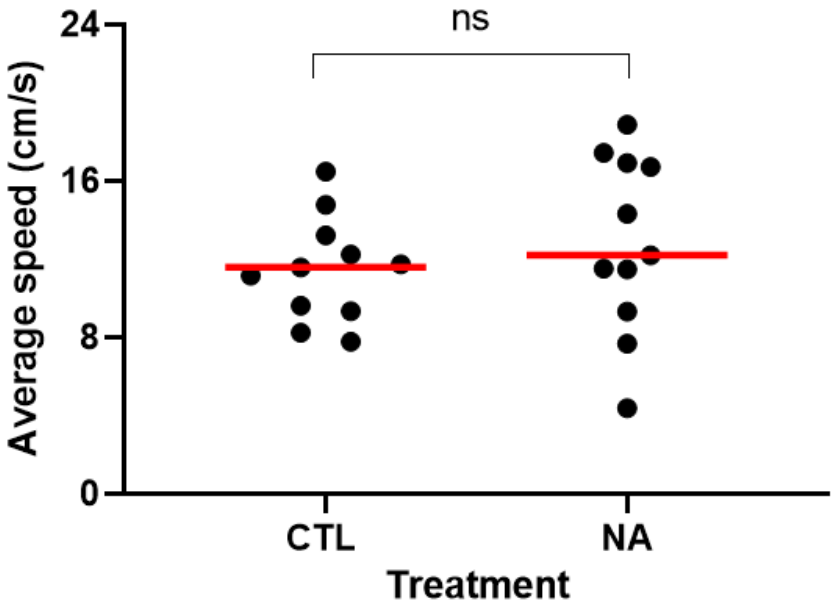

Figure 15. Effect of NA exposure on swimming speed. Individual data presented from pooled average of five swimming bursts per frog. Red lines indicate medians.

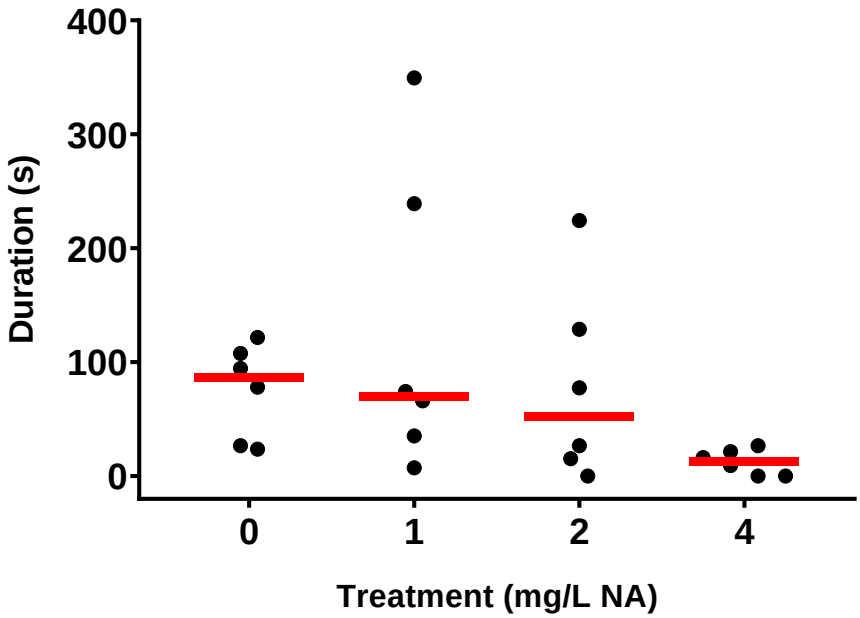

Figure 16. Total calling durations of males exposed to NAs. Individual data presented with red lines at medians. Effects are approaching significance, with p=0.053 for the difference between 0 and 4 mg/L NA (Tukey's post hoc test).

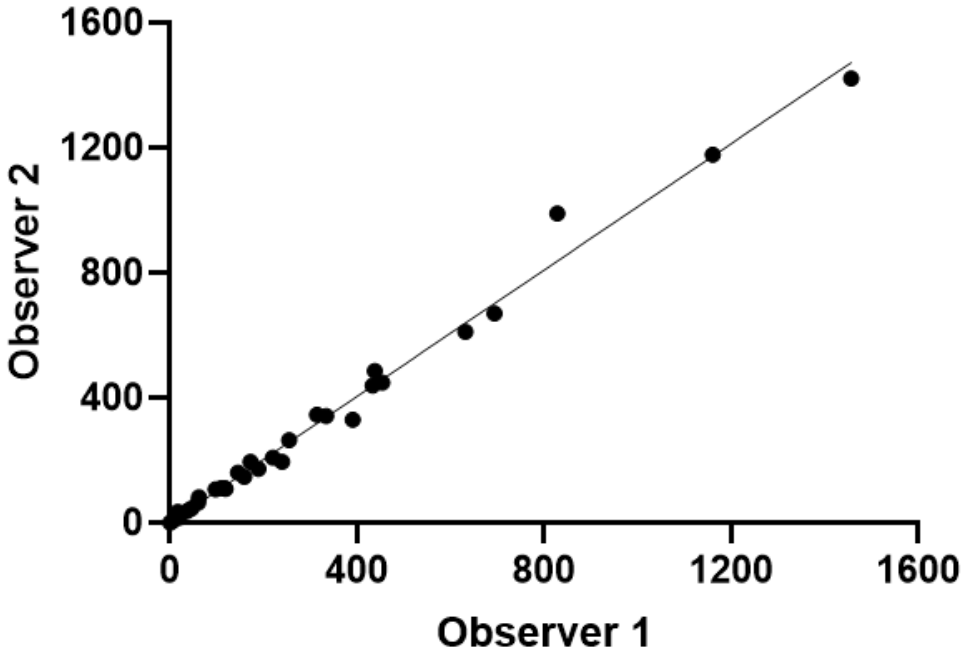

Figure 17. Inter-observer repeatability for vocalization assays. Correlation coefficient >0.99 for data obtained by two observers from the same recording.

Figure 18. Phonotaxis test apparatus, described in Section 3.5, Figure 14. A) Bird's eye view and B) front view of trap with C) infrared sensor.

Table 3. Effect of hCG and NAs on testicular steroidogenic gene expression. Gene expression data obtained by qPCR and analyzed with 2-way ANOVA for effects of hCG, NA, and hCG:NA interaction. Arrows indicate up or downregulation relative to saline injection and control exposure.

Gene	Product	Function	hCG	NA	Interaction
star	Steroidogenic acute regulatory protein	Transports cholesterol into the mitochondria	↑, <0.0001	↑, <0.0001	0.01
srd5α2	5α reductase	Converts testosterone to 5α-DHT	0.2	↓, 0.005	>0.9
srd5β	5β reductase	Converts testosterone to 5β-DHT	>0.9	0.3	0.8
cyp19	Aromatase	Converts androgens to estrogens	0.2	0.9	0.2
cyp17a1	Cytochrome P450 17a1	Involved in testosterone production	↑, 0.001	↑, <0.0001	0.8
rpl8	Ribosomal protein l8	Housekeeping gene	↑, <0.0001	0.2	0.4

R scripts

2 way ANOVA of vocal competition data with Tukey posthoc test

```
full.=lmer(dur ~ treat*phase + (1|frog)+(1|rep),
        data=comp,
        REML=TRUE) #full mixed model
leveneTest(dur~phase*treat,data=comp) #homoscedasticity
shapiro.test(residuals(full.)) #normality of residuals
anova(full.)
rand(full.) #random effects
simple.=lm(dur ~ treat*phase, data=comp) #without random effects
shapiro.test(residuals(simple.))
anova(simple.)
anova(full.,simple.) #compare full and simple models
lsmeans(simple.,pairwise ~ phase:treat,
      adjust="tukey") #posthoc multi comparisons
```

2 way ANOVA of vocalization after exposure and after recovery with Tukey posthoc test

```
mix.model=lmer(dur ~ treat*time + (1|frog),
            data=alone,
            REML=TRUE) #full model
rand(mix.model)
fix.model=aov(dur~treat*time,data=alone)
anova(mix.model,fix.model)
anova(mix.model)
leveneTest(dur~treat*time,data=alone)
shapiro.test(residuals(mix.model))
shapiro.test(residuals(fix.model))
lsmeans(mix.model,pairwise ~ time:treat,
      adjust="tukey
```

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