Time- and dose-related effects of a gonadotropin-releasing hormone agonist and dopamine antagonist on reproduction in the Northern leopard frog (*Lithobates pipiens*) and the Western clawed frog (*Silurana tropicalis*)

by

Maria Vu

Thesis submitted to the Faculty of Graduate and Postdoctoral Studies, University of Ottawa, in partial fulfillment of the requirements for the M.Sc. Degree in the Ottawa-Carleton Institute of Biology

Thèse soumise à la Faculté des études supérieures et postdoctorales, Université d'Ottawa, dans le cadre des exigences du programme de maîtrise en science de l'Institut de biologie d'Ottawa-Carleton

ACKNOWLEDGMENTS

As this adventure comes to an end, I would like to thank everyone who has been a part of it. First and foremost, thank you to my supervisor, Dr. Vance Trudeau, for embracing my interests in herpetology and conservation and for broadening my skills in molecular biology. Thank you for pushing me outside of my comfort zone and for your patience and understanding through the ups and downs of this project. I would like to extend my gratitude to my thesis advisory and evaluation committee: Dr. Gabriel Blouin-Demers, Dr. Steven J. Cooke, Dr. Emily Standen and Dr. Jan Mennigen, for their constructive advice and new perspectives they brought to this project.

To the past and present members of TeamENDO, I will look back on all of our memories, both in and outside of the lab, with a smile and am happy to have made a group of lifelong friends. Thanks for all the laughs! I'm looking forward to that ten year reunion! Dillon, my lab husband, thank you for guiding me through my molecular work and for being my social coach for the past two years (thanks for keeping me sane!). Marilyn, Kim, Lei, Juan and Crystal, thank you for your encouraging words and for always lending a helping hand through this learning curve. A big thank you to Léa for the spirit and energy you brought to the lab during your visit and for taking time to translate my thesis abstract.

I couldn't have asked for a better field team that helped me with frog collections for the past two years: our local herpetologist Fred Schueler and his wife Aleta Karstad, my "field slave" Brad Weiler who endured those long hours with me, Amanda Bennett and Jessica Longhi from Trent University, Javier Hilario Santos-Santos, Léa Fieschi-Méric, Jaime Graham and Clay Shearer. Michal, from day one you were always a great frog hunter and "taxi driver" (I think I

still owe you a Vietnamese meal). Those long days in the field will never be forgotten. Thanks for making them bearable and enjoyable!

My leopard frog spawning experiments at the Canadian National Wildlife Research Centre would not have been possible without the help of Dr. Stacey Robinson and Bruce Pauli who provided me with the space and equipment. Erin, Brad, Juan and Michal, thank you for offering your time and positive attitude on those long injection days. I would like to acknowledge Bill Fletcher and Christine Archer from the Aquatic Care Facility at the University of Ottawa for their ongoing efforts and for their advice on animal husbandry. To my friends who have stuck with me from the beginning to the end. A special thank you to Kim Bui who listened not only to my struggles, but also to my presentations on repeat. To my yoga family at Modern Body, thank you for giving me a space to breathe and flow. Finally, I cannot even begin to describe the never-ending love and support from my family. I am eternally grateful for this opportunity and for everyone who came along with me for the ride. It's been a blast. Namaste and save the frogs!

This project was funded by the University of Ottawa, the Ontario Graduate Scholarship and the Ottawa Field-Naturalists' Club.

ABSTRACT

The recent decline and disappearance of many amphibians around the world is thought to be the sign of an impending sixth mass extinction that is driven by disease, habitat loss and pollution. Reproductive technologies are now required to establish captive colonies followed by reintroduction into suitable habitats. The AMPHIPLEX method is a hormone mixture that has successfully stimulated spawning in several amphibians. However, its extensive application requires further experimentation and knowledge regarding the basic neuroendocrine control of reproduction in amphibians. The role of the catecholamine neurotransmitter dopamine in the regulation of spawning and gonadotropin synthesis was investigated using multiple time- and dose-related approaches in the field and laboratory. These end points were explored in two distantly-related frog species: the Northern leopard frog (*Lithobates pipiens*) and the Western clawed frog (*Silurana tropicalis*). Northern leopard frogs were injected during the natural breeding season with three doses of a gonadotropin-releasing hormone agonist (GnRH-A) (0.1 μg/g , 0.2 μg/g and 0.4 μg/g) alone and in combination with two doses of the selective dopamine receptor D2 antagonist metoclopramide (MET) (5 μg/g and 10 μg/g). Injected animals were allowed to breed in mesocosms in an outdoor field. Time to amplexus and oviposition were assessed, and egg mass release, incidences of amplexus, egg mass weight, total egg numbers and fertilization rates were measured. The results revealed no statistically significant interaction between GnRH-A and MET on amplexus and oviposition. A series of GnRH-A dose-response spawning studies were conducted in the Western clawed frog. The current findings indicate that partial ovulation, male sexual behavior and fertilization can be induced by 4 μg/g of GnRH-A alone and in combination with 10 μg/g of MET. This represents a first step towards understanding basic neuroendocrine reproductive mechanisms in this species. These spawning

results were paired with a second end point which explored the molecular mechanisms of gonadotropin synthesis in response to GnRH-A and MET alone and in combination. Pituitary gene expression results in the Northern leopard frog indicate a potentiating action of MET when combined with GnRH-A on the mRNA levels of gonadotropin subunits 36 hours following injection. The postulated mechanisms of action are through the upregulation of gonadotropin-releasing hormone receptor 1 and the downregulation of dopamine receptor D2. Such gene expression pathways were similarly explored in the Western clawed frog, however no significant changes in pituitary gonadotropin and receptor gene expression were present at 12 hours post-injection. The hypothesized inhibitory action of dopamine was supported by pituitary gene expression analysis, but not by spawning outcome. The results from this study provide a fundamental framework for future time- and dose-response investigations to improve current spawning methods in amphibians.

RÉSUMÉ

Le récent déclin et la disparition mondiale de nombreux amphibiens sont considérés comme les prémices d'une imminente sixième extinction de masse, entraînée par les maladies, la perte d'habitat et la pollution. Il devient nécessaire d'établir des techniques de reproduction afin de fonder des colonies en captivité qui puissent être réintroduites dans des habitats appropriés. La méthode AMPHIPLEX est un cocktail hormonal qui stimule la ponte chez plusieurs amphibiens. Cependant, des expérimentations et des connaissances plus poussées en ce qui concerne les fondements du contrôle neuroendocrinien de la reproduction des amphibiens sont nécessaires à la démocratisation de ce produit. Le rôle de la dopamine, neurotransmetteur de la famille des catécholamines, dans la régulation de la ponte et dans la synthèse de gonadotropines a été étudiée à l'aide de multiples approches, en fonction des doses et du temps sur le terrain et en laboratoire. Ces fonctions ont été investiguées chez deux espèces de grenouilles lointainement apparentées: la grenouille léopard (*Lithobates pipiens*) et le xénope tropical (*Silurana tropicalis*). Pendant leur saison de reproduction naturelle, les grenouilles léopard ont reçu trois doses d'un antagoniste de l'hormone de libération des gonadotrophines hypophysaires (GnRH-A) (0.1 μg/g , 0.2 μg/g et 0.4 μg/g), seul ou combiné avec deux doses de métoclopramide (MET), l'antagoniste sélectif du récepteur de type D2 de la dopamine (5 μg/g et 10 μg/g). Les animaux ayant reçu l'injection ont été placés dans des mésocosmes extérieurs, où ils pouvaient se reproduire. Les temps d'amplexus et d'oviposition ont été estimés, et la ponte, la fréquence des amplexus, le poids des masses d'œufs, le nombre total d'œufs et les taux de fertilisation ont été mesurés. Les résultats révèlent qu'il n'y a pas d'interaction statistiquement significatrice entre le GnRH-A et le MET pour l'amplexus et l'oviposition. Plusieurs études de la ponte en réponse à des doses de GnRH-A en ont été menées chez *Silurana tropicalis.* Les résultats présentés ici indiquent qu'une

ovulation partielle, un comportement sexuel chez le mâle, et une fertilisation des œufs peuvent être obtenus avec 4 µg/g de GnRH-A seul, et en combinaison avec 10 µg/g de MET. Cela constitue un premier pas vers la compréhension des fondements des mécanismes neuroendocriniens de la reproduction chez cette espèce. Ces résultats sur la ponte ont été couplés à un second aspect, qui explorait les mécanismes moléculaires de la synthèse de gonadotropines en réponse au GnRH-A et au MET, seuls ou appariés. L'analyse de l'expression des gènes hypophysaires chez la grenouille léopard indique un effet potentiateur du MET lorsqu'il est combiné avec le GnRH-A sur les taux d'ARNm de sous-unités de gonadotropines 36 heures après injection. Les mécanismes d'action supposés sont une augmentation des récepteurs de type-1 de l'hormone de libération des gonadotrophines hypophysaires et une inhibition des récepteurs de type D2 de la dopamine. Des voies moléculaires similaires ont aussi été étudiées chez *Silurana tropicalis* mais aucun changement dans l'expression génétique des gonadotrophines hypophysaires, ni des récepteurs, n'a été observé 12 heures après injection. L'hypothèse d'une action inhibitrice de la dopamine est soutenue par l'analyse de l'expression génétique hypophysaire mais ébranlée par les résultats de pontes. Les résultats de cette étude fournissent un cadre essentiel pour les futures recherches temps- et dose-dépendantes visant à améliorer les méthodes actuelles d'élevage des amphibiens.

Table of Contents

List of Tables

List of Figures

Abbreviations

17α-OHP_4	17α-hydroxyprogesterone
7α-OH-PREG	7α-hydroxypregnenolone
17β-HSD	17β-hydroxysteroid dehydrogenase
3β-HSD	3β-hydroxysteroid dehydrogenase
5α-DHT	5α-dihydrotestosterone
α-GSU	Glycoprotein alpha subunit
A4	Androstenedione
AArk	Amphibian Ark
ACAP	Amphibian conservation action plan
ANOVA	Analysis of variance
ART	Assisted reproductive technology
AVT	Arginine vasotocin
Bd	*Batrachochytrium dendrobatidis*
BLAST	Basic local alignment search tool
BO	Bidder's organ
Bsal	*Batrachochytrium salamandrivorans*
BWt	Body weight
cGnRH-II	Chicken gonadotropin-releasing hormone II
cDNA	Complementary DNA
CG	Chorionic gonadotropin
DA	Dopamine
DEPC	Diethylpyrocarbonate
DLS	Dorsal lymph sac
DNA	Deoxyribonucleic acid
DOM	Domperidone
DRD2	Dopamine receptor D2
E2	17β-estradiol
ECBs	Endocannabinoids
FSH	Follicle-stimulating hormone
FSHβ	Follicle-stimulating hormone beta subunit
GAA	Global amphibian assessment
GnIH	Gonadotropin-inhibitory hormone
GnIH-ir	GnIH-immunoreactive
GnRH	Gonadotropin-releasing hormone
GnRH-A	Gonadotropin-releasing hormone agonist
GnRHR	Gonadotropin-releasing hormone receptor
GnRHR1	Gonadotropin-releasing hormone receptor 1
GPCR	G protein-coupled receptor
GSI	Gonadosomatic index
hCG	Human chorionic gonadotropin
HPG	Hypothalamo-pituitary-gonadal
IP	Intraperitoneal
IUCN	International union for the conservation of nature
IVF	*In vitro* fertilization

LH	Luteinizing hormone
LHβ	Luteinizing hormone beta subunit
LHRH	Luteinizing hormone-releasing hormone
MEL	Melatonin
MET	Metoclopramide
mGnRH	Mammalian gonadotropin-releasing hormone
mRNA	Messenger ribonucleic acid
MT1	Melatonin type 1
NaCl	Saline
NCBI	National center for biotechnology information
NWRC	National wildlife research centre
P4	Progesterone
PACAP	Pituitary adenylate cyclase-activating polypeptide
PCR	Polymerase chain reaction
PIM	Pimozide
PMSG	Pregnant mare's serum gonadotropin
PRL	Prolactin
qPCR	Quantitative real-time PCR
QUIN	Quinpirole
RNA	Ribonucleic acid
rpl8	Ribosomal protein l8
sGnRH	Salmon gonadotropin-releasing hormone
SVL	Snout-vent length
T	Testosterone
TMS	Tricaine mesylate
VTG	Vitellogenin

CHAPTER 1

Thesis Rationale and Hypotheses

The development of amphibian reproductive technologies is a valuable conservation tool for the gradual recovery of vulnerable populations (Clulow et al., 2014; Kouba et al., 2012). This is especially important in species that may fail to respond to breeding attempts in captivity due to stress or insufficient environmental cues. Hormonal induction of spawning is a technique that involves the administration of exogenous hormones to promote the timed release of gametes for fertilization. One example, termed the AMPHIPLEX method, is a hormone mixture that has successfully stimulated ovulation and sperm release in several anurans and salamanders in the recent years (Trudeau et al., 2013; Trudeau et al., 2010). The extensive and effective application of these methods across amphibians, however, requires a basic understanding of the physiological mechanisms governing reproduction. Despite the immense diversity in reproductive modes that exist, there are nevertheless physiological systems that remain conserved to regulate reproduction across vertebrates. The principal and emerging factors involved in the neuroendocrine control of reproduction in amphibians were reviewed extensively in Chapter 2, followed by their practical applications for conservation.

In the AMPHIPLEX method, a gonadotropin-releasing hormone agonist is combined with a selective dopamine receptor D2 antagonist. Across vertebrates, the peptide gonadotropin-releasing hormone is a principal stimulator of pituitary luteinizing hormone and follicle-stimulating hormone synthesis and secretion that is required for sperm release and ovulation (Daniels and Licht, 1980; Schally, 1978). Extensive evidence in teleost fish demonstrates an inhibitory role of dopamine in the gonadotropin-releasing hormone-regulated release of

luteinizing hormone (Dufour et al., 2005; Popesku et al., 2008; Saligaut et al., 1999; Trudeau, 1997; Yaron et al., 2003), forming the basis to extrapolate these principles to amphibians. Emerging studies are suggesting that this is a conserved action in amphibians, therefore logically applying a dopamine antagonist to the AMPHIPLEX method. Despite this, the current evidence for this presumed inhibitory action of dopamine remains very limited. Specifically, further studies are required in which gonadotropin-releasing hormone agonists and dopamine antagonists are tested separately and in combination in order to confirm that dopamine is in fact interacting with GnRH-A in a potentiating manner. These physiological data will need to be confirmed in order to confidently apply the AMPHIPLEX method to a variety of amphibian species.

This thesis investigated the role of dopamine in the control of reproduction in two frog species: the Northern leopard frog (*Lithobates pipiens*) and the Western clawed frog (*Silurana tropicalis*) using multiple approaches both in the field and laboratory. These model organisms were selected due to their availability in the lab, their previous responsiveness to hormone treatments and they are recognized as least concern on the International Union for the Conservation of Nature (IUCN) Red List of Threatened Species. More importantly, these two species belong to two distantly-related anuran families: the Pipidae and Ranidae. Indeed, their last common ancestor can be traced back approximately 250 mA. This is crucial for if the inhibitory action of dopamine can be successfully confirmed in these two distantly-related frog species, then this would provide a strong foundation to extrapolate this physiological mechanism to other species within this clade.

It was hypothesized that dopamine exerts an inhibitory action on (1) spawning outcome and on (2) the synthesis of pituitary gonadotropins and their regulation through receptors. To test

the first hypothesis, several dose-response experiments were conducted in which a gonadotropin-releasing hormone agonist and the selective dopamine receptor D2 antagonist metoclopramide were injected separately and in combination at various doses in these two species. Following injection, spawning outcome including the incidences of amplexus, number of egg masses, egg mass weight, total egg numbers and fertilization rates were quantified. The relative time to amplexus and oviposition was also determined. The second hypothesis was tested through various time-response analyses of a gonadotropin-releasing hormone agonist and metoclopramide on the expression of pituitary genes including: luteinizing hormone, follicle-stimulating hormone, dopamine receptor D2 and gonadotropin-releasing hormone receptor 1 using quantitative real-time PCR. It was predicted that metoclopramide will exert a potentiating effect on spawning and the expression of gonadotropins when given in combination with the gonadotropin-releasing hormone agonist. These results are presented in Chapter 3 and Chapter 4 for the Northern Leopard Frog (*Lithobates pipiens*) and the Western Clawed Frog (*Silurana tropicalis*), respectively. The general discussion in Chapter 5 compares results in these two species for both hypotheses where spawning outcome was paired with gene expression analyses to unravel the reproductive role of dopamine. Future directions as well as contributions to amphibian conservation were also outlined in this final chapter. With amphibians now declining at such high rates alongside a rapidly changing climate and growing human populations, the implications of this research will contribute to not only expanding the current state of knowledge regarding the neuroendocrine control of amphibian reproduction, but will be important towards designing effective captive breeding strategies for the propagation other vulnerable or endangered species.

CHAPTER 2

Literature Review

This chapter was presented from:

Vu, M.[1] and Trudeau, V.L.[2] (2016). Neuroendocrine control of spawning in amphibians and its practical applications. *General and Comparative Endocrinology*, 234: 28-39.

Main contributions from each author:

[1] Principal author in manuscript preparation.

[2] Collected data for wood frog (*Lithobates sylvaticus*) spawning experiment and contributed to manuscript editing.

ABSTRACT

Across vertebrates, ovulation and sperm release are primarily triggered by the timed surge of luteinizing hormone. These key reproductive events are governed by the action of several brain neuropeptides, pituitary hormones and gonadal steroids which operate to synchronize physiology with behaviour. In amphibians, it has long been recognized that the neuropeptide gonadotropin-releasing hormone has stimulatory effects to induce spawning. Extensive work in teleosts reveals an inhibitory role of dopamine in the gonadotropin-releasing hormone-regulated release of luteinizing hormone. Preliminary evidence suggests that this may be a conserved function in amphibians. Emerging studies are proposing a growing list of modulators beyond gonadotropin-releasing hormone that are involved in the control of spawning including prolactin, kisspeptins, pituitary adenylate cyclase-activating polypeptide, gonadotropin-inhibitory hormone and endocannabinoids. Based on these physiological data, spawning induction methods have been developed to test on selective amphibian species. However, several limitations remain to be investigated to strengthen the evidence for future applications, especially for captive breeding of endangered amphibians.

2.1. INTRODUCTION

With an immense diversity spanning across three phylogenetic orders, amphibians exhibit a wide range of remarkable courtship rituals and reproductive strategies. This vertebrate class includes frogs and toads (Anura) that fertilize externally with a few exceptions, salamanders and newts (Caudata/Urodela) that primarily deposit spermatophores for females, and caecillians (Gymnophiona) where fertilization is strictly internal (Duellman and Trueb, 1986). Although the majority of amphibians lay and fertilize their eggs in moist environments where larval tadpoles progress through metamorphosis, there exists newly discovered cases of adaptive radiation (Duellman and Trueb, 1986; Haddad and Prado, 2005; Hödl, 1990). For instance, frogs in the family *Eleutherodactylidae* reproduce through direct development where eggs hatch as miniature adults, completely bypassing the tadpole stage (Hödl, 1990). In other species, egg incubation and development may take place in the male vocal sacs (*Rhinoderma darwinii*), female stomachs (*Rheobatrachus silus*) or be implanted in dorsal brood pouches (*Gastrotheca*). These reproductive events are driven by unique seasonal cycles and social stimuli that cue brain activity and courtship behaviours. Timing of successful reproduction is therefore complex, requiring a delicate coordination between several physiological, behavioural as well as environmental signals that are dependent on underlying interactions between the endocrine and nervous systems. This is especially relevant for spawning i.e., optimally timed release of gametes for fertilization.

The neuroendocrine control of ovulation and sperm release in amphibians is poorly understood and is largely limited to anurans (Figure 2.1). There is a notable lack in progress in this field compared to other vertebrates. Indeed, this subject has not been reviewed in any detail since the late 1980s (Moore, 1987). At that time, scientists were only beginning to explore the

roles of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Major breakthroughs have confirmed that amphibians share basic aspects of the hypothalamo-pituitary-gonadal (HPG) axis with other vertebrates such as neuropeptides, pituitary hormones and gonadal steroids. While it has long been assumed that the hypothalamic decapeptide gonadotropin-releasing hormone (GnRH) is the principal stimulatory system driving the LH surge, and subsequently ovulation and sperm release in amphibians (Daniels and Licht, 1980; McCreery and Licht, 1983a), that is far from being proven. There are new players emerging from research on mammals and teleosts that implicate numerous other systems that have not yet been examined in amphibians.

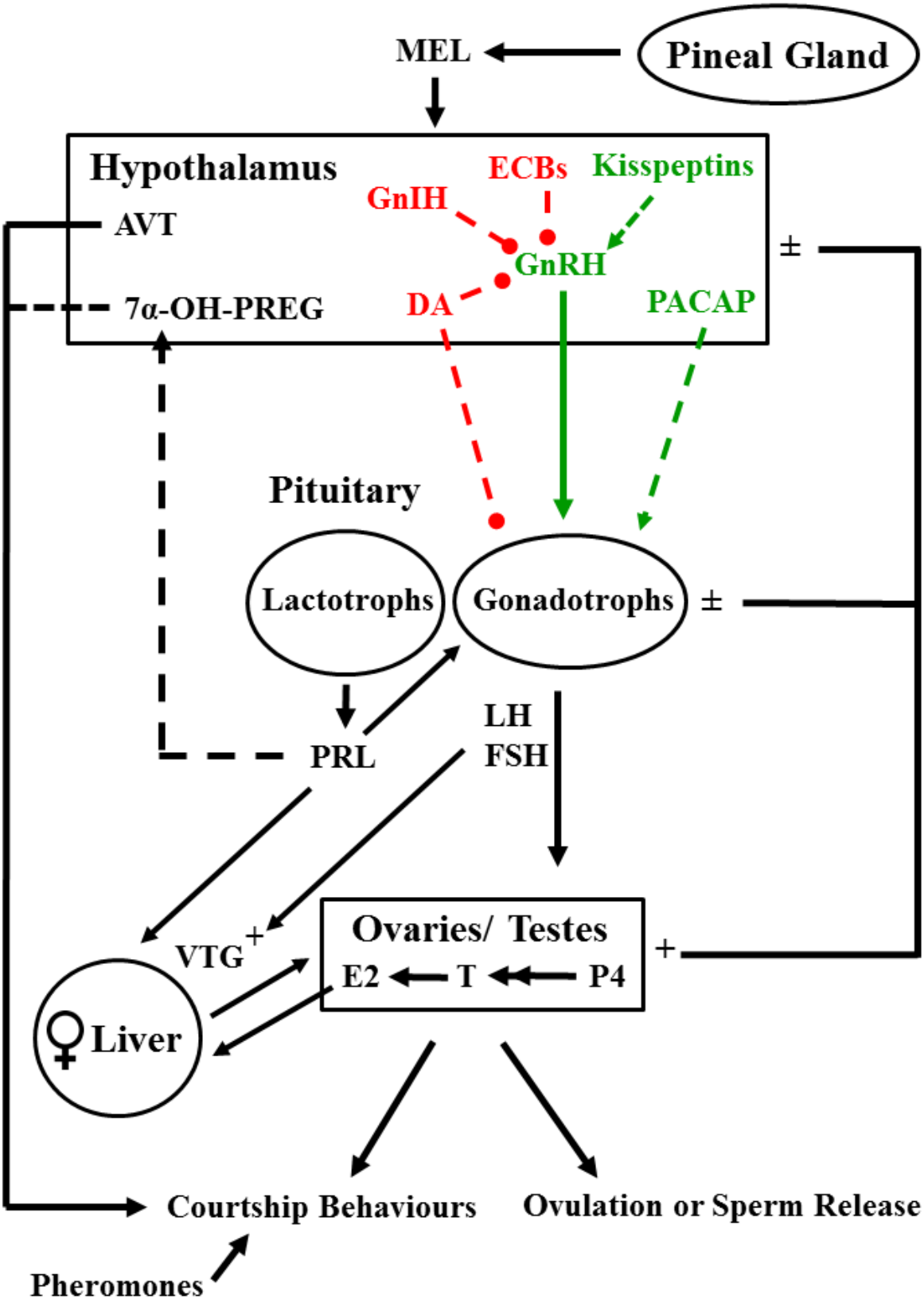

MEL
Pineal Gland
Hypothalamus
ECBs
Kisspeptins
GnIH
AVT
GnRH
±
7α-OH-PREG
DA
PACAP
Pituitary
Lactotrophs
Gonadotrophs
±
LH
FSH
PRL
VTG
+
Ovaries/ Testes
E2
T
P4
+
Liver
Courtship Behaviours
Ovulation or Sperm Release
Pheromones

Figure 2.1 Proposed model for the neuroendocrine control of spawning in amphibians. The principal stimulatory neuropeptide gonadotropin-releasing hormone (GnRH) is released from hypothalamic nerve terminals in the median eminence and transported to the anterior pituitary, where it acts on G-protein coupled GnRH receptors on gonadotrophs to synthesize the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH). The effects of GnRH are modulated by neurohormones with emerging functions including dopamine (DA), endocannabinoids (ECBs), GnRH-inhibitory hormone (GnIH), kisspeptins and pituitary adenylate cyclase-activating polypeptide (PACAP). It is possible that some of these factors have additional direct effects on the pituitary, but this has not been tested experimentally. The release of gonadotropins is enhanced by prolactin (PRL) secreted from pituitary lactotrophs. The gonadotropins act on their respective G protein-coupled receptors in the ovaries and testes to drive steroidogenesis and gamete release. Here, progesterone (P4) is converted through multiple enzymatic steps to testosterone (T) which is aromatized to estradiol (E2). Estradiol plays an additional role in stimulating the hepatic synthesis of the egg yolk vitellogenin (VTG) in females. The synthesis and release of VTG is additionally regulated by LH, FSH and PRL. Steroids are involved in gonadal development and reproductive behaviours that are mediated by arginine vasotocin (AVT), the neurosteroid 7α-hydroxypregnenolone (7α-OH-PREG) and pheromones. Melatonin (MEL) synthesis in the pineal gland is inhibited by light, is secreted diurnally during the dark period, and thus varies seasonally. Melatonin tends to negatively regulate reproductive processes in amphibians. Successful spawning is the result of these timed physiological mechanisms. Bold lines indicate functions supported by strong evidence and dashed lines indicate limited evidence in some species that will need further exploration. See text for additional details.

The global decline and disappearance of many amphibians around the world has persisted to become an imminent crisis with now an estimated 32.4 % of species that are categorized as threatened (IUCN). This is suspected to be driven primarily by disease, habitat loss and pollution (Bishop et al., 2012; Houlahan et al., 2000; Wake and Vredenburg, 2008). Consequently, there is now an urgent need to develop new amphibian reproductive technologies to establish captive colonies followed by reintroduction into suitable habitats. Hormone induction represents a powerful tool to circumvent the challenges often faced with spawning amphibians in captivity, with variable success cases that will be discussed. In order to successfully apply these methods, a fundamental understanding of how the HPG axis is regulated is required, providing a basis to design effective hormone treatments and other assisted reproductive technologies.

This review will focus on the principal neuroendocrine mechanisms that are known to govern the control of spawning in amphibians. Major breakthroughs throughout the past years will be outlined as will the major gaps in our knowledge of the endocrine control of amphibian reproduction. It is hoped that this review will provide a background for future research on hormone manipulations and captive breeding techniques for amphibian conservation in the face of rapid population declines.

2.2. THE HYPOTHALAMO-PITUITARY COMPLEX

The hypothalamo-pituitary complex in amphibians is composed of several identifiable regions that share structural similarities with fish, reptiles and mammals (Ball, 1981). Mapping out this neuroanatomical organization is key to understanding how the brain exerts control over the pituitary to translate neuronal inputs into hormone signals. The hypothalamus, chiefly the preoptic area, located in the ventral diencephalon is the central regulator of reproduction. Attached to the hypothalamus through an infundibular stalk is the pituitary gland that consists of

the neurohypophysis (posterior) and adenohypophysis (anterior). The amphibian neurohypophyseal peptides arginine vasotocin (AVT) and mesotocin are respectively the homologs of mammalian vasopressin and oxytocin that are released into systemic circulation. In amphibians, the adenohypophysis is controlled by hypothalamic neuropeptides and catecholamines released into and transported by the median eminence. It is specifically the cells of the *pars distalis* of the pituitary that biosynthesize and secrete hormones that are involved in growth, reproduction and stress response among many other functions (Ball, 1981; Moore, 1987). The particular group of pituitary hormones that are directly involved in gametogenesis, steroidogenesis and spawning are termed the gonadotropins.

2.3. GONADOTROPINS IN AMPHIBIANS

Two distinct forms of gonadotropins were initially isolated from the pituitaries of bullfrogs (*Lithobates catesbeianus*) and Northern leopard frogs (*Lithobates pipiens*) that closely resembled mammalian LH and FSH (Farmer et al., 1977; Licht and Papkoff, 1974; Papkoff et al., 1976). The basic structure of these glycoproteins is a heterodimer consisting of a common alpha subunit and a unique beta subunit that confers specificity of action as in other tetrapods. These gonadotropins are biosynthesized by gonadotrophs and are stored in secretory vesicles either separately or together in the *pars distalis* (Gracia-Navarro and Licht, 1987). Both LH and FSH receptors have been partially characterized in bullfrog testes (Yamanouchi and Ishii, 1990) and liver (Kubokawa and Ishii, 1987), in addition to newt *(Cynops pyrrhogaster)* testes (Kubokawa and Ishii, 1980). Early data on the biological actions of amphibian LH and FSH are somewhat confusing, likely related to the lack of specificity of bioassays. Consequently, the development of radioimmunoassays with specific antisera against LH and FSH was a valuable tool towards assessing these separate gonadotropic functions (Daniels et al., 1977). Critically, progress on the

roles of LH and FSH has been severely hampered by a limited availability of purified amphibian LH and FSH. Nevertheless, the gonadal steroidogenic functions of LH and FSH are conserved across the vertebrate classes, with notable differences in teleosts (Levavi-Sivan et al., 2010; Polzonetti-Magni et al., 1998). Final oocyte maturation and testicular androgen production in frogs is regulated primarily by LH, whereas FSH is classically associated with promoting early follicular development. Importantly, both LH and FSH have an additional role in female frogs to stimulate the hepatic synthesis and uptake of the egg yolk protein vitellogenin directly, and indirectly through increased 17β-estradiol (E2) (Licht, 1979; Polzonetti-Magni et al., 1998). Across vertebrates, it has been established the timed surge of LH is a critical mechanism to induce ovulation and sperm release (Herbison et al., 2008; Tischkau et al., 2011). The multifactorial control of LH and FSH will be reviewed.

2.4. CONTROL OF GONADOTROPIN SYNTHESIS AND RELEASE

2.4.1 Gonadotropin-releasing hormone

A series of extensive *in vivo* and *in vitro* pioneering studies conducted by Licht and his colleagues have established the stimulatory effects of gonadotropin-releasing hormone (GnRH) on the secretion of LH and FSH from the anterior pituitary of anurans (Daniels and Licht, 1980; McCreery and Licht, 1983a, b; McCreery et al., 1982; Stamper and Licht, 1990). Since its initial isolation from porcine (Matsuo et al., 1971) and ovine (Amoss et al., 1971) hypothalami as LHRH (luteinizing hormone-releasing hormone) before its actions on FSH were discovered, more than 20 GnRH subforms have been identified across vertebrates with diverse functions ranging beyond its primary role in gonadotropin release. The general structure of this decapeptide is characterized as pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, where variants are distinguished by amino acid substitutions (Millar et al., 2004). Despite sharing this

structural similarity, these variants are derived from unique genes that contain highly conserved coding regions (Chow et al., 1998).

Phylogenetic analyses have classified this suite of GnRH subforms into three main branches: GnRH-I, GnRH-II and GnRH-III (Millar et al., 2004). Two or three forms are typically present in a single species whose names are derived from the species in which the subform was first discovered. Early studies reported a GnRH-like substance in the amphibian brain that was structurally identical to that in mammals (Rivier et al., 1981). Since then, HPLC and immunological analysis have identified several GnRH variants in anurans (*Lithobates pipiens, Hyla regilla, Xenopus laevis*) and urodeles (*Taricha granulosa, Ambystoma gracile*) including mammalian GnRH (mGnRH/ GnRH-I), chicken GnRH-II (cGnRH-II/ GnRH-II) and salmon GnRH (sGnRH/ GnRH-III) (King and Millar, 1986; Sherwood et al., 1986). The two most common forms that occur in amphibian brains are mGnRH and cGnRH-II (Licht et al., 1994).

The differential distribution of GnRH subforms has been characterized in the central nervous system of a few anurans. Studies in *Pelophylax ridibundus, Lithobates pipiens* and *Pelophylax esculentus* have revealed that cGnRH-II is widely distributed in the brain and spinal cord and is therefore suspected to be involved in neuromodulatory control and sexual behaviours. The expression of mGnRH, however, is restricted to the anterior pre-optic area of the hypothalamus and the median eminence, suggesting that mGnRH is the primary hypophysotropic form stimulating the synthesis and release of gonadotropins (Collin et al., 1995; Licht et al., 1994).

This neuropeptide is synthesized from prepro-GnRH that is subsequently cleaved by prohormone convertases and exopeptidases. Active peptides are stored and transported in secretory granules in hypothalamic neurons and are released through axon terminals in the

median eminence where they exert their actions through membrane bound G protein-coupled GnRH receptors (GnRHRs) found on gonadotrophs. Three distinct GnRHRs have been identified in the bullfrog that exhibit differential ligand selectivity and intracellular signalling (Acharjee et al., 2002; Wang et al., 2003; Wang et al., 2001). Important here is that GnRHR-1 is the prominent subtype in the amphibian pituitary, whereas GnRHR-2 and -3 mRNAs were expressed in the brain, suggesting additional roles to control reproductive behaviour (Wang et al., 2001). Rank potencies for natural GnRHs based in a heterologous transfection system on COS-7 cells indicate that GnRHR-1 has the highest affinity for cGnRH-II, followed by sGnRH and mGnRH (Wang et al., 2001). The significance of this *in vivo* regulation has yet to be explored. The stimulating actions of GnRH on reproduction have made this neuropeptide a suitable target to treat hormone-dependent diseases (Millar et al., 2004). Specific analogs used for amphibian spawning induction will be discussed in the application section of this review. These key physiological aspects of GnRH will be essential to improve current reproductive technologies.

2.4.2 Dopamine

Emerging evidence suggests an inhibitory role of the catecholamine dopamine (DA) in the control of spawning in amphibians. In teleost fish, DA acts to inhibit the release of gonadotropins from the pituitary and to attenuate the actions of GnRH (Dufour et al., 2005). This mechanism is suspected to be evolutionarily conserved in amphibians. A series of studies conducted in hibernating grass frogs (*Rana temporaria*) have confirmed the existence of inhibitory hypothalamic control where LH release and ovulation were induced following electrolytic nucleus infundibularis ventralis lesions (Sotowska-Brochocka, 1988; Sotowska-Brochocka and Licht, 1992). The administration of the DA antagonist metoclopramide (MET) in hibernating grass frogs triggered ovulation, whereas the DA agonist bromocriptine reduced the

release of LH (Sotowska-Brochocka et al., 1994). Recent studies by Creighton and colleagues have suggested that DA is involved in social performances such as male motor behaviours and vocalization. It was reported that male green tree frogs (*Hyla cinerea*) displayed decreased calling rates and were less likely to engage in activities such as climbing following injections of the specific dopamine receptor D2 agonist quinpirole (Creighton et al., 2013; Creighton and Wilczynski, 2014). These data strongly suggest that DA is involved in inhibiting spawning and mate advertisement in amphibians.

To further test the hypothesis that DA may be regulating reproduction in frogs, female and male wood frogs (*Lithobates sylvaticus*) were treated with quinpirole (Table 2.1). Behavioural observations revealed no differences in the number of pairs engaging in amplexus. The time to first amplexus was highly variable and was not affected by treatments. However, the time between amplexus and oviposition, and the number of egg masses produced was affected by quinpirole injections. When females alone or both females and males were treated, the same results were obtained; injection of the D2 agonist delayed or inhibited oviposition. Consequently, the number of egg masses obtained was lower in these groups. In marked contrast, when males alone were injected, no significant effects on any of the parameters measured were observed. These data indicate that quinpirole is inhibiting ovulation in wood frogs in a sex-dependent manner during the breeding season.

Treatment	# Pairs in amplexus (%)	Median time (min.) to amplexus (Range)	Median time (min.) to ovulation (Range)[a,b]	# Egg masses* (%)
Control	8/8 (100 %)	10 (10-780)	690[a] (160-960)	8/8 (100 %)
Quin-F	7/8 (87.5 %)	45 (10-2880)	2880[b] (1270-2880)	2/8 (p=0.004) (25 %)
Quin-M	8/9 (88.8 %)	70 (10-2880)	340[a] (90-1270)	8/9 (p=0.265) (88.8 %)
Quin-F+Quin-M	7/8 (87.5 %)	30 (10-2880)	2880[b] (360-2880)	2/8 (p=0.019) (25 %)

Table 2.1 Dopaminergic inhibition of spawning activity in wood frogs (*Lithobates sylvaticus*). Animals were collected at breeding ponds in Bishop's Mills, Ontario at the time of spring spawning migration in early April, and then housed in the laboratory. Females and males were kept separately at 4 °C for approximately 2 weeks prior to experimentation. The experimental groups were as follows: 1) Control (8 females and 8 males injected with saline); 2) QUIN-F (8 females injected with quinpirole and 8 males injected with saline); 3) QUIN-M (9 males injected with quinpirole and 9 females injected with saline); 4) QUIN-F+M (8 females injected with quinpirole and 8 males injected with quinpirole). On the day of experimentation, animals were warmed slowly from 4 °C to 12 °C in preparation for injections. At 18:00-20:00 h, both females and males were injected with saline (0.7 % NaCl; 10 µl/g body weight) and kept in water (12 °C) for 12 hr until they received the second injection. Similarly both females and males were injected with the specific dopamine receptor D2 agonist quinpirole-HCL (Tocris) (15 µg/g in saline vehicle; 10 µl/g body weight). At the time of the second injection, 3 females and 3 males were placed in plastic spawning tanks (121 L TuffStore Storage Box). The water (~50 L) was 20 cm deep and temperature ranged from 12-14 °C over the course of the test period. Each tank contained 1 m of yarn and 2 pieces of 18 X 18 cm green fence mesh to serve as spawning substrate. Animals in each tank were checked at 10 minute intervals for the first 9 hrs, then hourly thereafter for a total of 48 hrs. The time to amplexus (min.), time of oviposition from amplexus (min.) and number of egg masses were recorded. The behavioural data were not normally distributed (Shapiro-Wilk test; $p < 0.05$), therefore Kruskal-Wallis one-way analysis of variance on ranks followed by Dunn's test (a,b; median values with different superscripts are significantly different, $p < 0.05$) were performed. When no eggs were obtained, the maximum duration of the experiment (48 h; 2880 min.) was used for data analysis. The number of egg masses obtained was analysed by Fisher's 1-sided Exact Test (* p values compared to control are indicated).

Anatomically, dopaminergic pathways have been mapped in the amphibian brain to support these findings. Fluorescent monoaminergic neuronal tracts in *Rana temporaria* indicate the possibility for delivery of DA from the pre-optic area to the median eminence (Rao and Hartwig, 1974). Further characterization of this system through immunohistochemistry has illustrated the distribution of the enzyme tyrosine hydroxylase along with the protein DARPP-32 in the diencephalon, demonstrating the presence of both DA producing and responsive cells in the amphibian brain (Chu and Wilczynski, 2002; Gonzalez and Smeets, 1991; Lopez et al., 2010; O'Connell et al., 2010). It has been speculated that this inhibitory action is modulated through DA receptor D2 which are known to be expressed in the bullfrog brain and pituitary (Nakano et al., 2010a; Nakano et al., 2010b).

Phylogenetically, the inhibitory role of DA is evolutionarily and differentially conserved in invertebrates (Chen et al., 2003) and numerous vertebrates including teleosts (Dufour et al., 2005), birds (Sharp et al., 1989), sheep (Bertrand et al., 1999), rabbits (Dailey et al., 1978) and humans (Huseman et al., 1980). Therefore, there is a significant possibility that similar mechanisms exist in at least some amphibians, although current evidence is very limited. Consequently, this provides a framework for future research on DA in amphibians, which lags significantly in comparison with what is known for teleosts (Dufour et al., 2005; Popesku et al., 2008; Trudeau, 1997; Yaron et al., 2003). In particular, it will be important to determine the role of DA and DA receptors in the control of GnRH, LH and FSH synthesis and secretion in a range of amphibian species, since it is likely to vary across different families. The role of DA in amphibian reproduction has not been functionally established.

2.4.3 New players: gonadotropin-inhibitory hormone, kisspeptin, pituitary adenylate cyclase-activating polypeptide and endocannabinoids

Over the last decade, several new neuropeptides and other factors have emerged with regulatory actions on gonadotropin release. Gonadotropin-inhibitory hormone (GnIH) is one example of such modulators that was initially discovered in the quail hypothalamus in 2000. Since then, extensive studies in birds have been devoted to deciphering the localization and function of GnIH. Several lines of evidence in quail and sparrows both *in vivo* (Osugi et al., 2004) and *in vitro* (Tsutsui et al., 2000) have demonstrated that GnIH suppresses the release of pituitary LH as well as reduces the mRNA levels of its common α and unique β subunits (Tsutsui et al., 2010a; Ubuka et al., 2006). This inhibitory effect is suspected to be mediated through GnRH neurons. Visualization of GnIH-immunoreactive (GnIH-ir) neurons in the avian brain revealed high co-localization with GnRH fibers in the paraventricular nucleus and median eminence (Bentley et al., 2003). These neuropeptides are part of the RF-amide peptide family where multiple orthologues have been documented in other vertebrates, including amphibians (Tsutsui et al., 2010a). Double-label immunohistochemistry revealed that the GnIH and GnRH systems overlap in the anterior preoptic area in the frog *Pelophylax esculentus*, such that, some GnIH-ir fibers were in close proximity to mGnRH-ir cell bodies (Pinelli et al., 2015). However, the limited evidence available suggests that this does not affect LH release, but rather is a growth hormone-releasing peptide in frogs (Koda et al., 2002).

Shortly after the discovery of GnIH, structurally similar kisspeptins were recognized for their action on suppressing tumor metastasis in mammals. Current data point toward their key stimulatory role on LH secretion in mammals through the activation of GnRH neurons, which may be a likely explanation for the hypogonadotropic hypogonadism condition that is observed

in mice and humans with mutations and impairments in the kisspeptin system (Roa et al., 2008). Kisspeptins are encoded by the *KiSS-1* gene where its 145 amino acid product (K-145) is cleaved into shorter peptides (K-54, -10, -13, -14) that exert their actions through GPR54 receptors (Kotani et al., 2001). Variants of the *KiSS-1* gene and its receptors have been cloned in several mammals and fish. Recent molecular investigations have identified three isoforms of kisspeptin ligands and GPR54 receptors (*KiSS-1a*, *-1b*, *-2*; *GPR54-1a*, *-1b*, *-2*) in the brain of *Silurana tropicalis.* The *KiSS-1* and *KiSS-2* genes are products of a genome duplication event early in vertebrate evolution. It is suspected that an additional round of duplication may have led to the *KiSS-1a* and *KiSS-1b* genes found in amphibians. (Lee et al., 2009; Meccariello et al., 2013; Tena-Sempere et al., 2012). High levels of GPR54 mRNA have been detected in the bullfrog hypothalamus and pituitary (Moon et al., 2009), suggesting a critical role for the kisspeptin/ GPR54 system in the neuroendocrine regulation of reproduction, although this has yet to be tested experimentally. Moreover, GPR54 has been cloned in the testes of *Pelophylax esculentus*, indicating a new site of action for kisspeptins (Chianese et al., 2013). While it is tempting to speculate that the kisspeptin system will play a stimulatory role in amphibian reproduction as it does in mammals, data in teleosts suggest extreme phylogenetic variation (Trudeau, 2015). For example, in the bass *Morone saxilis*, kisspeptin antagonists evoked LH and FSH secretion (Zmora et al., 2015), while complete knockout of kisspeptins and receptors in the zebrafish *Danio rerio* did not affect reproduction (Tang et al., 2015). Additional studies will be required to establish the functional and evolutionary role of kisspeptins in the reproductive axis of amphibians.

An early study suggested that pituitary adenylate cyclase-activating polypeptide (PACAP) may stimulate gonadotrophs by enhancing calcium mobilization in dispersed cells

from *Pelophylax ridibundus* (Gracia-Navarro et al., 1992). Although evidence is limited in frogs, it appears that PACAP (Grey and Chang, 2013; Halvorson, 2014) and PACAP-related peptide (Tam et al., 2011) stimulate LH in other vertebrates. The role of PACAP in amphibian reproduction should be further investigated. Presently, most studies have merely focused on the stimulatory effects of GnRH on LH synthesis and release given the importance of this gonadotropin surge. Future studies will need to further explore the roles of these emerging neuropeptides in the control of spawning.

Recent evidence suggests a critical role for endocannabinoids (ECBs) in the regulation of reproduction in non-mammalian vertebrates including teleosts and amphibians. Endocannabinoids are amides, esters and ethers of long-chain polyunsaturated fatty acids that act on cannabinoid receptors distributed in the brain, peripheral tissues and gonads (Meccariello et al., 2013). The negative effects of ECBs on reproductive function in humans and other mammals including the suppression of LH release and the stimulation of DA, have been widely documented (Pagotto et al., 2006). However, their involvement can only be postulated for amphibians based on the neuroanatomical distribution of the endocannabinoid receptors CB1 and CB2. In amphibians, CB1 has been cloned in the central nervous system of *Taricha granulosa* (Soderstrom et al., 2000), *Xenopus laevis* (Cottone et al., 2003) and *Pelophylax esculentus* (Meccariello et al., 2007), while the CB2 gene has been identified in *Silurana tropicalis* (Cottone et al., 2013). Immunoreactivity of CB1-like cells have also been observed in the gonadotrophs, lacototrophs and thyrotrophs of *Xenopus laevis* (Cesa et al., 2002). The presence of CB1 receptors was detected in the gonads of *Xenopus laevis* and *Pelophylax esculentus* (Cottone et al., 2008). In male *Taricha granulosa*, injections with the cannabinoid receptor agonist levonantradol significantly decreased clasping behaviour (Soderstrom et al., 2000). Given the

evolutionarily conserved function of GnRH, it is assumed that ECBs may be interfering with GnRH secretion, as in other vertebrates. Indeed, mGnRH immunoreactive neurons have been co-localized with CB1 expressing neurons in the telencephalic septum and pre-optic areas of *Pelophylax esculentus* and *Xenopus laevis* (Cottone et al., 2008; Meccariello et al., 2008). Furthermore, levels of mGnRH and CB1 mRNA during the annual reproductive cycle in the diencephalon and telencephalon of male *Pelophylax esculentus* follow opposite patterns. In the same study, incubation of diencephali with the mGnRH agonist buserelin induced the transcription of CB1 and decreased mRNA levels of mGnRH. Treatments with the ECB anandamide downregulated the mRNA levels of mGnRH (Cottone et al., 2013; Meccariello et al., 2013; Meccariello et al., 2008). These lines of evidence suggest that ECBs may be acting primarily through the CB1-modulated inhibition of mGnRH to suppress reproductive functions, however this remains to be fully investigated. There are opportunities to examine the *in vivo* and *in vitro* effects of the endocannabinoid system on amphibian reproduction.

2.4.4 Prolactin

Prolactin (PRL) is synthesized and released from pituitary lacotrophs and is best known for its role in stimulating milk production in mammals. Limited evidence in amphibians indicates a paracrine role of PRL in enhancing LH release from gonadotrophs. In 1997, Oguchi et al. reported a potentiating effect of PRL on the responsiveness of LH cells to GnRH from bullfrog pituitaries *in vitro* (Oguchi et al., 1997). Furthermore, PRL has been shown to induce hepatic VTG release *in vitro* in *Pelophylax esculentus* (Carnevali et al., 1993). The diverse reproductive roles of PRL have been elucidated predominantly in urodeles given the unique expression of courtship behaviours in this order. In male Japanese red-bellied newts (*Cynops pyrrhogaster*), PRL has been shown to induce the migration to water for courtship and oviposition, including its

corresponding structural changes from terrestrial to aquatic skin types (Iwata et al., 2000; Kikuyama et al., 2009; Toyoda et al., 1996). Moreover, males that were administered PRL displayed a greater frequency of tail vibrations – a distinct mating cue seen in this species (Toyoda et al., 1993; Toyoda et al., 1996). It has been established that the synthesis and release of the female-attracting pheromone, sodefrin, from the abdominal glands of male *Cynops pyrrhogaster* is enhanced by PRL, in addition to its detection in the female olfactory organ (Iwata et al., 2000; Kikuyama et al., 2009). These effects are suspected to be governed through the synthesis of 7-alpha-hydroxypregnenolone (7α-OH-PREG) in the amphibian brain (Haraguchi et al., 2010). The function of this neurosteroid in seasonal courtship behaviours will be discussed in the following section on sex steroids. Current data suggest that PRL plays a critical role in facilitating these key courtship events in urodeles, however the complete mechanisms governing these behaviours remain to be elucidated. Further studies will be required to examine the importance of paracrine signalling by PRL and other anterior pituitary factors on gonadotropin synthesis and release in amphibians.

2.5. GONADAL STEROIDOGENESIS AND ROLE OF STEROID HORMONES

Sex steroids are essential for sexual behaviours, gonadal maturation and the development of secondary sexual characteristics in adults. Steroidogenesis is controlled largely by the actions of LH and FSH on the testes or ovaries. In female amphibians, three classes of sex steroids, the estrogens, progesterone and androgens, are primarily synthesized by ovarian follicles that are composed of thecal and granulosa cell layers. The two-cell model proposes differential steroidogenic capacities for these two regions that have been well-established in the anurans *Rana nigromaculata* (Kwon and Ahn, 1994)*, Rana dybowskii, Lithobates catesbeianus* and *Bombina orientalis* (Ahn et al., 1999). The high activity of the steroid-metabolizing enzymes

17α-hydroxylase and $C_{17, 20}$ lyase in the granulosa cells promotes the synthesis of progesterone to 17α-hydroxyprogesterone (17α-OHP_4) and androstenedione (A4) that are subsequently converted to testosterone (T) in the thecal layer by 3β-hydroxysteroid dehydrogenase (3β-HSD). These granulosa cells have an additional role in converting T to E2 through the actions of aromatase. Consequently, the effective production of sex steroids in females is driven by the bi-directional communication between these follicular layers (Ahn et al., 1999; Kwon and Ahn, 1994; Ogielska, 2009). The main steroidogenic tissues found in male amphibians are the interstitial Leydig cells and Sertoli cells, in addition to lobule boundary cells that occur exclusively in urodeles. Predominantly, it is the androgens 5α-dihydrotestosterone (5α-DHT) and T that are synthesized and secreted by the Leydig cells, and are aromatized to E2 in the Sertoli cells (Ogielska, 2009).

This multicellular model is a classical view of gonadal steroidogenesis that is similar across the main vertebrate taxa. However, recent data indicate an additional source of sex steroids with newly-discovered steroidogenic capacities called Bidder's organ (BO). Of amphibians belonging to the family *Bufonidae*, Bidder's organ is a unique structure that differentiates as a rudimentary ovary at the anterior end of both ovaries and testes and interestingly, possesses cortical germ cells resembling diplotene oocytes that proliferate during metamorphosis (Ogielska, 2009; Sassone et al., 2015). Previous studies in *Bufo melanostictus* and *Bufo woodhousii* have confirmed the presence of the steroidogenic enzymes 3β-HSD and 17β-hydroxysteroid dehydrogenase (17β-HSD) in the follicles of BO (Ghosh et al., 1984; Pancak-Roessler and Norris, 1991). Recent investigations in the toad *Rhinella arenarum* provide additional evidence for similar enzymatic activities in both adult males and larval tadpoles. Enzymatic activity in the follicles of BO was detected through immunohistochemical studies

which revealed the expression of CYP17, 3β-HSD/I, aromatase and 5α-reductase (Sassone et al., 2015; Scaia et al., 2011). These studies indicate that BO is able to synthesize E2 from endogenous substrates and is therefore a potential new source for E2 in male anurans. Indeed, the estrogen receptor ERβ has recently been identified in previtellogenic and vitellogenic follicles in BO of *Rhinellum arenarum* using immunohistochemistry, suggesting a possible autocrine mechanism for E2 on BO (Scaia et al., 2015). In light of these recent findings, little is actually known regarding the differentiation, structure, function and regulation of this organ. It would be important to know if LH, FSH or other pituitary hormone receptors are expressed in cells of BO.

Both negative and positive sex steroid feedback effects have been reported *in vivo* and *in vitro* for several amphibian species. Direct evidence for negative feedback exerted by sex steroids on pituitary gonadotropin release derives from studies in gonadectomized adult bullfrogs. An increase in plasma LH and FSH was observed in males and females following gonadectomy, the effects of which were suppressed following replacements with the steroids E2 and 5α-DHT (McCreery and Licht, 1984). These effects are consistent with *in vitro* studies in which secretion of LH and FSH was elevated in cultured pituitaries derived from gonadectomized male and female Northern leopard frogs (Pavgi and Licht, 1989; Stamper and Licht, 1991). Moreover, sexually mature gonadectomized male Northern leopard frogs implanted with 5α-DHT and E2 had significantly suppressed levels of plasma and pituitary LH (Tsai et al., 2005) and lower levels of both LHβ and FSHβ transcripts (Zhang et al., 2007). Positive feedback from sex steroids on GnRH-induced LH and FSH secretion has been reported in juvenile bullfrogs. Females treated with 5α-DHT and a GnRH agonist enhanced gonadotropin secretion both *in vivo* and *in vitro* (Stamper and Licht, 1994). Therefore, steroidal regulation of

gonadotropins may be exerted through positive or negative feedback mechanisms, the nature of which is dependent on dose and specific times during the seasonal or life cycle.

Closer examination reveals that sex steroids exert their effects at the hypothalamic level by influencing GnRH neuronal activity and morphology. An increase in GnRH immunoreactivity was observed in the anterior preoptic area of gonadectomized female and male frogs (*Pelophylax esculentus*) supplemented with 5α-DHT and E2 (Iela et al., 1994). These two steroids have also been reported to enlarge GnRH soma size in female Northern leopard frogs that negatively correlated with LH release and gonad size, suggesting that increased GnRH neuronal soma size may reflect suppressed reproductive activity (Tsai and Jones, 2005). These results attest to the both inhibitory and stimulatory actions of steroids on the amphibian HPG axis.

The ability to ovulate and release sperm is dependent on the development of mature gametes, a process that is highly reliant on sex steroids. Oocyte development and maturation has been studied extensively in *Xenopus laevis* given the large availability of eggs that remain arrested in meiosis until triggered by steroids. This unique response allows the possibility to study steroid-induced oocyte maturation. Early *in vitro* studies conducted in *Xenopus laevis* proposed that progesterone (P4) and its derivatives were the principal stimulatory factors for oocyte maturation and ovulation. However, subsequent *in vivo* studies following gonadotropin administration have suggested otherwise. Female frogs injected with human chorionic gonadotropin (hCG) had significantly higher levels of A4 and T in their ovaries and serum than P4. Since then, it has been established that androgens play a major role in promoting oocyte maturation in frogs (Deng et al., 2009; Rasar and Hammes, 2006).

Sex steroids are also critically important in courtship displays in amphibians such as male vocalizations, female phonotaxis and amplectic clasping that are sequentially exhibited during

the breeding season. In amphibians, mating is visually marked by a copulatory embrace called amplexus in which a male grasps onto a female with his forelimbs that can last from minutes to days until spawning is accomplished. Despite being such an observable and common reproductive behaviour in frogs and some urodeles, little is known about the hormonal regulation of amplexus. Studies have proposed the role of androgens in the control of amplectic clasping in both anurans and urodeles, however there exists controversial conclusions across species that will need to be resolved (Moore et al., 2005).

Studies have established a clear positive relationship between testicular androgens and male sexual activity, a generalization that holds true for both anurans and urodeles. Comparable studies involving castration followed by replacement with T or 5α-DHT have demonstrated that amplectic clasping and mate advertisement calls are highly dependent on circulating androgen levels. These effects have been reported in *Xenopus laevis* (Kelley and Pfaff, 1976; Wetzel and Kelley, 1983) and *Taricha granulosa* (Deviche and Moore, 1988; Moore, 1978). Apart from advertisement calls, release calls are performed by both sexually unreceptive males and females. There exists sex-specific differences in the characteristics of these calls that are suspected to be controlled by sex steroids, although there remains to be a lack of direct evidence (Boyd, 1992; McClelland and Wilczynski, 1989). The role of androgens can be further extrapolated to urodele species that display reproductive behaviours beyond amplectic clasping. Interestingly, these androgen-dependent effects on courtship behaviours in anurans and urodeles are enhanced by AVT. This hormone has been shown to facilitate advertisement calls, aggressive calls, amplexus in urodeles, tail vibrations and the secretion of the peptide pheromone sodefrin (Moore et al., 2005). Emerging data are revealing the interaction of the neurosteroid 7α-OH-PREG with AVT, PRL and androgens in stimulating locomotor activity in breeding male red-bellied newts

(Toyoda et al., 2015). Behaviours such as tail vibrations were elicited in a dose-dependent manner following intracerebroventricular injections of 7α-OH-PREG. These effects are suspected to be mediated by the dopaminergic system.(Matsunaga et al., 2004; Toyoda et al., 2012; Tsutsui et al., 2010b).

Reproductive behaviours in females are similarly triggered by sex steroids. One of the most common behaviours exhibited by female amphibians is phonotaxis. In response to male advertisement calls, females will move towards these signals and strong evidence suggests that this receptive behaviour is mediated primarily by E2. Elevated phonotactic response has been reported in female túngara frogs (*Physalaemus pustulosus*) injected with E2, implicating that E2 influences female neuronal responses to promote phonotaxis (Chakraborty and Burmeister, 2009, 2015; Wilczynski and Lynch, 2011). Similarly, gray treefrogs (*Hyla versicolor*) injected with P4 displayed phonotaxis and increased plasma E2 levels in comparison to Ringers' saline-injected or uninjected females (Gordon and Gerhardt, 2009). Currently, there is great emphasis on male amphibian courtship behaviours, however the neuroendocrine regulation of female behaviours is an avenue that remains to be further explored.

Apart from stimulating reproductive behaviours, steroids are equally important in suppressing these displays when a behavioural shift is required. These effects are primarily mediated by the stress axis. In response to stress, corticotropin-releasing factor is secreted from the hypothalamus to trigger the release of adrenocorticotropic hormone from the pituitary and corticosterone from the interrenal tissues. In the newts *Taricha granulosa* and *Notophthalmus viridescens*, corticosterone has been shown to inhibit amplexus within a few minutes (Davis et al., 2015; Moore and Miller, 1984). Importantly, the high correlation between stress and captivity is well-established in amphibians. Indeed, plasma corticosterone concentrations in wild female

whistling frogs (*Litoria ewingii*) and bullfrogs significantly increased with capture time (Coddington and Cree, 1995; Licht et al., 1983). Moreover, corresponding gonadotropin and sex steroid levels are highly sensitive to stresses induced by capture and handling. In *Taricha granulosa, Lithobates catesbeianus* and *Bufo marinus,* confinement has been consistently accompanied with a suppression of circulating androgens and gonadotropins (Licht et al., 1983; Mendonça et al., 1985; Moore and Zoeller, 1985; Orchinik et al., 1988). Such data suggest the potential stress induced by capture could have negative effects on reproduction in wild amphibians in captive breeding programs.

2.6. COURTSHIP BEHAVIOUR AND SOCIAL SIGNALS REGULATE GONADOTROPIN RELEASE

Gonadotropin release is regulated primarily by hypothalamic factors, however external social cues and courtship behaviours such as those discussed are key players in their secretion as well. Although the hormonal regulation of amplexus remains unclear, amphibians are induced ovulators and it has been established that gonadotropin release is the result of this amphibian reproductive behaviour. In a study where male toads (*Bufo japonicus*) were placed with real females or an artificial female model, plasma LH and FSH were elevated following amplexus in both scenarios, confirming that amplexus is a sufficient stimulus to generate the LH surge required for sperm release (Ishii and Itoh, 1992). It has also been demonstrated that endocrine responses can be elicited through mating calls. Female túngara frogs (*Engystomops pustulosus*) exposed to natural mate choruses exhibited significantly elevated levels of plasma E2 (Lynch and Wilczynski, 2006), while male green tree frogs (*Hyla cinerea*) exposed to a mating chorus had significantly greater GnRH-immunoreactive cells (Burmeister and Wilczynski, 2005).

Breeding involves the coordination of physiological events not only within individuals, but also between males and females in a timely manner. A classic form of communication is male calling behaviour. Most amphibians participate in explosive breeding where individuals gather in breeding ponds at high densities with intense male-to-male competition. In these events, mate recognition becomes crucial where individuals depend on more than acoustic signals. These courtship behaviours displayed by amphibians are further orchestrated by a set of chemosignals called pheromones that are emitted through specialized glands as a form of exocrine communication during the breeding season. Chemicals like these are well-studied in urodeles such as salamanders and newts. Chemosignalling systems represent a relatively new field with opportunities for future insights (Janssenswillen et al., 2014; Woodley, 2015). More studies will need to be conducted to elucidate the relationship between behaviour and spawning.

2.7. SEASONALITY AND SPAWNING

Given the wide global distribution of amphibians, the modes and timing of reproduction are highly variable and dependent on environmental conditions. The most diverse amphibian assemblages can be found in Neotropical areas such as Brazil, with a reported 932 species. In some tropical amphibians, continuous gametogenesis permits reproduction to take place year-round, while in other species breeding will only occur during a well-characterized wet season (Cynthia et al., 2005; Donnelly and Guyer, 1994). By contrast, most temperate-zone species have distinct seasonal breeding cycles and will often remain in prolonged states of aestivation or brumation, followed by a short breeding period that is triggered by specific environmental events, most commonly increasing temperatures, photoperiod and rainfall. In mammals and birds, it has been established that the effects of temperature and photoperiod on reproduction is primarily mediated through the activity of the pineal gland. The main pineal hormone, melatonin

(MEL), is synthesized nocturnally and has been linked to suppressed GnRH-regulated LH release (Skinner and Robinson, 1997; Vanecek, 1999). In *Rana perezi*, high temperatures have been linked to pronounced MEL rhythms in the pineal gland, lateral eyes and plasma with levels peaking at night. In addition to synthesis, the transduction of MEL signals has been found to be temperature-dependent (Delgado and Vivien-Roels, 1989; Isorna et al., 2004). In female anurans, impaired ovarian function has been induced by artificially modified photoperiods. Significant reductions in previtellogenic follicles have been observed in MEL-injected *Rana perezi* (Alonso-Bedate et al., 1990), in addition to a high incidence of oocyte atresia in *Bufo bufo* exposed to low light:dark cycles (Horseman et al., 1978). Likewise in males, MEL appears to exert negative effects on Leydig cells in the testes. Both *in vitro* and *in vivo* MEL treatments in the frog *Pelophylax esculentus* inhibited androgen production (d'Istria et al., 2004) and the proliferation of primary spermatogonia (d'Istria et al., 2003).

As previously described, AVT is a critical modulator of reproductive behaviours and social performances in amphibians. Emerging studies in *Hyla cinerea* suggest sex and seasonally-dependent interactions between AVT and MEL. Reproductively active males implanted with MEL-filled silastic capsules had significantly lower AVT immunoreactive cells in the nucleus accumbens, supriachiasmatic nucleus, caudal striatum and amygdala than females (Lutterschmidt and Wilczynski, 2012). Moreover, these effects are likely regulated by melatonin type 1 (MT1) receptors. Similarly, reproductively active males displayed a higher level of MT1-immunoreactive cells in these brain regions than non-reproductive males or reproductive females during the summer breeding season (Howard and Lutterschmidt, 2015). Perhaps the sensitivity of male sexual behaviours to photoperiod is regulated by the effects of MEL on the AVT system.

Timing of reproduction is complex where the mechanisms that drive seasonal rhythms remain poorly understood in the diverse amphibian classes.

Physiologically, pituitary content, gonadosomatic index (GSI) and gonadal sex steroids have been shown to fluctuate according to these temporal cycles. These patterns have been traced in only a selective number of species. In the toad *Bufo japonicus* (Itoh et al., 1990), the bullfrog (Licht et al., 1983) and the frog *Pelophylax esculentus* (Polzonetti-Magni et al., 1998), LH and FSH peak just prior to the breeding period in March-April and in the autumn, where androgen levels rise in parallel with LH. Moreover, the steroids E2 and P4 are greatest in the autumn and correlates with an increase in GSI. Pituitary-gonadal cycles are likely to be species-specific, however understanding these general patterns is a crucial component to effectively implement captive breeding strategies. Researchers should aim to either mimic natural breeding cycles and conditions, or target these periods for hormone administration.

2.8. PRACTICAL APPLICATIONS AND LIMITATIONS

2.8.1 The need for assisted reproduction

The number of new amphibian species being discovered has been steadily increasing over the last few decades (http://amphibiaweb.org/). For example there was a 26.3 % global increase of new species from 1992 to 2003 (Köhler et al., 2005). Many of these are elusive species found in tropical and mountainous areas. Some newly distinguished species include the lungless frog from Indonesia (*Barbourula kalimantanensis*) (Bickford et al., 2008) and seven miniature frogs in Southern Brazil (*Brachycephalus spp.*) (Ribeiro et al., 2015) which display unique life histories and traits that contribute to expanding our knowledge of amphibian evolution. However, these exciting discoveries are accompanied by major amphibian population declines linked to habitat loss, disease and pollution, pushing this class towards extinction at a rate that far

surpasses background extinction rates traced in the fossil record (Alroy, 2015; McCallum, 2007; Wake and Vredenburg, 2008). One example illustrates the crisis and need for multiple conservation approaches, including captive breeding. The charismatic Indian purple borrowing frog (*Nasikabatrachus sahyadrensis*) was first reported in 2003 (Biju and Bossuyt, 2003), yet it is already on the IUCN list of endangered species (http://www.iucnredlist.org/details/58051/0). It will be necessary to create solutions that will help amphibians thrive alongside these rapidly emerging threats (Harding et al., 2015).

In an attempt to sustain these populations, *ex-situ* conservation strategies have been put forward by forming captive assurance colonies. Since 2006, the Amphibian Ark (AArk) (http://www.amphibianark.org) has collaborated with zoos and institutions to establish an array of captive breeding programs in response to the IUCN Amphibian Conservation Action Plan (ACAP) (Bishop et al., 2012). Unfortunately, it has been reported that the global estimate of species that can be raised under captive conditions is approximately 50 (Bishop et al., 2012), which is in marked contrast to the ~2030 species that are categorized as threatened. Regardless of these limitations, this conservation strategy represents a crucial step towards slowing down this extinction crisis (Harding et al., 2015). Some of these species have been introduced into well-controlled artificial environments that attempt to simulate their natural habitats. When it is possible to breed them in captivity, colonies are raised until conditions are favourable to permit reintroduction into suitable habitats in the wild (Griffiths and Pavajeau, 2008). Despite protecting selected amphibian species from immediate threats, the effectiveness of captive breeding remains debatable. Many amphibians do not reproduce naturally or easily in captivity. Animals may fail to mature or may lack environmental and behavioural cues, exhibit selective mate choice, or suffer poor nutrition and elevated stress from handling (Kouba et al., 2012).

One approach is to investigate and establish the optimal environmental conditions for captive breeding of a given species. This requires considerable investment, space and time, and cannot be applied across all species. Often, little is known about the breeding ecology of endangered amphibian species. Effective and broadly applicable reproductive technologies are therefore required. In this regard, hormone-induced spawning shows great promise because some of the neuroendocrine systems controlling reproduction are evolutionarily conserved. This approach has been used in selective amphibian species, with variable success.

2.8.2 Current methods and future directions

Hormone-based spawning induction can be classified into two approaches: hypophyseal and hypothalamic. The gonadotropin surge required for ovulation or sperm release may be mimicked by the injection of exogenous gonadotropins (hypophyseal) or by promoting the release of endogenous gonadotropins through manipulations of the HPG axis (hypothalamic) (Yaron, 1995). Hypophyseal approaches have been applied to induce spawning in amphibians that include crude pituitary extracts, purified gonadotropins and human chorionic gonadotropin (hCG). However, these classic approaches are most often accompanied by several disadvantages. Purified pituitary homogenates harvested from adults was the first method employed to stimulate gamete release in live animals. The effectiveness of these extracts is compromised by the possible introduction of transmissible diseases and the necessity to sacrifice whole animals, therefore defeating the purpose of sustaining live captive populations. Variations in pituitary size and seasonal gonadotropin contents also make it difficult to achieve accurate doses (Clulow et al., 2014). The presence of other, potentially inhibitory hormones in crude extracts may also reduce their effectiveness. Mammalian chorionic hormones such as hCG and pregnant mare's serum gonadotropin (PMSG) have been used to effectively stimulate spawning in some *Bufonids*

and *Xenopus*, with highly variable success in other species. Recent phylogenetic analyses have revealed that chorionic gonadotropins such as hCG and PMSG are distantly related to amphibian LHβ and FSHβ subunits (Clulow et al., 2014). The application of these chorionic gonadotropins (CGs) therefore tends to require high doses given frequently. Low efficacy of CGs most likely relates to the relatively low affinity of amphibian gonadal LH receptors in many species (Clulow et al., 2014; Licht, 1995). On the other hand, these human gonadotropin preparations are easy to obtain, and some species will respond.

The so-called hypothalamic approach (Yaron, 1995) has been most successful in teleosts, and has been applied to amphibians, but to a lesser extent. Several synthetic GnRH analogs have been tested (Clulow et al., 2014), and des-Gly^{10}-His(Bzl)6-GnRH-ethylamide and des-Gly^{10}, D-Ala^{6}, Pro^{6}-GnRH-ethylamide are most commonly used. In some species, GnRH agonists alone can effectively stimulate some aspects of reproduction, yet in others they do not (Clulow et al., 2014). To some extent, this likely reflects species differences in GnRH receptor affinities (Seong et al., 2003). Regardless, there is considerable scope for research on synthetic GnRH analogs that could be used to enhance gonadotropin release and spawning in frogs, given the range of GnRHs that bind to the 3 known amphibian GnRHR subtypes (Acharjee et al., 2002; Wang et al., 2003; Wang et al., 2001). Understanding GnRH ligand-receptor interactions is crucial to effectively carry out applications. Future works should target those GnRH agonists with the highest potency for GnRHR-1, the most abundant receptor subtype in the amphibian pituitary (Wang et al., 2001).

Emerging evidence suggests a conserved inhibitory role of DA on LH release in amphibians, as seen in teleosts. The use of GnRH agonists in combination with DA antagonists has been successfully applied in fish aquaculture through the commercial use of OVAPRIM.

This is a mixture of a sGnRH analog and the DA receptor D2 antagonist domperidone as a co-treatment to potentiate LH release. Over a wide range of teleosts, especially cyprinids, OVAPRIM has a reported success rate of 90 % and greater (Hill et al., 2009; Peter et al., 1988).

Through evolutionary extrapolation, we have developed a similar approach for amphibians (Trudeau et al., 2013; Trudeau et al., 2010). These studies revealed that the most effective combination in frogs were the GnRH agonist des-Gly^{10}, D-Ala^{6}, Pro-GnRH ethylamide and the DA receptor D2 antagonist metoclopramide. Female and male leopard frogs that were injected once during their natural breeding season with this mixture produced fertilized egg masses and healthy tadpoles. However, it was noted that frogs that were bred out of season in a laboratory environment that simulated artificial hibernation required a priming dose of the GnRH agonist. This has been named the AMPHIPLEX method for spawning induction, which derives from the words "Amphibian" and "Amplexus" (Trudeau et al., 2010). Healthy tadpoles have been obtained in four species of Argentinian frogs (*Ceratophrys ornata*, *Ceratophrys cranwelli*, *Odontophrynus americanus* and *Lepidobatrachus llanensis*) (Costa et al., 2015; Trudeau et al., 2010) using this method. Unpublished data (Trudeau, V.L., Thoney, D., Dancosse, J. and McGinnity, D.) indicate that the AMPHIPLEX mixture is highly effective in endangered species such as the Oregon spotted frog (*Rana pretiosa;* see Figure 2.2), the boreal chorus frog (*Pseudacris maculata*) and Eastern hellbender salamander (*Cryptobranchus alleganiensis*). Induced spawning therefore has high potential in amphibian conservation.

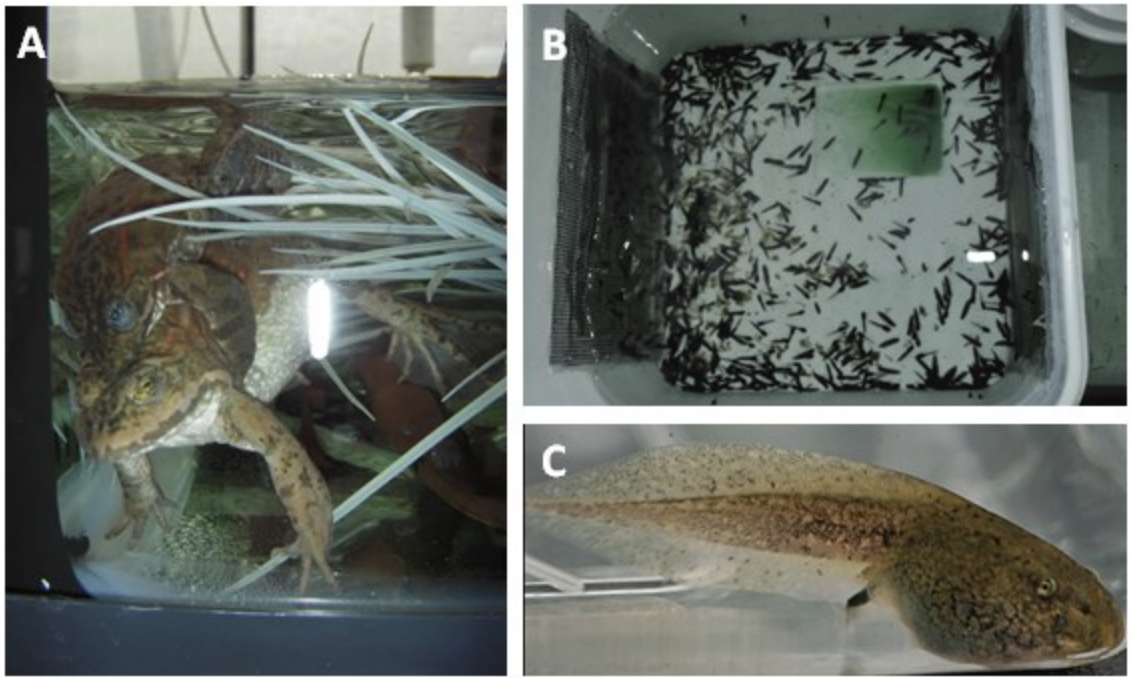


Figure 2.2 Successful induction of spawning in captive Oregon Spotted Frogs (*Rana pretiosa*), an endangered species listed under the Canadian Species at Risk Act (http://www.registrelep-sararegistry.gc.ca/default.asp?lang=En&n=6AC28F91-1). (A) Co-injection of a GnRH agonist and a DA antagonist following the AMPHIPLEX method (Trudeau et al. 2010) induced amplexus. The male (top) is clasping the female (bottom). (B) Spawning was successful and *Rana pretiosa* tadpoles at Gosner stage 25-28 are shown. (C) Close-up of a *Rana pretiosa* tadpole at Gosner stage 30. Photos are courtesy of Kris Rossing, Darren Smy and Dennis Thoney at the Vancouver Aquarium (British Columbia, Canada).

2.9. CONCLUSIONS

To move forward with assisted reproductive technologies in amphibians, a number of issues will need to be addressed. It has long been established that GnRH has a conserved stimulatory action on gonadotropin release and spawning, however it is clear that this neuropeptide is not the only modulator (Figure 2.1). Emerging studies on other systems such as GnIH, kisspeptin, PACAP and ECBs are opening opportunities for further investigations that would heighten the physiological support required for successful spawning induction practices. Moreover, it is unclear how and if DA negatively regulates LH and FSH synthesis and release in amphibians. The few studies available have only examined dopaminergic effects on LH release and ovulation, with little exploration of the basic mechanisms of action at the level of the brain and pituitary. Progress is hampered by the lack of purified or recombinant LH and FSH molecules and specific radioimmunoassays in various amphibians. Such molecular studies would be required for closer examination of gonadotropin synthesis and release. Experiments will need to be carried out to determine effective doses, frequencies and timing of hormone treatments. Future strategies should focus on factors that have highly conserved actions to regulate gonadotropin biosynthesis and release *in vivo*, and thus have high potential to enhance gonadal steroidogenesis, pheromone synthesis and spawning in a wide range of species.

It is evident that the neuroendocrine control of spawning in amphibians is far from being resolved, given the complex interplay between neuropeptides, pituitary hormones and sex steroids that exist to regulate the diverse reproductive modes exhibited by amphibians. With such diversity, amphibian neuroendocrinology is a fascinating and exciting field with many windows of opportunity for new discoveries and applications.

CHAPTER 3

Time- and dose-related effects of GnRH agonist and DA antagonist injections on spawning and pituitary gene expression in the Northern leopard frog (*Lithobates pipiens*)

3.1. Introduction

Applying conservation tools is now critical to alleviate amphibian population declines. In particular, hormonal induction of spawning has proven to be an effective strategy to facilitate captive breeding in some amphibian species. Importantly, a fundamental understanding of the physiological control of spawning is necessary to successfully move forward with these new conservation approaches. One example of a hormone mixture that has been recently applied to some amphibian species consists of a combination of a GnRH agonist and the selective DA receptor D2 antagonist metoclopramide. This is called the AMPHIPLEX method for spawning induction (Trudeau et al., 2013; Trudeau et al., 2010). AMPHIPLEX is a combination of the words amphibian and amplexus, reflecting the sexual behaviours of spawning amphibians. This method was based on limited knowledge of spawning control in amphibians and from evidence in teleost fish through evolutionary extrapolation. Across vertebrates, GnRH is considered the principal stimulator of gonadotropin release, particularly LH (Schally, 1978; Vu and Trudeau, 2016). In teleost fish, there are multiple levels of evidence supporting the inhibitory role of DA on reproduction (Dufour et al., 2005), therefore forming the foundation to apply a DA antagonist to the AMPHIPLEX method. Currently, there is limited evidence regarding the inhibitory action of DA in amphibians where the potentiating effect of metoclopramide in this spawning method is

only assumed. Therefore, despite some success, there are challenges to the application of this hormone mixture that remain to be resolved, including the effective dose of this GnRH agonist. The goal of this study was to investigate this inhibitory dopaminergic effect on reproduction in amphibians through various time- and dose-response experiments where a GnRH agonist and DA antagonist were tested separately and in combination. These effects were examined through spawning and gene expression analysis of pituitary gonadotropins. This was achieved through multiple experimental approaches in the field and laboratory. It was predicted that both spawning and gonadotropin synthesis will be potentiated by the addition of a DA antagonist to a GnRH agonist. These two end points were paired to elucidate the hypothesized inhibitory role of dopamine.

To understand the actions of DA and GnRH on spawning and gonadotropin regulation, these questions were addressed in this study using the Northern leopard frog (*Lithobates pipiens*) as a model. The Northern leopard frog is a species that has previously benefitted from the AMPHIPLEX method. Studies have demonstrated that thousands of fertilized eggs could be produced from a single intraperitoneal injection (Trudeau et al., 2013; Trudeau et al., 2010). Over the recent years, the gradual recovery of Northern leopard frog populations has been made possible by this *ex-situ* conservation strategy. Currently, recovery efforts in British Colombia have applied this method to successfully breed Northern leopard frogs in captivity at the Vancouver Aquarium.

The iconic Northern leopard frog represents a classic example of a species that is locally abundant, however is peripherally declining. Endangered species are theoretically associated with low population densities and restricted geographical ranges (Cooper et al., 2008). However, it is important to recognize that declines are simultaneously affecting common amphibian

populations that do not necessarily fit these criteria. The Northern leopard frog is categorized as least concern on a global scale according to the IUCN Red List of Threatened Species and historically, is considered to be one of the most widespread amphibian species in North America with populations extending from British Colombia to Labrador, and the southwestern and central United States (Rogers and Peacock, 2012). Although populations in eastern Canada continue to persist, a closer look at their historical range map indicates that Northern leopard frogs are indeed experiencing dramatic declines at the western end of their distribution in Canada and the United States. These patterns are most clear in British Colombia and Alberta where extant populations have been restricted to certain historical corners of the province (Leonard et al., 1999). Northern leopard frog populations have disappeared completely in some areas that they once occupied (Corn and Fogleman, 1984; Werner, 2003). These declines were first documented in the early 1970s and recent surveys now reveal that what was once a common species is now of conservation concern. Introduction of invasive species such as bullfrogs, emerging lethal diseases including chytridiomycosis and the creation of habitats that are no longer supportive of such populations are only some of the factors threatening the Northern leopard frog (Hayes and Jennings, 1986; Johnson et al., 2011). Furthermore, the elimination of certain native populations means that remaining populations now have a heightened vulnerability for genetic isolation (Rogers and Peacock, 2012; Wilson et al., 2008). Northern leopard frogs not only play a critical role in ecosystem functioning as both predators and prey, but have been used extensively as a model species for research in the fields of physiology, ecotoxicology, developmental biology and genetics (Gibbs et al., 1971). Careful attention and attempts to sustain their populations are now required, followed by close monitoring in the future. The results from this study would have

implications towards the development of strategic spawning methods for the recovery of closely-related ranid species.

3.2. Effects of GnRH-A and MET injections on spawning

3.2.1. Materials and methods

3.2.1.1. Animal collections and husbandry

Wild adult Northern leopard frogs were collected in and around Bishop's Mills and Kemptville, Ontario during the spring migration in late March to mid-April in 2015 and 2016. Animals were primarily caught as they were emerging from deep ponds or creeks and crossing roads to shallower breeding ponds. Generally, warm air temperature and precipitation will draw frogs out of their overwintering sites. Mass movements were witnessed starting at dusk on nights that were characterized by rain and a minimum air temperature of 14 °C. Sexually mature individuals were selected and were distinguished by thick male thumb pads and gravid females that had a snout-vent length of 70 mm or greater. Alternatively, some individuals were caught during the day in their breeding ponds with dip nets or in holes and ditches by the sides of the road by hand. Frogs were placed in bags with air and were kept on ice following capture.

Upon arrival at the University of Ottawa Aquatic Care Facility, animals were immediately stored at 4 °C to allow their metabolism to slow down. Frogs were housed in 121 L Tuff Store plastic tote bins with a tight-fitting lid in which holes were drilled to permit gas exchange. Animals were separated according to sex and approximately 50 frogs were placed in each bin containing 4 °C dechlorinated water. Each habitat consisted of an additional one or two bricks which served as a platform for males and females, respectively. The use of moss was avoided to prevent any possible growth of bacteria that may introduce diseases to the frogs. Routine water changes were performed three times a week by transferring frogs over to new tote

bins with fresh 4 °C dechlorinated water. Containers that previously housed frogs were cleaned with hot water and disinfected with ACCEL Prevention Concentrate. Animals were kept under these conditions for three weeks until the start of the experiment.

3.2.1.2. Mesocosm and experimental design

This spawning experiment was carried out in an outdoor field at the Canadian National Wildlife Research Centre (NWRC) on the Carleton University Campus (Ottawa, ON). Each mesocosm consisted of a 300 L Rubbermaid stock tank that was filled with 200 L of water. An artificial spawning habitat was recreated in each tank, consisting of a floating styrofoam board (30 cm x 60 cm) to allow for a basking platform, and tree branches tied down to a brick with jute twine to provide surface area for egg masses to attach to during spawning. Variations between branch sizes and patterns were kept to a minimum (Figures 3.1 and 3.2). Prior to transporting housed animals to the NWRC, the water was aged for at least 48 hours to allow the temperature to stabilize and for free chlorine to evaporate. Frogs were then transferred into these tanks according to sex and were allowed to acclimate outdoors for one to two weeks. Two mesh lids were secured to each tank with bungee rope to prevent escape and predation by birds or raccoons. In 2015, five acclimating tanks were prepared (3 females and 2 males), however this resulted in overcrowding which risked compromising the animal's health. Therefore, ten tanks (5 female and 5 male) were used to acclimate frogs in the following experiment in 2016. Daily surveys were performed in which water temperature was measured and the health status was assessed. All animal care procedures were compliant with protocols in the University of Ottawa Animal Care and Veterinary Service.

Figure 3.1 Experimental layout at the Canadian National Wildlife Research Centre (Ottawa, ON).

Figure 3.2 Artificial spawning habitat created in each mesocosm for *Lithobates pipiens* in 2015 and 2016. Each tank consisted of a styrofoam basking platform and branches as spawning substrate submerged by a brick in dechlorinated water.

3.2.1.3. Chemicals and injections

Chemicals used in this experiment were the GnRH agonist (GnRH-A) (Des-Gly^{10}, D-Ala^{6}, Pro-NHEt^{9})-LHRH acetate salt (BACHEM, Item #4012028.0025, CAT #H-4070.0025), and the selective dopamine receptor D2 antagonist metoclopramide hydrochloride (MET) (Sigma-Aldrich, CAT #M0763-25G) which have previously been shown to be biologically active in frogs (Browne et al., 2006; Michael et al., 2004; Waggener and Carroll, 1998). Hormones were weighed using a microbalance (Sartorius M2P) and diluted in 0.7 % saline (NaCl) on the day of injection. Various combinations of doses were tested for this study based on previous findings in frogs (Trudeau et al., 2013; Trudeau et al., 2010) and goldfish (*Carassius auratus*) (Chang and Peter, 1983; Peter, 1980; Peter et al., 1987; Sokolowska et al., 1985). The treatments selected for the experiment in 2015 were as follows: Control (0.7 % NaCl), GnRH-A Low (0.2 μg/g), GnRH-A High (0.4 μg/g), MET Low (5 μg/g), MET High (10 μg/g), and the combinations GnRH-A Low and MET Low, GnRH-A Low and MET High, GnRH-A High and MET Low and GnRH-A High and MET High. For the experiment repeated in 2016, the low GnRH-A dose was decreased to 0.1 μg/g.

In the first experiment, 36 tanks were set up where each treatment group consisted of 4 tanks (n=4). The first three replicates held a sex ratio of 4 females and 5 males, whereas the last replicate contained 3 males and 3 females, for a total of 15 females and 18 males per treatment. In 2016, 45 tanks were set up, each treatment containing 5 tanks (n=5) with a male to female sex ratio of 5:3 in each, for a total of 25 males and 15 females per treatment. The injection day was selected based on weather conditions, particularly water temperature. This spawning experiment is ideally carried out in a water temperature of 16-17 °C which is typical during the breeding season. At 8:00, frogs were removed from their acclimating tanks and processed successively

according to treatment group. Body weights (BWt) and snout-vent lengths (SVL) of each individual were recorded, followed by an intraperitoneal (IP) injection using a 25 gauge x 5/8 inch needle attached to a disposable 1 mL tuberculin syringe (2 μL/g BWt). A unique toe clip was then performed on each individual using surgical scissors cleaned with 70 % ethanol for easy recognition. Toe clipping amphibians is a standard and accepted method used to identify individuals by removing a unique combination of digits. Despite some debate, this procedure is considered safe in amphibians where most studies have reported no or few adverse effects (Perry et al., 2011; Phillott et al., 2007). This protocol was adapted from the USGS National Wildlife Health Center (SOP No. 110). Following injection, females and males were placed in their respective tanks. Injections for all individuals ended at 22:00 and tanks were left overnight to be surveyed the next day.

3.2.1.4. Survey of tanks and egg masses

Each day following injections between 12:00 and 15:00, tanks were individually surveyed for water temperature, the number of couples in amplexus, new egg masses and the health of frogs. It should be noted that amplexus frequencies were based on these surveys conducted once a day where individuals were not tracked. New egg masses were tagged with a different colour of thread each day to facilitate identification and egg screening. Mortalities were noted and removed. Daily air temperature and weather conditions were also reported. At three days post-fertilization, egg masses were removed from tanks and processed. Individual egg masses from each treatment were weighed and five subsamples were removed. Subsamples were weighed and screened under a dissecting microscope to determine the number of eggs and the fertilization rate. The total number of eggs in each egg mass was calculated from the weight of the egg mass and the average number of eggs per gram of subsample. Fertilization rate was

determined in the same manner. Post-ovulatory body weights of females were recorded at the end of the experiment. Frogs were sacrificed at the end of this experiment in a water bath containing an overdose of TMS (tricaine mesylate), also known as MS-222 (2 g/ L), and sodium bicarbonate (2 g/ L).

3.2.1.5. Statistical analysis

Egg mass numbers were calculated as the average number released per female for each tank replicate. The incidence of amplexus was calculated as an average over the number of days in the experiment for each tank replicate. Averages for each tank were obtained for egg mass weight, number of eggs per mass and fertilization rate. These data were analyzed using a two-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple pairwise comparisons and to test for interactions between GnRH-A and MET. Data that did not meet parametric assumptions were analyzed on ranks. Statistically significant differences were denoted by a p value < 0.05. Statistical analyses and graphs were generated using the statistical software SigmaPlot (version 11) and GraphPad Prism (version 6).

3.2.2. Results

3.2.2.1. Spawning 2015

Morphometrics and spawning conditions

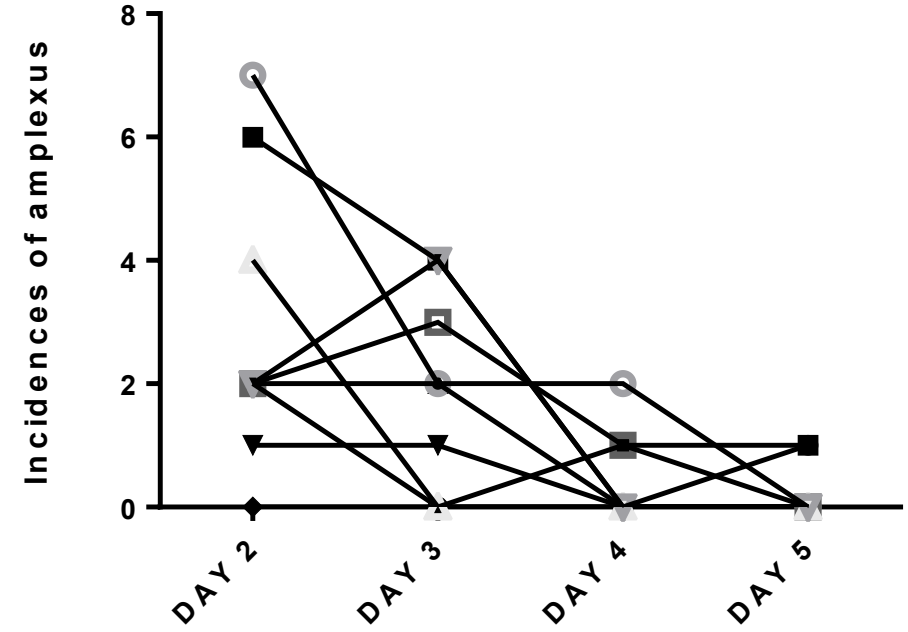
Mean (± SD) body weights for females and males at the time of injection were 42.47 g (± 11.56 g) and 26.00 g (± 6.33 g), respectively. Mean (± SD) snout-vent lengths for females and males were 73.86 mm (± 7.09 mm) and 64.62 mm (± 5.31 mm), respectively. Conditions during the acclimation and experimental period, including water and air temperature are presented in Table 3.1.

Date (2015)	Day	Water Temperature (°C)	Air Temperature (°C)
May 1	Acclimation	15	20
May 3	Acclimation	15	22
May 4	Acclimation	-	28
May 5	Day 1 (Injections)	17.8 – 22.4	24
May 6	Day 2 (Survey)	18.5 – 21.6	31
May 7	Day 3 (Survey)	17.2 – 21.6	27
May 8	Day 4 (Survey)	18.8 – 22.7	28
May 9	Day 5 (Survey)	22.3 – 25.1	31

Table 3.1 Air temperature range (°C) and water temperature in mesocosms (°C) during the acclimation and experimental periods in May 2015. Daily measurements were recorded between 12:00 and 15:00, with the exception of injection day where values were recorded between 11:00 and 21:00.

Intraperitoneal injections of GnRH-A alone and in combination with MET increase spawning in *Lithobates pipiens*

Behavioural data illustrating the timeline to amplexus and oviposition in this experiment are presented in Figure 3.3. Under the conditions of observation and data collection presented, the exact timing of amplexus and oviposition are not known. These values were approximated in terms of number of days. Therefore, based on these surveys conducted once a day, the incidences of amplexus recorded were highest 24 hours after injection and the greatest number of egg masses released occurred after 48 hours.


Incidences of amplexus
8
6
4
2
0
DAY 2
DAY 3
DAY 4
DAY 5

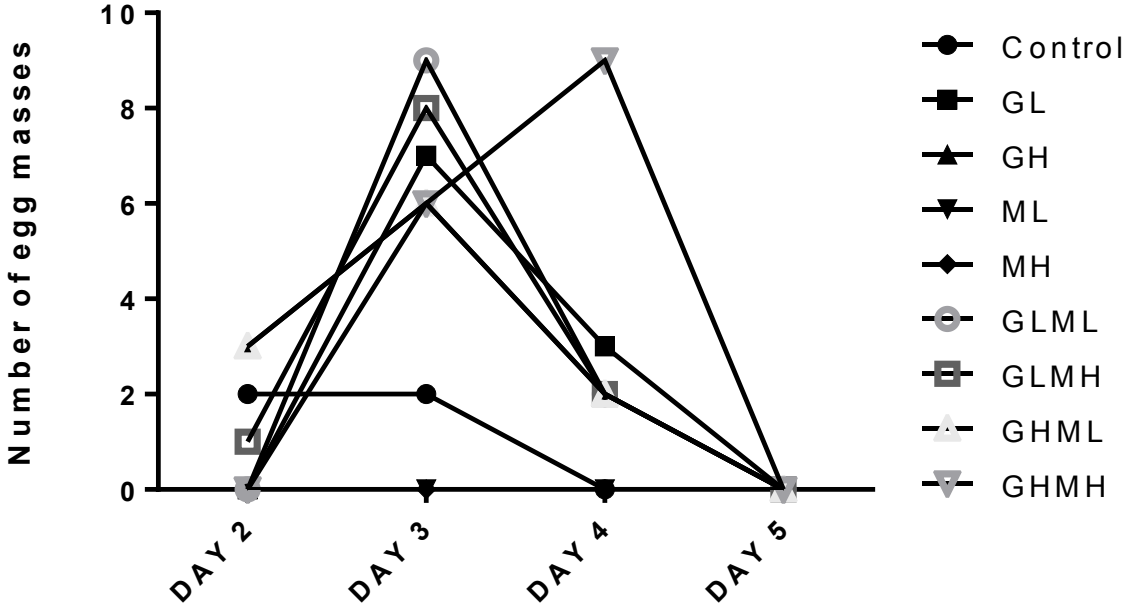

Number of egg masses
10
8
6
4
2
0
DAY 2
DAY 3
DAY 4
DAY 5
Control
GL
GH
ML
MH
GLML
GLMH
GHML
GHMH

Figure 3.3 Timeline of amplexus occurrence and egg mass release over four days post-injection in May 2015 for *Lithobates pipiens*. A GnRH agonist (G) at two doses [High (H) = 0.4 µg/g, Low (L) = 0.2 µg/g)] was administered alone and in combination with two doses of the dopamine antagonist metoclopramide (M) [High (H) = 10 µg/g, Low (L) = 5 µg/g].

The average incidence of amplexus reported per day in May 2015 across treatments is presented in Figure 3.4. There was a statistically significant effect of GnRH-A on the average incidence of amplexus [$F(2) = 8.432$, $p < 0.05$]. The incidence of amplexus increased significantly when the low dose of GnRH-A was delivered with both high ($p < 0.05$) and low ($p < 0.05$) doses of MET. However, there was no overall effect from MET [$F(2) = 0.436$, $p > 0.05$] and therefore, no statistically significant interactions between GnRH-A and MET [$F(4) = 0.769$, $p > 0.05$] on amplexus.

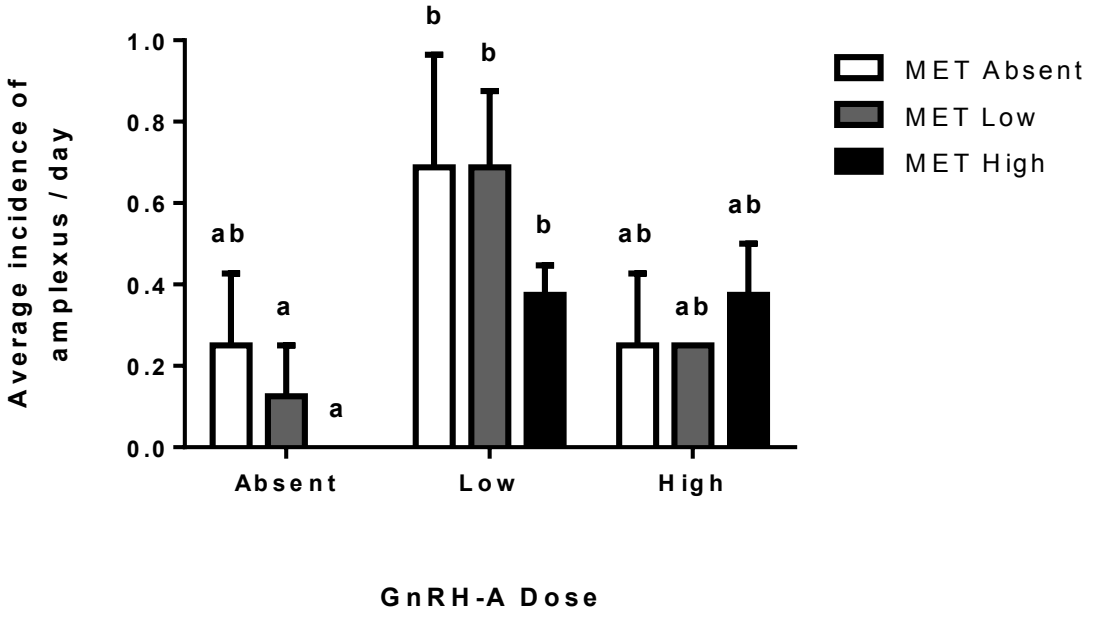


Figure 3.4 The average incidence of amplexus occurring per day in May 2015 following injections in *Lithobates pipiens*. Means +SEM are presented (n = 4). A GnRH agonist (G) at two doses [High (H) = 0.4 µg/g, Low (L) = 0.2 µg/g)] was administered alone and in combination with two doses of the dopamine antagonist metoclopramide (M) [High (H) = 10 µg/g, Low (L) = 5 µg/g]. Statistically significant differences ($p < 0.05$) are marked by unique letters.

Four out of fifteen possible egg masses were released in the control groups, although this was most likely due to an unexpected increase in water temperature. These egg masses were mostly disorganized and unfertilized. Indeed, some females were already releasing eggs on their own before handling and injections. No egg masses were produced in couples injected with metoclopramide. Females in this group remained gravid throughout the course of this experiment. Couples injected with the high doses of GnRH-A (0.4 μg/g) and MET (10 μg/g) in combination produced the greatest number of egg masses (15/15). The remaining three hormone mixtures produced equal number of egg masses (11/15). Interestingly, there was no difference in spawning effectiveness between GnRH-A administered alone at these doses or in combination with MET (Supplementary Table 3.1).

The average number of egg masses released per female in 2015 is presented in Figure 3.5. Note that a value of 1 indicates 100 % spawning success for a given treatment group. There was a statistically significant effect of GnRH-A on the average number of egg masses released per female [$F(2) = 34.507$, $p < 0.05$]. However, there was no effect from MET [$F(2) = 0.560$, $p > 0.05$] and therefore, no statistically significant interactions between GnRH-A and MET [$F(4) = 1.560$, $p > 0.05$] on egg mass release. Multiple comparisons revealed a significant increase in egg mass release when the high dose of GnRH-A was delivered in the absence of MET ($p < 0.05$), with the low dose of MET ($p < 0.05$) and with the high dose of MET ($p < 0.05$). Additionally, the low dose of GnRH-A increased egg mass release when administered with both low ($p < 0.05$) and high ($p < 0.05$) doses of MET. Importantly, 100 % spawning success was only obtained in groups injected with high GnRH-A and high MET doses in combination.

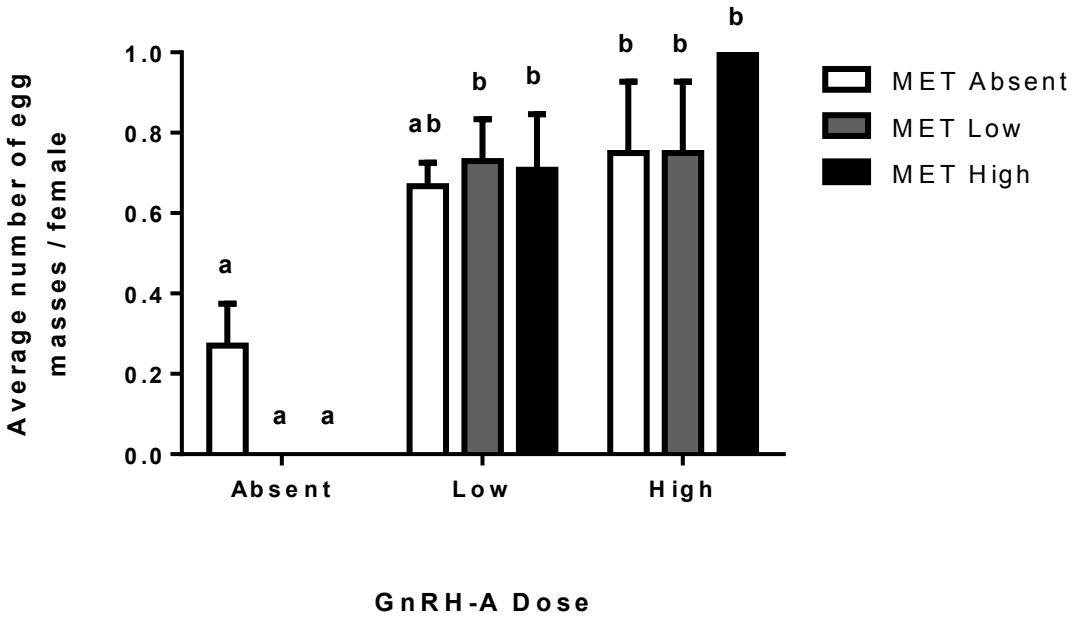


Figure 3.5 The average number of egg masses released per female following injections in *Lithobates pipiens* in May 2015. Means +SEM are presented (n = 4). A GnRH agonist (G) at two doses [High (H) = 0.4 μg/g, Low (L) = 0.2 μg/g)] was administered alone and in combination with two doses of the dopamine antagonist metoclopramide (M) [High (H) = 10 μg/g, Low (L) = 5 μg/g]. Statistically significant differences ($p < 0.05$) are marked by unique letters.

It was not a possibility to quantify egg mass weight, total egg numbers and fertilization rates in this experiment in 2015 due to the rapid development of eggs into tadpoles from unusually high temperatures. Under these conditions, tadpoles were hatching even after two days post-fertilization. A quick screening revealed that the majority of eggs (roughly over 90 %) were developing in a healthy manner, although accurate averages could not be calculated. The average post-spawn weight lost (%) and range for females on day 10 across treatment groups is presented in Table 3.2. These data indicate that all females that spawned also fully ovulated. Females in control groups lost a lower amount of weight (17.24 %), most likely attributed to the release of satellite egg masses under the influence of heat.

Treatment	Average Female Weight Lost (%) (Min.-Max.)
Control (0.7 % NaCl)	17.24 (7.24 – 35.06)
GnRH-A Low (0.2 μg/g)	26.37 (12.53 – 52.98)
GnRH-A High (0.4 μg/g)	28.76 (16.89 – 45.58)
MET Low (5 μg/g)	-
MET High (10 μg/g)	-
GnRH-A Low + MET Low	27.22 (14.07 – 48.35)
GnRH-A Low + MET High	26.01 (11.82 – 36.97)
GnRH-A High + MET Low	29.15 (15.02 – 63.48)
GnRH-A High + MET High	26.49 (8.53 – 34.62)

Table 3.2 Average post-spawn female weight loss (%) with minimum and maximum range on day 10 in May 2015. Mean values are presented for only females that released egg masses in each treatment.

3.2.2.2. Spawning 2016

Morphometrics and spawning conditions

Mean (± SD) body weights for females and males at the time of injection were 47.76 g (± 12.16 g) and 24.86 g (± 8.18 g), respectively. Mean (± SD) snout-vent lengths for females and males were 76.51 mm (± 6.24 mm) and 64.13 mm (± 6.74 mm), respectively. Water and air temperature during the acclimation period and the experimental week are presented below in Tables 3.3 and 3.4, respectively.

Date (2016)	Water Temperature (°C)	Air Temperature (°C)
April 19	-	10
April 20	10.4 – 12.8	19
April 21	13.4 – 16.8	25
April 22	13.8 – 15.1	16
April 23	7.7 – 9.3	11
April 24	7.2 – 9.2	13
April 25	7.3 – 9.4	11
April 26	8.6 – 10.2	16
April 27	5.7 – 7.3	7
April 28	8.6 – 10.7	11
April 29	8.2 – 10.6	14
April 30	8.3 – 10.3	14
May 1	10.1 – 11.3	8
May 2	8.7 – 9.3	10

Table 3.3 Air temperature (°C) and water temperature range in mesocosms (°C) during the acclimation period in April to May 2016. Daily measurements were recorded between 12:00 and 15:00.

Date (2016)	Day	Water Temperature (°C)	Air Temperature (°C)
May 3	Day 1 (Injections)	7.8 – 18.8	16
May 4	Day 2 (Survey)	12.3 – 15.3	13
May 5	Day 3 (Survey)	17.5 – 20.0	17
May 6	Day 4 (Survey)	16.7 – 19.6	17
May 7	Day 5 (Survey)	18.7 – 20.9	18
May 8	Day 6 (Survey)	11.7 – 13.5	6
May 9	Day 7 (Survey)	8.7 – 9.7	8

Table 3.4 Air temperature (°C) and water temperature range in mesocosms (°C) during the experimental period in May 2016. Daily surveys were conducted between 12:00 and 15:00. Injection day values were recorded between 9:00 and 23:00.

Intraperitoneal injections of GnRH-A alone and in combination with MET increase spawning in *Lithobates pipiens*

Behavioural data illustrating the timeline to amplexus and oviposition in this experiment are presented in Figure 3.6. Under the conditions of observation and data collection presented, the exact timing of amplexus and oviposition are not known. These values were approximated in terms of number of days. Therefore, based on the spawning conditions during 2016 and the surveys conducted once a day, the incidences of amplexus recorded were highest 24 to 48 hours after injection and the greatest number of egg masses released occurred after 72 to 96 hours. Furthermore, the results from this spawning experiment in 2016 demonstrated that amplexus and oviposition are closely related in the breeding events in the mesocosms and under the surveying conditions of this experiment (Supplementary Figure 3.2).

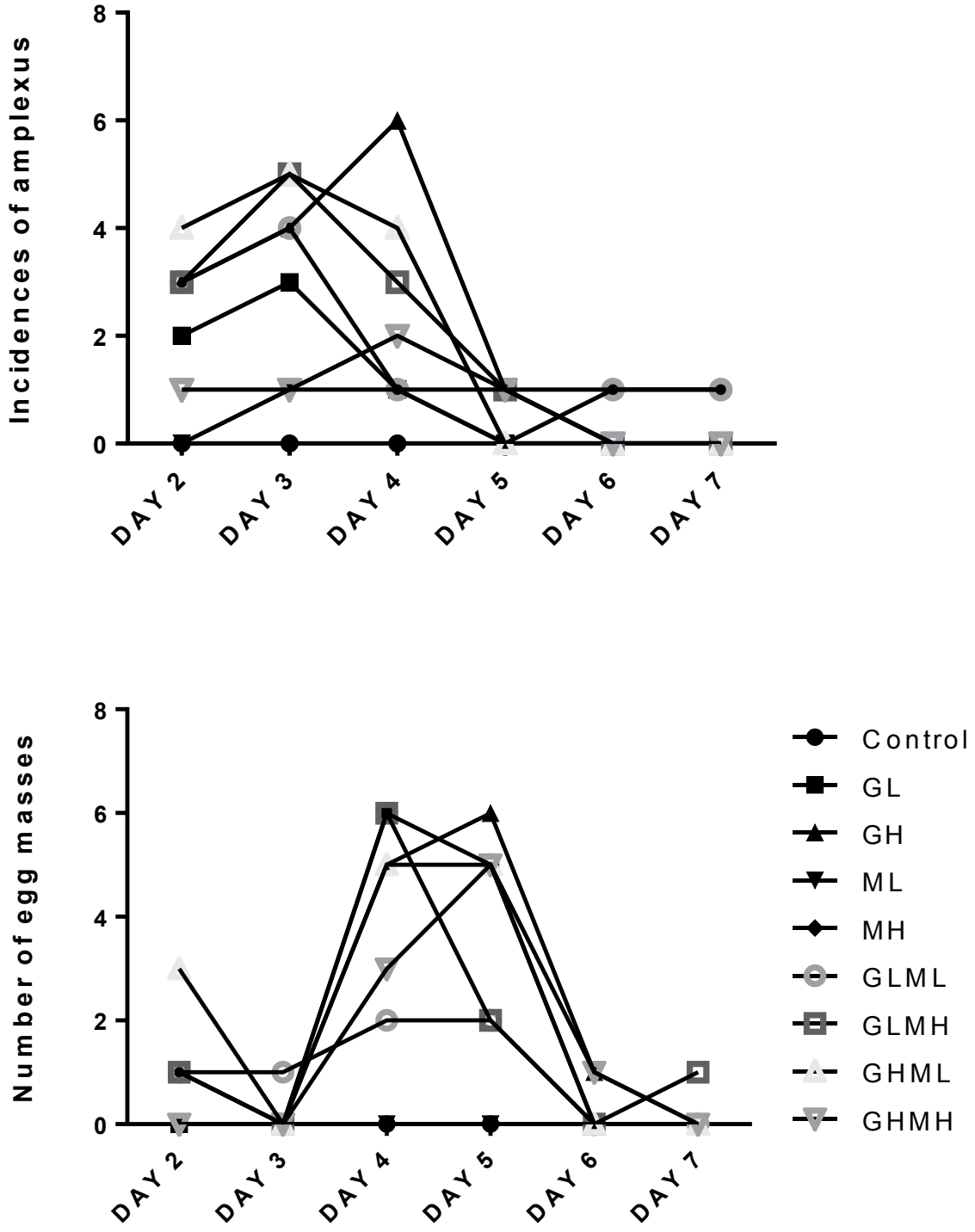

Incidences of amplexus
8
6
4
2
0
DAY 2
DAY 3
DAY 4
DAY 5
DAY 6
DAY 7
Number of egg masses
8
6
4
2
0
DAY 2
DAY 3
DAY 4
DAY 5
DAY 6
DAY 7
Control
GL
GH
ML
MH
GLML
GLMH
GHML
GHMH

Figure 3.6 Timeline of amplexus occurrence and egg mass release over six days post-injection in May 2016 for *Lithobates pipiens*. A GnRH agonist (G) at two doses [High (H) = 0.4 µg/g, Low (L) = 0.1 µg/g)] was administered alone and in combination with two doses of the dopamine antagonist metoclopramide (M) [High (H) = 10 µg/g, Low (L) = 5 µg/g].

The average incidence of amplexus reported per day in May 2016 across treatments is presented in Figure 3.7. There was a statistically significant effect of GnRH-A on the average incidence of amplexus [$F(2) = 11.693$, $p < 0.05$]. However, there was no effect from MET [$F(2) = 0.732$, $p > 0.05$] and therefore, no statistically significant interactions between GnRH-A and MET [$F(4) = 1.048$, $p > 0.05$] on amplexus. Multiple comparisons revealed a significant increase in amplexus when the high dose of GnRH-A was injected in the absence of MET ($p < 0.05$) and when delivered with the low dose of MET ($p < 0.05$).

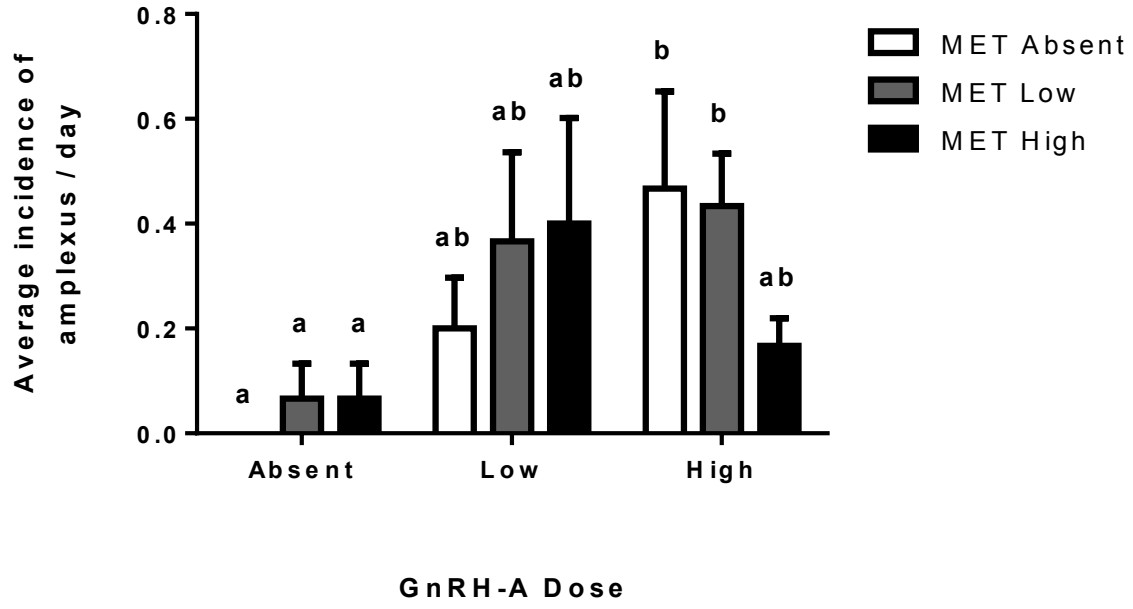


Figure 3.7 The average incidence of amplexus occurring per day in May 2016 following injections in *Lithobates pipiens*. Means +SEM are presented (n = 5). A GnRH agonist (G) at two doses [High (H) = 0.4 µg/g, Low (L) = 0.1 µg/g)] was administered alone and in combination with two doses of the dopamine antagonist metoclopramide (M) [High (H) = 10 µg/g, Low (L) = 5 µg/g]. Statistically significant differences ($p < 0.05$) are marked by unique letters.

The average number of egg masses released per female in 2016 is presented in Figure 3.8. There was a statistically significant effect of GnRH-A on the average number of egg masses released per female [$F(2) = 62.637$, $p < 0.05$], while there was no effect from MET [$F(2) = 1.177$, $p > 0.05$]. A statistically significant interaction was detected between GnRH-A and MET [$F(4) = 2.711$, $p < 0.05$] on egg mass release. Multiple comparisons revealed statistically significant increases in egg mass release when the high dose of GnRH-A was injected in the absence of MET ($p < 0.05$) and with both low ($p < 0.05$) and high ($p < 0.05$) doses of MET. Similarly, the low dose of GnRH-A significantly increased egg mass release in the absence of MET ($p < 0.05$) and with both low ($p < 0.05$) and high ($p < 0.05$) doses of MET. Interestingly, the high dose of GnRH-A resulted in significantly greater egg mass release compared to the low dose of GnRH-A ($p < 0.05$) when injected with the low dose of MET. The average number of egg masses released was significantly lower in groups that received the low dose of MET in combination with the low dose of GnRH-A relative to the low dose of GnRH-A on its own ($p < 0.05$).

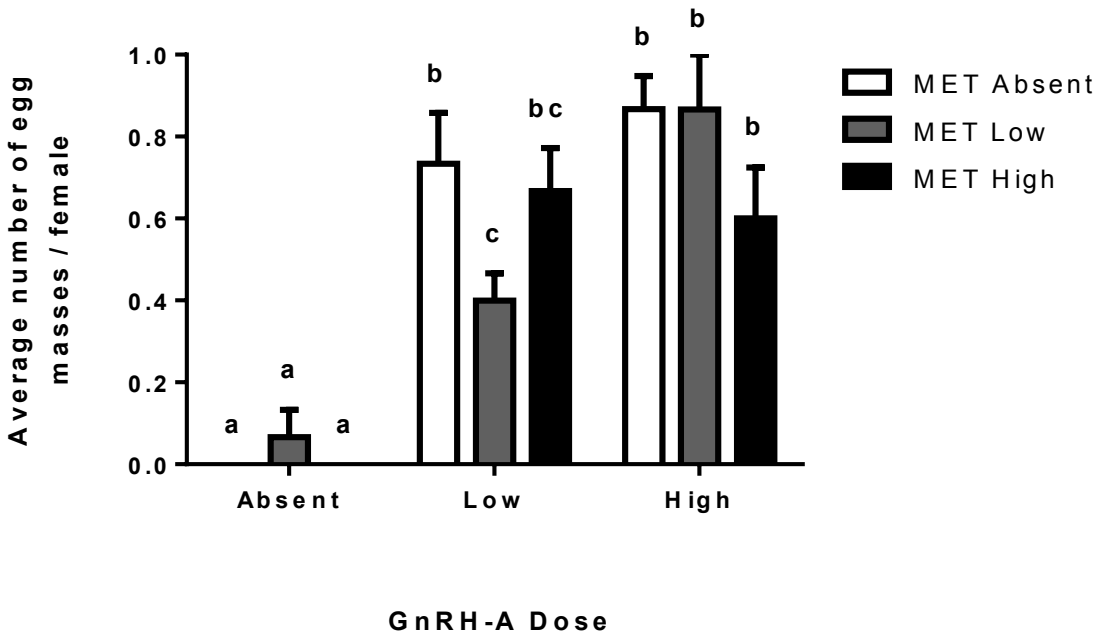


Figure 3.8 The average number of egg masses released per female following injections in *Lithobates pipiens* in May 2016. Means +SEM are presented (n = 5). A GnRH agonist (G) at two doses [High (H) = 0.4 µg/g, Low (L) = 0.1 µg/g)] was administered alone and in combination with two doses of the dopamine antagonist metoclopramide (M) [High (H) = 10 µg/g, Low (L) = 5 µg/g]. Statistically significant differences ($p < 0.05$) are marked by unique letters.

Egg mass weight, total number of eggs per egg mass and fertilization from induced spawning in May 2016 are presented in Table 3.5. There was a statistically significant effect of GnRH-A on egg mass weight [$F(2) = 34.175$, $p < 0.05$], total number of eggs [$F(2) = 41.588$, $p < 0.05$] and fertilization [$F(2) = 46.972$, $p < 0.05$]. However, there were no effects from MET on these measurements: [$F(2) = 1.060$, $p > 0.05$], [$F(2) = 3.072$, $p > 0.05$] and [$F(2) = 0.260$, $p > 0.05$], respectively. No significant interactions between GnRH-A and MET were detected on egg mass weight [$F(4) = 1.212$, $p > 0.05$] and fertilization [$F(4) = 266$, $p > 0.05$]. However, there was a statistically significant interaction between these two drugs on the total number of eggs per mass [$F(4) = 3.085$, $p < 0.05$].

Treatment	Egg Mass Weight (g)	Total Number of Eggs/ Mass	Fertilization (%)	Total Number of Eggs
Control (0.7 % NaCl)	0.00^{a}	0.00^{a}	0.00^{a}	0.00
GnRH-A Low (0.1 µg/g)	158.83 ± 31.52^{b}	2894.47 ± 578.02^{cd}	89.55 ± 2.34^{b}	29560.85
GnRH-A High (0.4 µg/g)	166.46 ± 15.60^{b}	3170.23 ± 146.82^{d}	90.28 ± 3.54^{b}	40929.00
MET Low (5 µg/g)	27.44 ± 27.44^{a}	585.72 ± 585.72^{ab}	0.00 ± 0.00^{a}	2928.59
MET High (10 µg/g)	0.00^{a}	0.00^{a}	0.00^{a}	0.00
GnRH-A Low + MET Low	106.23 ± 26.24^{b}	2060.97 ± 441.93^{bc}	70.57 ± 16.00^{b}	12938.13
GnRH-A Low + MET High	157.35 ± 23.78^{b}	3277.09 ± 167.06^{d}	80.00 ± 8.45^{b}	32319.33
GnRH-A High + MET Low	126.72 ± 8.61^{b}	2627.85 ± 117.50^{c}	92.43 ± 2.05^{b}	33345.86
GnRH-A High + MET High	171.71 ± 29.37^{b}	3036.88 ± 202.23^{cd}	87.19 ± 5.62^{b}	26377.29

Table 3.5 Egg mass weight, egg numbers and fertilization rates three days post-fertilization in *Lithobates pipiens*. Adults were injected with a GnRH agonist (GnRH-A) alone and in combination with two doses of the dopamine antagonist metoclopramide (MET). Mean values (n = 5) are presented ± standard error. Significant differences ($p < 0.05$) are indicated by unique letters. The total number of eggs produced in each treatment is reported.

No egg masses were produced in control groups in this spawning experiment, which was the expectation based on previous research on this species (Trudeau et al., 2013; Trudeau et al., 2010). One egg mass was released at the low dose of metoclopramide (5 μg/g), however a quick screening revealed that the quality of this egg mass was poor. All eggs in this clutch were dead or unfertilized and exhibited a disorganized texture. The greatest number of egg masses was produced in animals injected with the high dose of GnRH-A alone and in combination with the low dose of MET (13/15) (Supplementary Table 3.2). Complete ovulation occurred in females that did spawn as indicated by their consistent weight lost (%) in Table 3.6.

Treatment	**Average Female Weight Lost (%) (Min.-Max.)**
Control (0.7 % NaCl)	-
GnRH-A Low (0.1 μg/g)	31.04 (23.36 – 37.98)
GnRH-A High (0.4 μg/g)	29.87 (21.69 – 36.05)
MET Low (5 μg/g)	35.66
MET High (10 μg/g)	-
GnRH-A Low + MET Low	32.81 (31.54 – 34.23)
GnRH-A Low + MET High	28.65 (17.58 – 49.97)
GnRH-A High + MET Low	29.73 (16.54 – 39.27)
GnRH-A High + MET High	23.83 (14.40 – 33.63)

Table 3.6 Average post-spawn female weight loss (%) with minimum and maximum range on day 10 in May 2016. Mean values are presented for only females that released egg masses in each treatment.

3.3. Effects of GnRH-A and MET injections on pituitary gene expression

3.3.1. Materials and methods

3.3.1.1. Animals and husbandry

Sexually mature female Northern leopard frogs (*Lithobates pipiens*) were obtained from Anilab Enr (Québec, QC) in the fall and were housed at the University of Ottawa Aquatic Care Facility under a natural photoperiod (10L: 14D). Animals were shipped overnight in cardboard boxes containing holes, damp paper towel and crumpled paper to buffer against movements during transportation. Frogs were placed in sixteen 70 L tanks previously cleaned with hot water and disinfected with ACCEL Prevention Concentrate, and covered with a secure and ventilated net screen as a lid. To minimize stress, garbage bags were placed to cover half of the lids. Frogs were held in an open system where a PVC standpipe was at the center of each tank to allow excess waste and water to be continuously drained. Each tank housed six animals for a total of ninety six frogs. An artificial habitat was designed in each tank consisting of a styrofoam board as a platform to which a petri dish was attached for feeding. Frogs were fed two superworms (*Zoophobas morio*) (RECORP INC.) each, three times a week. Superworms were gut loaded with oatmeal, corn, apple and potato slices, and dusted with calcium and vitamin D_3 powder (Repti Calcium, Zoomed, CAT #A34-8) prior to feeding them to leopard frogs. Moss or any substantial substrates were eliminated to reduce the buildup of unwanted parasites. A continuous stream of 13.1-13.3 °C dechlorinated water was supplied to the tank and air temperature ranged from 14.8-17.5 °C. These conditions were sufficient for the chosen acclimation period of one to two weeks. Frogs were surveyed and monitored closely every day where their health was evaluated along with the condition of their enclosure. Daily spot cleaning of the tank and its furnishings consisted of the removal of unconsumed worms and visible feces. Dead or animals

exhibiting signs of disease (red leg, septicemia, tetany, paralysis, slouched posture, erythema) were removed and sacrificed immediately, followed by disinfection of the enclosure to prevent the spread of potential diseases and accumulation of nitrogenous waste. Biosafety practices were employed in all procedures to avoid the spread of pathogens. All animal care procedures were compliant with protocols in the University of Ottawa Animal Care and Veterinary Service.

3.3.1.2. Injections and sampling

As previously described, chemicals used in this experiment were the GnRH agonist (Des-Gly^{10}, D-Ala^{6}, Pro-NHEt^{9})-LHRH acetate salt and the selective dopamine receptor D2 antagonist metoclopramide hydrochloride. Hormones were weighed using a microbalance and diluted in 0.7 % NaCl on the day of injection. Four treatment groups were selected for this study based on the original doses reported in the AMPHIPLEX method (Trudeau et al., 2013; Trudeau et al., 2010): Control (0.7 % NaCl), GnRH (0.4 μg/g), MET (10 μg/g) and the AMPHIPLEX mixture (0.4 μg/g GnRH and 10 μg/g MET). Following acclimation, frogs were injected I.P. using a 31 gauge, 8 mm needle attached to a 0.3 CC insulin syringe (2 μL/g BWt). A unique toe clip was taken for each individual for identification, a protocol adapted from the USGS National Wildlife Health Center. Following toe clipping, any remaining blood was removed with a cotton swab and the wound was sprayed with the antiseptic Bactine®. This formula is the only safe over-the-counter product to use on amphibians for it does not contain oil or alcohol that may remove essential mucus or be toxic to the animal (Martin and Hong, 1991). The air temperature of each tank was recorded using a digital thermometer probe (ZooMed, CAT #TH-24).

Animals were euthanized in a water bath containing 2 g/ L of MS-222 buffered with 2 g/ L sodium bicarbonate. Pituitaries were collected at three time points: 24 hours (n = 7) in 2015 and 12 hours (n = 6) and 36 hours (n = 6) in 2016. Two pituitaries were pooled into one RNase-

free safelock tube (Eppendorf, CAT #022600044) and preserved on dry ice and for long term storage in -80 °C until molecular analysis. Dissection tools were rinsed with 0.3 % hydrogen peroxide, followed by two washes of 0.1 % diethylpyrocarbonate (DEPC) water to inhibit RNAses. Sampling methods were approved by the University of Ottawa Animal Care and Veterinary Service.

3.3.1.3. Total RNA isolation and cDNA synthesis

Total ribonucleic acid (RNA) from adult pituitaries was extracted using the RNeasy Microkit (Qiagen, CAT #74004). The extracted RNA was re-suspended in 14 µL of RNase-free water and concentrations of total RNA, the ratio of absorbance at 260 nm and 280 nm and the ratio of absorbance at 260 and 230 nm were measured using a spectrophotometer (NanoDrop 2000, Thermo Scientific). Samples were stored at -80 °C. The integrity of RNA samples was verified by a 1 % (w/v) agarose electrophoresis gel. Two distinct bands representing 18S and 28S were present and RNA quality was confirmed in these samples. Total complementary DNA (cDNA) of pituitary samples was synthesized from 1 µg of total RNA using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific, CAT #K1642). The thermal cycling parameters were as follows: an initial cycle at 25 °C for 10 minutes, followed by 50 °C for 30 minutes and a final cycle at 85 °C for 5 minutes. Each 20 uL cDNA reaction was stored at -20 °C. All cDNA for each experiment was synthesized at the same time. Protocols for RNA extraction and cDNA synthesis followed the manufacturer's guidelines.

3.3.1.4. Primer design and gene expression analysis by quantitative real-time PCR

To examine changes in messenger RNA (mRNA) levels in response to these treatments, quantitative real-time PCR (qPCR) was performed. Specific primers for the following genes: luteinizing hormone beta subunit (*LHβ*), follicle-stimulating hormone beta subunit (*FSHβ*),

glycoprotein alpha subunit (*α-GSU)*, dopamine receptor D2 (*DRD2*), gonadotropin-releasing hormone receptor 1 (*GnRHR1*) and the reference gene ribosomal protein l8 (*rpl8*), were designed according to specific conditions using the program PRIMER 3. These genes were selected because of their central importance for ovulation and sperm release, and the regulation of these receptors may provide possible mechanisms of action for these treatment effects. Sequences for *LHβ* (GenBank: DQ054791.1), *FSHβ* (GenBank: DQ054790.1) and *rpl8* (GenBank: EF370416.1) in *Lithobates pipiens* were obtained from the National Center for Biotechnology Information (NCBI) nucleotide database. Primers for *α-GSU* were designed based on conserved areas in the known sequence reported for *Rana japonica* (GenBank: AB178055.1). The CLUSTAL multiple sequence alignment tool was used to design primers for *GnRHR1*and *DRD2* by comparing conserved regions in sequences from closely-related species. Sequences for *Rana catesbeiana* (GenBank: AB440160.1), *Notophthalmus viridescens* (GenBank: HQ660498.1) and *Xenopus tropicalis* (GenBank: XM_002937825.2) were compared for *DRD2*, and sequences for *Rana ridibunda* (GenBank: AY260153.1), *Rana esculenta* (GenBank: JF522233.1), *Rana catesbeiana* (GenBank: AF144063.1) and *Rana dybowskii* (GenBank: AF236879.2) were aligned for *GnRHR1*. Conserved areas used for these primers did not span the 7-transmembrane G protein-coupled receptor domain to ensure primer specificity. Sequence and primer specificity were verified with the Basic Local Alignment Search Tool (BLAST). Primers were supplied by Integrated DNA Technologies. Optimal primer annealing temperatures for qPCR was determined by a thermal gradient (55-65 °C) using cDNA products diluted 1:10. Primer sequences, amplicon location, size and annealing temperatures for each gene are presented in Table 3.7. Thermal gradient qPCR products were run on a 2 % (w/v) agarose electrophoresis gel for 45 minutes. Bands were stained with SYBR Safe DNA Gel Stain (Thermo Fisher Scientific, CAT #S33102)

where single products represented a specific primer pair. Bands were excised under UV light and DNA was extracted and purified using the NucleoSpin Gel and PCR Clean-up kit (Machery-Nagel CAT #740609.50). The identity of the amplicon was confirmed by sequencing purified PCR products at StemCore Laboratories, Ottawa Hospital Research Institute. These sequence analyses were used to confirm the specificity of these products. Primer specificity was also verified by a single distinct melting peak in qPCR analysis.

Quantitative real-time PCR was performed using Maxima SYBR Green qPCR Master Mix (Thermo Scientific, CAT #K0252) following the manufacturer's protocol. Each sample was run in duplicates on a 96-well plate, each plate representing one gene. No template and reverse transcriptase controls were included for each plate to confirm the absence of genomic contamination and the specificity of target cDNA amplification. Each 25 uL reaction consisted of 5 uL of cDNA template and 20 uL of Master Mix containing 100 nM specific primers, 1X SYBR Green qPCR Master Mix and nuclease-free water. Thermal cycling parameters using the Bio-Rad CFX96 qPCR system were: an initial cycle of Taq activation at 95 °C for 3 minutes, followed by 40 amplification cycles of denaturation at 95 °C for 10 seconds and primer annealing for 30 seconds at 61 °C or 63 °C . Denaturing occurred at 95 °C for 10 seconds and a melt curve was generated at 65 °C to 95 °C for 30 seconds, increasing 0.5 °C every 5 seconds. Standard curves for each gene were generated from serial dilutions of pooled cDNA from all samples (2-fold dilutions for *DRD2* and *GnRHR1*, and 5-fold dilutions for *LHβ*, *FSHβ*, *α-GSU* and *rpl8*). The efficiency for all standard curves were within 90-110 % with an r^2 value of 0.980 or greater (Taylor et al., 2010).

3.3.1.5. Statistical analysis

Relative mRNA levels were calculated using the relative standard curve method based on Cq values and data were normalized using the algorithm NORMA-Gene (Heckmann et al., 2011). Fold-change of mRNA levels relative to control was calculated for each sample. Mean fold change in mRNA levels was the average of biological replicates. Normality and homogeneity of variance were verified for each data set using Shapiro-Wilk and Levene's tests, respectively ($p > 0.05$). A one-way ANOVA followed by Tukey's HSD post-hoc test for pairwise comparisons were performed. Gene expression data that did not meet parametric assumptions were analyzed using a non-parametric Kruskall-Wallis test, also known as a one-way ANOVA on ranks. To determine significant changes between specific pairwise treatments, Dunn's post-hoc test was performed ($p < 0.05$). Data were expressed as median values presented in Tukey's box and whisker plots, where boxes extend from the 25^{th} to 75^{th} percentiles, whiskers show the minimum and maximum values, and single points represent outliers. Statistical significance was denoted by a p-value < 0.05. Statistical analyses and graphs were generated using the statistical software SigmaPlot (version 11) and GraphPad Prism (version 6).

3.3.2. Results

Gene	Element	Primer Sequence (5'-3')	Amplicon location and size (bp)	Annealing (°C)
LHβ	Forward	TACCGTGGAGAGCAGTGAA	405-547 (143)	61
	Reverse	TTAGCAGATGAAAGCAAAGG		
FSHβ	Forward	TACGGGCTGGTTCTGTTTTG	49-232 (184)	63
	Reverse	CACCTTGCTTCTCCGATTTCT		
α-GSU	Forward	CTGAAGCGAAGTGCTGTGTG	351-585 (235)	61
	Reverse	GGTAAAGGGAAAAGTGTGCTTC		
DRD2	Forward	TGCTCCTTACCCTCCTTATCTT	224-415 (192)	63
	Reverse	TAAACCTCCACTCGCCAACC		
GnRHR1	Forward	ACAGTCCACCACACACATCC	1011-1148	61
	Reverse	TCGGCGTCTGATGTCCTTCT	(138)	
rpl8	Forward	GTGTAGAAGAGAAGCCAGGTGAT		
	Reverse	GGATTGTGGGAGATGACGGTAG	74-152 (79)	61

Table 3.7 Quantitative real-time PCR primer sets and reaction conditions for *Lithobates pipiens*.

3.3.2.1. The effects of intraperitoneal injections on pituitary *LHβ, FSHβ, α-GSU, DRD2* and *GnRHR1* expression in *Lithobates pipiens* after 12 hours

At 12 hours post-injection, there was a significant treatment effect on the mRNA levels of the gonadotropin subunits *LHβ* [$F(3) = 11.998$, $p < 0.05$)], *FSHβ* [$F(3) = 6.447$, $p < 0.05$)] and *α-GSU* [$F(3) = 7.183$, $p < 0.05$)]. Specifically, females injected with the dopamine receptor D2 antagonist metoclopramide had statistically significantly decreased mRNA levels of *LHβ* ($p < 0.05$), *FSHβ* ($p < 0.05$) and *α-GSU* ($p < 0.05$) compared to control. These respectively corresponded to 45.1 %, 57.3 % and 40.6 % decreases in mRNA levels compared to control (Figure 3.9a-c). Furthermore, there was a statistically significant decrease in the mRNA level of *FSHβ* by 43.3 % in females injected with the mixture GnRH-A and MET ($p < 0.05$). There was a significant treatment effect on the mRNA level of the receptor *DRD2* [$F(3) = 7.139$, $p < 0.05$)]

where a decrease with treatment was observed, however a statistically significant decrease compared to control was detected in the group injected with both GnRH-A and MET ($p < 0.05$). Here, mRNA levels decreased by 36.7 % (Figure 3.9d). The results at 12 hours post-injection indicated a clear treatment effect on the mRNA levels of the receptor *GnRHR1* [$F(3) = 40.522$, $p < 0.05$)]. A statistically significant increase in the mRNA levels of *GnRHR1* compared to control was detected in females injected with GnRH-A ($p < 0.05$) and when GnRH-A was delivered in combination with MET ($p < 0.05$). These corresponded to 170.5 % and 144.4 % increases, respectively. In addition, the mRNA levels of *GnRHR1* was statistically significantly decreased in the MET treatment ($p < 0.05$) by 44.4 % compared to control (Figure 3.9e).

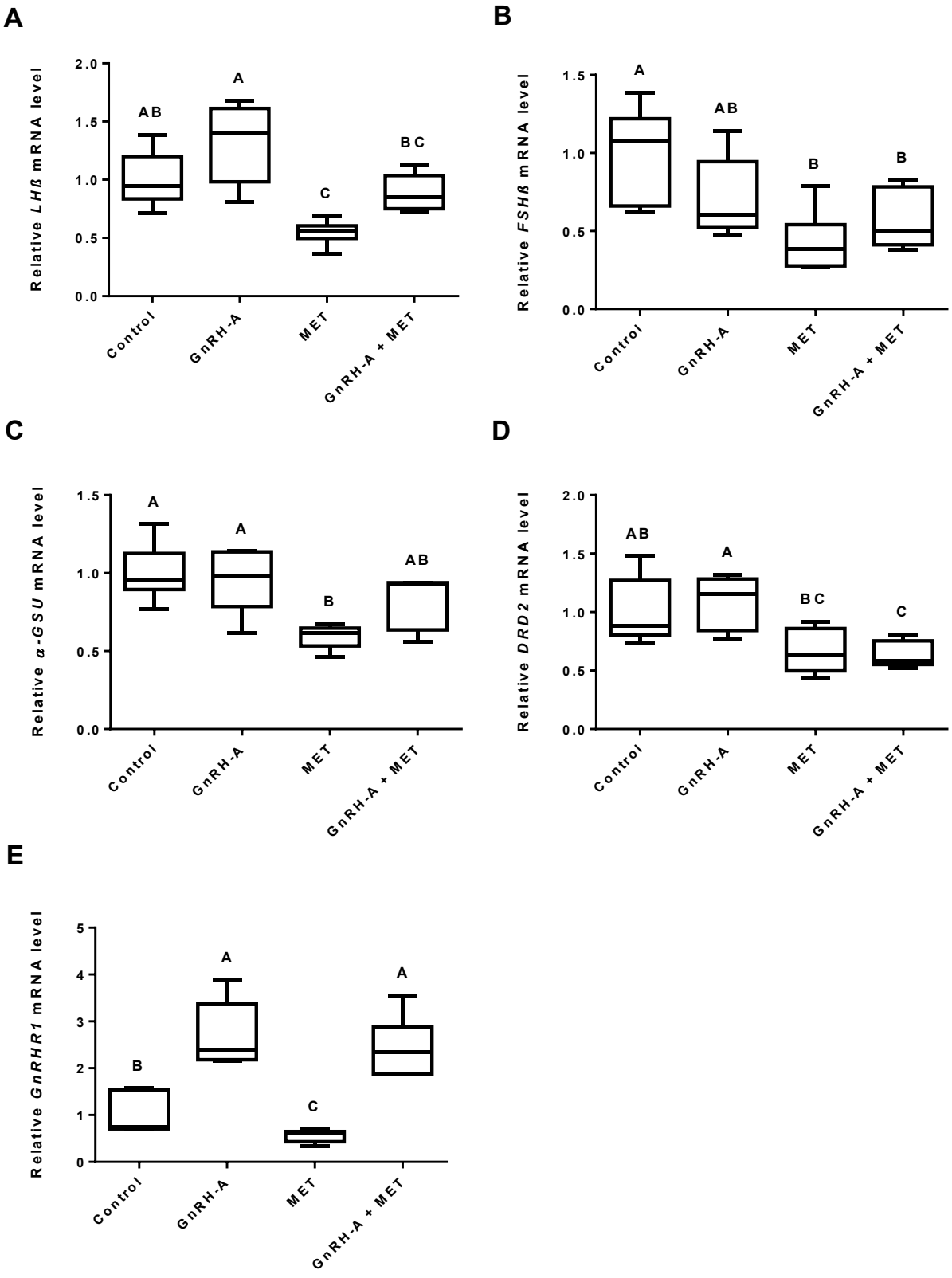

A
Relative LHß mRNA level
2.0
1.5
1.0
0.5
0.0
AB
A
C
BC
Control
GnRH-A
MET
GnRH-A + MET
B
Relative FSHß mRNA level
1.5
1.0
0.5
0.0
A
AB
B
B
Control
GnRH-A
MET
GnRH-A + MET
C
Relative α-GSU mRNA level
1.5
1.0
0.5
0.0
A
A
B
AB
Control
GnRH-A
MET
GnRH-A + MET
D
Relative DRD2 mRNA level
2.0
1.5
1.0
0.5
0.0
AB
A
BC
C
Control
GnRH-A
MET
GnRH-A + MET
E
Relative GnRHR1 mRNA level
5
4
3
2
1
0
B
A
C
A
Control
GnRH-A
MET
GnRH-A + MET

Figure 3.9 Quantitative real-time PCR analyses illustrating the effects of GnRH-A (0.4 µg/g) and MET (10 µg/g) delivered separately and in combination on the mRNA levels of pituitary reproductive genes in *Lithobates pipiens*. Females were injected intraperitoneally and the mRNA levels of *LHβ* (A), *FSHβ* (B), *α-GSU* (C), *DRD2* (D) and *GnRHR1* (E) were measured after 12 hours. Data are presented as fold change relative to control. Median values (n = 6) are presented in box and whisker plots, where boxes extend from the 25th to 75th percentiles and whiskers show the minimum and maximum values. Different letters indicate statistically significant differences ($p < 0.05$) between groups.

3.3.2.2. The effects of intraperitoneal injections on pituitary *LHβ, FSHβ, α-GSU, DRD2* and *GnRHR1* expression in *Lithobates pipiens* after 24 hours

There was no statistically significant change in the mRNA levels of the gonadotropin subunits *LHβ* [$F(3) = 0.799$, $p > 0.05$)] and *FSHβ* [$F(3) = 1.794$, $p > 0.05$)] after 24 hours (Figure 3.10ab). However, a statistically significant difference was detected in the mRNA levels of *α-GSU* [$F(3) = 6.892$, $p < 0.05$)]. Here, post-hoc analysis revealed statistically significant differences in *α-GSU* mRNA levels compared to GnRH-A in females injected with MET ($p < 0.05$) and GnRH-A + MET ($p < 0.05$) (Figure 3.10c). Interestingly, there was a significant decrease in the mRNA levels of the receptor *DRD2* [$F(3) = 5.402$, $p < 0.05$)] as observed after 12 hours. The results illustrated a statistically significant decrease in *DRD2* mRNA levels by 56.9 % in females injected with MET ($p < 0.05$) and by 63.5 % in females injected with both GnRH-A and MET ($p < 0.05$) relative to control (Figure 3.10d). No statistically significant change in *GnRHR1* mRNA levels was detected at this time point [$H(3) = 8.417$, $p > 0.05$)] (Figure 3.10e).

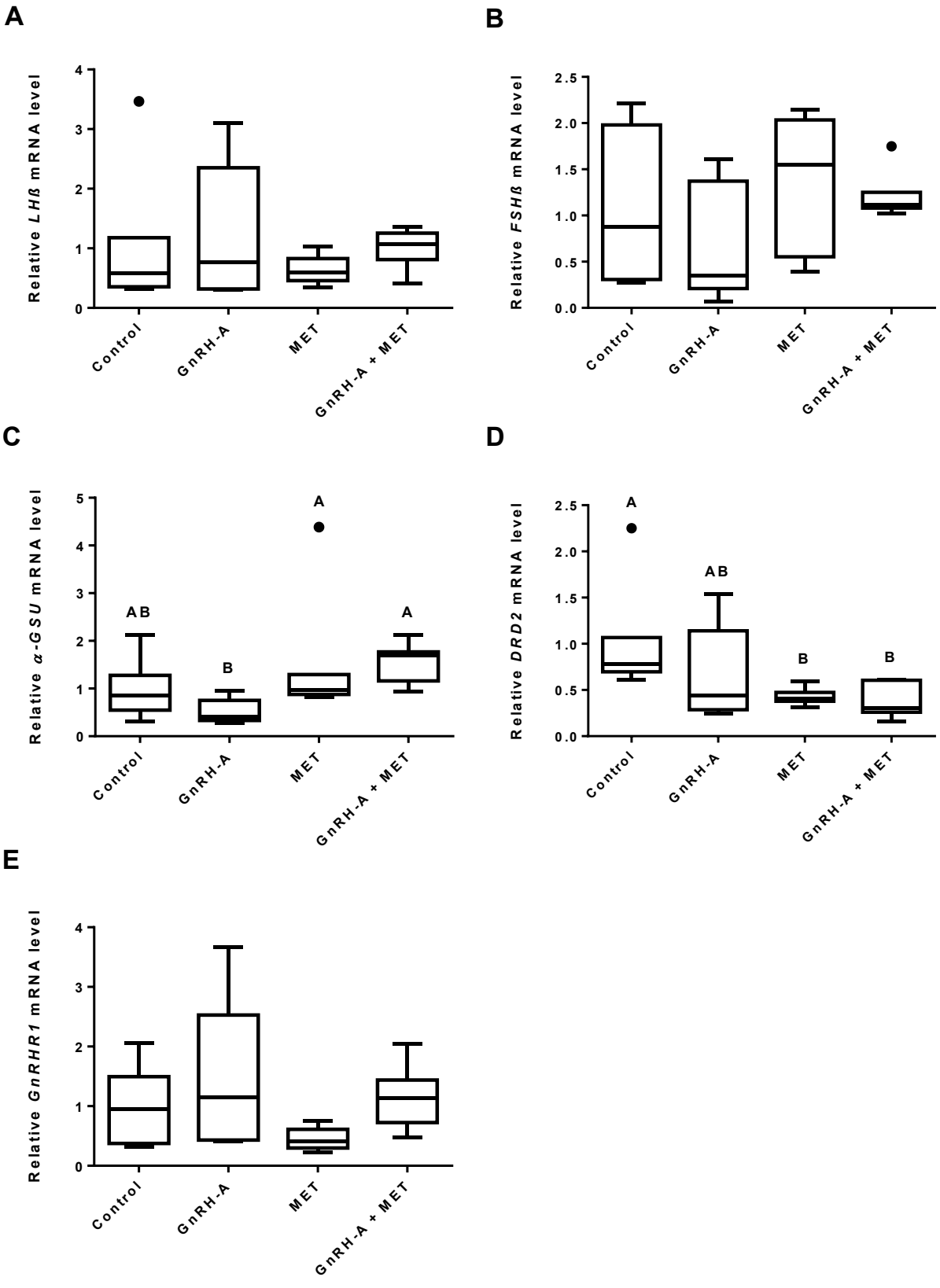

A
Relative LHß mRNA level
4
3
2
1
0
Control
GnRH-A
MET
GnRH-A + MET
B
Relative FSHß mRNA level
2.5
2.0
1.5
1.0
0.5
0.0
Control
GnRH-A
MET
GnRH-A + MET
C
Relative α-GSU mRNA level
5
4
3
2
1
0
AB
B
A
A
Control
GnRH-A
MET
GnRH-A + MET
D
Relative DRD2 mRNA level
2.5
2.0
1.5
1.0
0.5
0.0
A
AB
B
B
Control
GnRH-A
MET
GnRH-A + MET
E
Relative GnRHR1 mRNA level
4
3
2
1
0
Control
GnRH-A
MET
GnRH-A + MET

Figure 3.10 Quantitative real-time PCR analyses illustrating the effects of GnRH-A (0.4 μg/g) and MET (10 μg/g) delivered separately and in combination on the mRNA levels of pituitary reproductive genes in *Lithobates pipiens*. Females were injected intraperitoneally and the mRNA levels of *LHβ* (A), *FSHβ* (B), *α-GSU* (C), *DRD2* (D) and *GnRHR1* (E) were measured after 24 hours. Data are presented as fold change relative to control. Median values (n = 7) are presented in box and whisker plots, where boxes extend from the 25th to 75th percentiles, whiskers show the minimum and maximum values, and single points represent outliers. Different letters indicate statistically significant differences ($p < 0.05$) between groups.

3.3.2.3. The effects of intraperitoneal injections on pituitary *LHβ, FSHβ, α-GSU, DRD2* and *GnRHR1* expression in *Lithobates pipiens* after 36 hours

Clear changes in the mRNA levels of gonadotropin subunits and pituitary receptors in response to these treatments were observed in females at 36 hours following injection. Statistically significant treatment effects were detected on the mRNA levels of the gonadotropin subunits *LHβ* [$F(3) = 33.699$, $p < 0.05$)], *FSHβ* [$F(3) = 14.916$, $p < 0.05$)] and *α-GSU* [$F(3) = 67.963$, $p < 0.05$)]. The mRNA levels of *LHβ* were statistically significantly greater relative to control in females injected with GnRH-A ($p < 0.05$) and when GnRH-A was delivered in combination with MET ($p < 0.05$). These corresponded to 139.7 % and 264.8 % increases, respectively (Figure 3.11a). The mRNA levels of *FSHβ* were statistically increased relative to control in females injected with both GnRH-A and MET ($p < 0.05$) by 234.6 % (Figure 3.11b). Tukey's post-hoc analysis revealed statistically significant differences across all pairwise comparisons for *α-GSU*. Most notably, the mRNA levels of *α-GSU* were statistically increased relative to control in females injected with GnRH-A ($p < 0.05$) and when GnRH-A was combined with MET ($p < 0.05$). These corresponded to 257.4 % and 430.8 % increases, respectively (Figure 3.11c). These results suggest that at 36 hours post-injection, there was a clear potentiating action on gonadotropin gene expression when MET was delivered in combination with GnRH-A.

Consistent with previous time points (12 and 24 hours), there was a significant decrease in the mRNA levels of *DRD2* with treatment [$F(3) = 7.017$, $p < 0.05$)]. However, a statistically significant decrease in this receptor relative to control by 59.0 % was only detected in females injected with MET ($p < 0.05$) (Figure 3.11d). There was a significant treatment effect on the mRNA levels of the receptor *GnRHR1* [$F(3) = 57.500$, $p < 0.05$)]. As seen at 12 hours post-injection, statistically significant increases in mRNA levels of *GnRHR1* relative to control was demonstrated in females injected with GnRH-A alone ($p < 0.05$) and when delivered with MET ($p < 0.05$). These corresponded to 210.5 % and 364.6 % increases, respectively (Figure 3.11e).

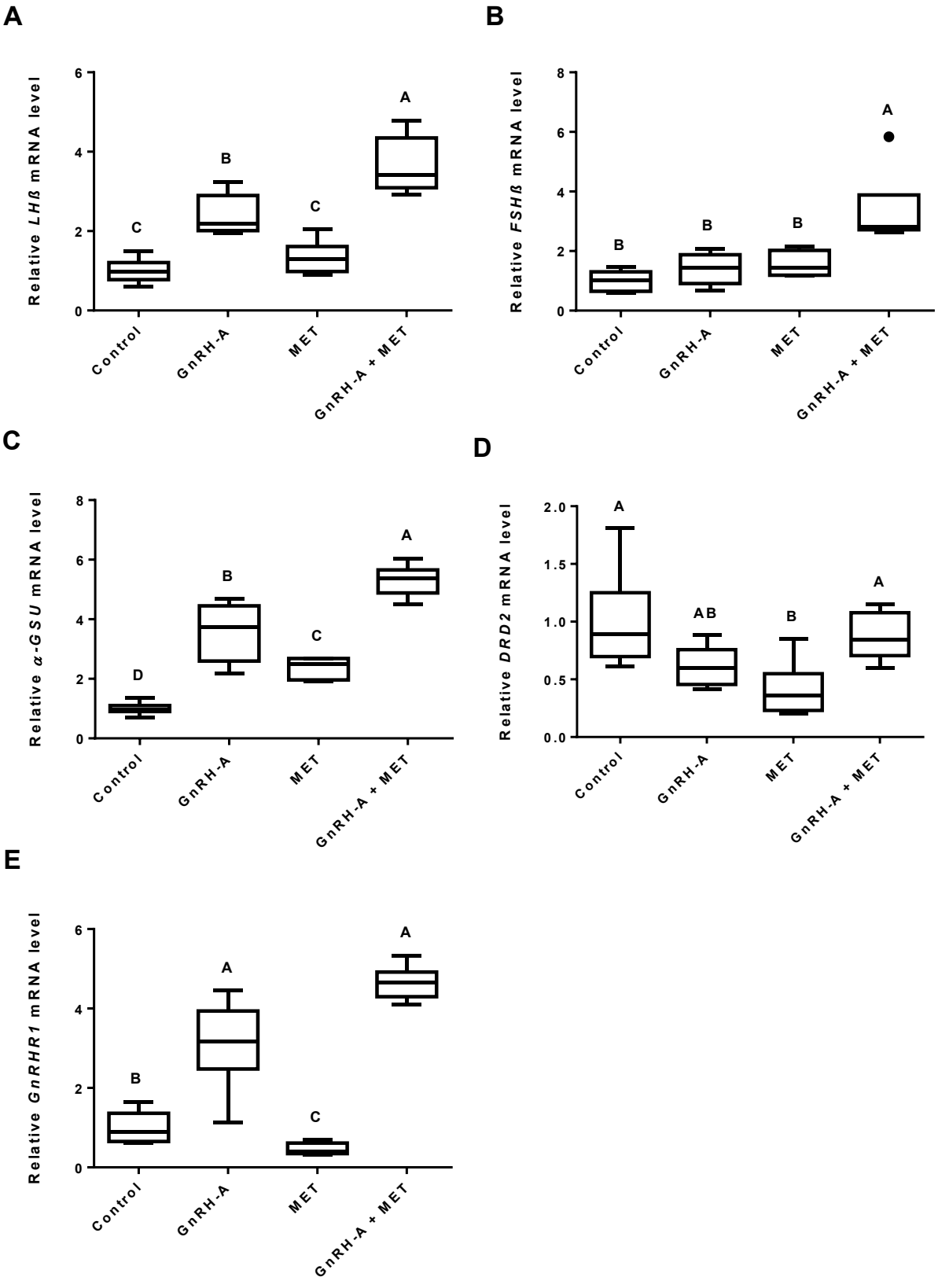

A
Relative LHß mRNA level
6
4
2
0
C
B
C
A
Control
GnRH-A
MET
GnRH-A + MET
B
Relative FSHß mRNA level
8
6
4
2
0
B
B
B
A
Control
GnRH-A
MET
GnRH-A + MET
C
Relative α-GSU mRNA level
8
6
4
2
0
D
B
C
A
Control
GnRH-A
MET
GnRH-A + MET
D
Relative DRD2 mRNA level
2.0
1.5
1.0
0.5
0.0
A
AB
B
A
Control
GnRH-A
MET
GnRH-A + MET
E
Relative GnRHR1 mRNA level
6
4
2
0
B
A
C
A
Control
GnRH-A
MET
GnRH-A + MET

Figure 3.11 Quantitative real-time PCR analyses illustrating the effects of GnRH-A (0.4 μg/g) and MET (10 μg/g) delivered separately and in combination on the mRNA levels of pituitary reproductive genes in *Lithobates pipiens*. Females were injected intraperitoneally and the mRNA levels of *LHβ* (A), *FSHβ* (B), *α-GSU* (C), *DRD2* (D) and *GnRHR1* (E) were measured after 36 hours. Data are presented as fold change relative to control. Median values (n = 6) are presented in box and whisker plots, where boxes extend from the 25th to 75th percentiles, whiskers show the minimum and maximum values, and single points represent outliers. Different letters indicate statistically significant differences ($p < 0.05$) between groups.

3.4. Discussion

3.4.1. Effects of GnRH-A and MET injections on spawning

The results from both spawning studies conducted in the Northern leopard frog revealed that the AMPHIPLEX method for spawning induction is primarily driven by the effects of GnRH-A. The data from these studies indicate that at these doses of GnRH-A (0.4 μg/g, 0.2 μg/g and 0.1 μg/g), the hypothesized potentiating effect from the dopamine receptor D2 antagonist MET was not observed. Animals injected with MET alone in these studies did not enter amplexus or release fertilized egg masses. In contrast, GnRH-A administered alone and in combination with MET were equally effective at stimulating spawning in this species. These results demonstrate that GnRH-A can stimulate spawning alone. These findings revealed no significant interaction between GnRH-A and MET with respect to the average incidences of amplexus per day in both years, and the average number of egg masses released per female in 2015. A significant interaction was present between GnRH-A and MET on egg mass release and the number of eggs per mass in 2016. This can attributed to the lower dose of GnRH-A that was selected in the second year. It is now evident that the Northern leopard frog will respond effectively to a GnRH-A dose as low as 0.1 μg/ g. This is consistent with the dose used in goldfish when the inhibitory action of DA on gonadotropin release was initially unraveled (Chang and Peter, 1983; Peter, 1980; Peter et al., 1987; Sokolowska et al., 1985). This dose is four times lower than the one originally used in the spawning induction studies for the Northern

leopard frog (Trudeau et al., 2013; Trudeau et al., 2010). The current spawning data at hand do not strongly support the hypothesis that DA is exerting an inhibitory role in amphibian reproduction.

The results demonstrate that the Northern leopard frog is a responsive species that will generate thousands of fertilized eggs in three to five days from a single intraperitoneal injection. There are indications nevertheless that DA does have a role in reproduction, for instance, if the biological significance is compared across treatments in the first spawning study. With respect to total number of egg masses released, there is a clear 27 % increase in spawning success between GnRH-A High (11/15) and GnRH-A High + MET High (15/15) (Supplementary Table 3.1). Such an increase would represent approximately 12,000 more fertilized eggs produced in the mixture compared to GnRH-A alone. This increase in success rate along with the spawning timeline provided in this study is significant from a conservation and management point of view. The results from this study have applications towards developing and improving spawning induction methods in other ranids with respect to dose and timing.

The environmental conditions were notably different between the two spawning studies, particularly with respect to temperature. In 2015, an unexpected increase in air and water temperature resulted in a few animals spawning in an uncontrolled manner. This was most evident in the control groups that released four unfertilized egg masses. Despite this, applying what is known about the positive effects of temperature on spawning to conservation management is unlikely to be effective. Animals may release their eggs pre-maturely before amplexus, sperm release and fertilization, and it would be costly to establish a heating system for this purpose.

Further studies are required to elucidate the inhibitory role of DA in amphibian reproduction with regards to spawning output. It appears that the doses of GnRH-A used were maximal, therefore overriding a potential dopaminergic inhibitory control over spawning. Given these data, future dose-response studies should test lower doses of GnRH-A both separately and in combination with several doses of MET. A long term study should focus on the development, metamorphic rate and survival of tadpoles in these treatment groups, building onto previous studies which have begun to explore these effects (Trudeau et al., 2013). Different GnRH and DA agonists and antagonists should be tested in the same context. The reproductive output measured in these studies were primarily female-driven, therefore future studies should be strictly directed towards males. Recent studies in the Panamanian golden toad (*Atelopus zeteki*) are further unravelling the inhibitory role of DA on spermic urine release where the highest sperm concentration in this species was obtained using the AMPHIPLEX method (Della Togna et al., 2017). These emerging studies provide an avenue for future research to analyze parameters such as sperm motility and morphology in spawn-induced males.

3.4.2. Injection of GnRH-A and MET alters the expression of reproductive genes in the pituitary

In contrast to the spawning studies, the results from gene expression analysis support the hypothesis that DA is exerting an inhibitory role in amphibian reproduction. A time-course analysis was conducted on reproductive genes in the pituitary in response to GnRH-A and MET at doses used in the original AMPHIPLEX method. Gonadotropin subunits were not significantly altered in response to GnRH-A and GnRH-A + MET after 12 hours, however these genes were significantly decreased in females injected with MET compared to control. At 24 hours post-injection, no significant changes in gonadotropin subunits were observed in response to all

treatments. The results were most clear at 36 hours post-injection where a predicted potentiating effect of MET on GnRH-A was observed with respect to *LHβ*, *FSHβ* and *α-GSU*. The mRNA levels of the gonadotropin subunits *LHβ* and *α-GSU* were significantly increased compared to control following injection with GnRH-A by 139.8 % and 257.3 %, respectively. Interestingly, the mRNA levels of these genes were further increased in the GnRH-A plus MET group. This was represented by 264.8 % and 430.8 % increases in *LHβ* and *α-GSU* mRNA levels relative to control, respectively. Furthermore, the effects of these treatments on *FSHβ* only reached statistical significance when GnRH-A and MET were injected together. The timeline of this study was selected based on the sequence of events observed in the spawning experiments (Figure 3.3 and 3.6). The time course of spawning and gene expression follow the timing of peak sperm and egg release in the Southern Corroboree frog at 36 hours following injection of GnRH-A (Byrne and Silla, 2010).

The results from this study also provide insight into the possible mechanisms of action for these stimulatory effects of GnRH-A and MET. This was indicated by changes in the mRNA levels of gonadotropin-releasing hormone receptor 1 and dopamine receptor D2. At 12 and 36 hours post-injection, GnRH receptor 1 notably increased in animals injected with GnRH-A and the mixture GnRH-A + MET. This upregulation in GnRH receptors is a response that has been previously documented in cultured pituitaries of mice (Cheon et al., 1999) and carp (Lin et al., 1994), and in ovariectomized ewes (Kirkpatrick et al., 1998) following treatment with GnRH. The observed effects support the principles of GnRH self-priming that have been applied to breed amphibians in captivity (Porter and Licht, 1985; Silla, 2011; Trudeau et al., 2013). In contrast, across all time points, the mRNA levels of dopamine receptor D2 decreased with GnRH-A and MET alone and in combination. The results suggest that metoclopramide is not

only blocking D2 receptors in the pituitary, but is exerting additional autoregulatory actions on this receptor. These results propose that the alterations in gonadotropin mRNA levels observed are controlled through the upregulation of GnRH receptor 1 primarily by GnRH-A and simultaneously, the downregulation of dopamine receptor D2 in the pituitary. Importantly, there is a possibility that these treatments may be simultaneously exerting effects on other cell types apart from gonadotrophs in the pituitary. For example, *DRD2* may also be expressed in lactotrophs and melanotrophs secreting prolactin and α-MSH, respectively. Cell-specific localization of *DRD2* and *GnRHR1* in the amphibian pituitary should be further explored through *in-situ* hybridization or immunocytochemical studies. These are the first studies to examine gene expression changes in pituitary gonadotropins following GnRH agonist and DA antagonist treatment in any amphibian species.

It is clear that this study in the Northern leopard frog supports the hypothesis of DA inhibition of gonadotropin gene expression, but was not well-supported in the spawning results. These differences may be related to the presence or absence of a male during the different experiments. It has been previously proposed that amplexus promotes the LH surge in amphibians (Ishii and Itoh, 1992). Therefore, although treatment with GnRH-A will stimulate gonadotropin cells in the pituitary, LH may also be under a positive influence from amplexus. This serves as a potential explanation for overriding the dopaminergic inhibitory control observed in the spawning studies. The absence of a male may have allowed subtle dopaminergic effects to be observed where MET potentiated GnRH-A on the expression of gonadotropins in the female pituitary.

There exist limitations to this study that future experiments should aim to address. One set back is the lack of information regarding the history of female leopard frogs prior to arriving

in the laboratory. Another area of investigation would be to explore seasonality i.e. animals with varying gonadotropin profiles across the annual cycle. Measurement of gonadotropins in the blood in response to these treatments would complement the current gene expression studies. However, this requires the development of antibodies, recombinant hormones and assays for amphibian pituitary hormones. Finally, the effects of these treatments on gonadotropin gene expression should also be examined in the male pituitary.

3.5. Conclusion

This study investigated the effects of GnRH-A and MET injections on spawning response and the expression of reproductive genes in the pituitary of the Northern leopard frog. Various dose- and time-response experiments were conducted using these two approaches. The current results of this study in the Northern leopard frog lend partial support to the hypothesis that dopamine has an inhibitory role in amphibian reproduction. A clear potentiating effect was observed on gonadotropin subunit mRNA levels with the addition of the dopamine antagonist MET to GnRH-A. Although the spawning data do not indicate any interaction between these drugs, there may be practical significance to the addition of MET to GnRH-A given the highest spawning success was obtained with this combination. This represents a pioneering study towards establishing future GnRH-A dose-response studies in this species to determine an effective dose as well as providing molecular insights into the regulation of reproductive genes. Specifically, the regulation of FSH is particularly novel in the field of amphibian reproductive endocrinology. The results from this study represent the first steps towards fine-tuning spawning induction methods for Northern leopard frogs and determining a role for dopamine in the control of amphibian reproduction.

CHAPTER 4

Time- and dose-related effects of GnRH agonist and DA antagonist injections on spawning and pituitary gene expression in the Western clawed frog (*Silurana tropicalis*)

4.1. Introduction

The establishment of captive colonies for conservation and research goals can be greatly hampered when an amphibian species is unresponsive to breeding attempts in captivity. This failure to respond may be attributed to factors such as missing environmental cues, selective mate choice or stress, among several others. In this situation, the administration of exogenous hormones to stimulate spawning is required. Hormone induction methods have been previously applied to native species as well as distantly-related ones. This conservation strategy is especially relevant in non-native species that have complex and unknown breeding ecologies. A classic model is frogs in the family Pipidae, for example the African clawed frog (*Xenopus* laevis) and its relative the Western clawed frog (*Silurana tropicalis,* formerly *Xenopus tropicalis*). The Western clawed frog does not exhibit any sexual activity in the laboratory without hormone injections (Waring et al., 1941). A commonly used and highly effective method to stimulate spawning in this species is through injections of human chorionic gonadotropin (hCG) into the dorsal lymph sac. In the majority of studies where this spawning method has been employed, full clutches of fertilized eggs were obtained following injection of a low priming dose and an ovulatory dose 20 to 24 hours later (Duarte-Guterman et al., 2010; Hirsch et al., 2002; Khokha et al., 2002; Langlois et al., 2010). Priming is typically required when eggs have not matured or

when animals are kept out of season. The hCG method lends its success to early studies on the *Xenopus* pituitary in the 1930s by Lancelot Hogben who initially revealed the high responsiveness of this species to heterologous gonadotropic hormone preparations. This formed the basis to use these animals in pregnancy tests where ovulation was induced following injection of pregnant female urine. It was later discovered that the substance responsible for evoking this response was hCG, leading to its purification and commercial use (Gurdon and Hopwood, 2003).

Silurana tropicalis are suitable experimental model organisms for toxicological research and have provided great insights into vertebrate developmental systems. Fertilized eggs are relatively easy to obtain year round using the hCG injection protocol. Genetic analysis is possible since it is the only diploid in the family Pipidae and is the first amphibian with a fully sequenced genome (Hellsten et al., 2010; Kashiwagi et al., 2010). In addition, a detailed and standard developmental staging chart has been described (Nieuwkoop and Faber, 1994). Spawned *S. tropicalis* and *X. laevis* eggs are therefore widely used in research to study development and to address the effects of endocrine-disrupting chemicals. Consequently, most if not all spawning experiments in the lab using this model organism have been directed towards simply obtaining fertilized eggs for these goals. In this regard, there is a lack of progress towards understanding the basic neuroendocrine mechanisms controlling reproduction in this particular species compared to other anuran models, especially compared to frogs in the family Ranidae. Alternative methods for spawning induction have not been extensively tested in members of the family Pipidae. Although this hypophyseal approach using hCG is frequently and effectively applied in anurans from the genus *Xenopus* and *Bufonidae*, it is not ideal for studying the physiological control of spawning *in vivo* given that it is a human chorionic gonadotropin. A

high dose is typically required and it does not interact with the amphibian LH receptor with as high affinity (Clulow et al., 2014). Moreover, physiological mechanisms governing gonadotropin synthesis and release in *S. tropicalis* remain to be intensively examined, including those that are presumed to be well-conserved across vertebrates such as the GnRH peptide system (Chapter 2, section 2.4.1). One way to unravel such mechanisms is through evolutionary extrapolation and by applying the hypothalamic approach to induce spawning. Here, animals are administered superactive agonists and antagonists to stimulate the release of endogenous hormones to induce egg and sperm release. The AMPHIPLEX method for spawning induction described by Trudeau et al. is one example of such hormone mixtures. Here, a gonadotropin-releasing hormone agonist is injected in combination with a dopamine receptor D2 antagonist (Trudeau et al., 2013; Trudeau et al., 2010). The principles of applying a dopamine antagonist to this method were based on data in teleost fish suggesting the inhibitory role of dopamine on reproduction (Dufour et al., 2005). The current data in Chapter 3 suggest that this inhibitory system is somewhat conserved in *Lithobates pipiens* with partial support for spawning and strong evidence with respect to the expression of pituitary gonadotropins. This in combination with other emerging studies in amphibians propose similar inhibitory effects from dopamine (Vu and Trudeau, 2016).

The primary goal of this study was to investigate the possible inhibitory role of dopamine in the control of reproduction in *S. tropicalis*. While addressing this question, the effect of GnRH on reproduction in this species was simultaneously explored. This was achieved through several dose-response studies in which a gonadotropin-releasing hormone agonist and the dopamine antagonist metoclopramide were tested separately and in combination. The effects of these treatments were measured through spawning outcome and the expression of reproductive genes in the pituitary. It was predicted that spawning and gonadotropin synthesis would be potentiated

by the combined actions of the gonadotropin-releasing hormone agonist and dopamine antagonist. The findings from this study would provide the first steps towards uncovering the neuroendocrine regulation of egg and sperm release in this species.

4.2. Effects of GnRH-A and MET injections on spawning

4.2.1. Materials and methods

4.2.1.1. Animal care and husbandry

Silurana tropicalis were obtained from the Trudeau lab breeding colony at the University of Ottawa Aquatic Care Facility under a 12:12 light:dark cycle i.e. light from 7:00 to 19:00 each day. Eggs were collected from adults injected using a standardized hCG protocol. The hCG (Millipore, CAT #230734-1MG) was suspended in 0.7 % NaCl and was injected into the dorsal lymph sac (D.L.S) (100 µL/ frog). A priming injection (12.5 IU) administered with the sexes separated was followed by a boosting dose (100 IU) 24 hours later (Duarte-Guterman et al., 2010; Langlois et al., 2010). Fertilized eggs were obtained after 4 to 6 hours on the second day and were treated with 2 % L-cysteine to remove the jelly coat.

Tadpoles were raised in a static and closed system comprising of 23-24 °C dechlorinated water in 15 L glass aquaria with air stones and regular 75 % water changes every three days. Each egg mass was divided into multiple tanks with an initial density of 130 tadpoles/ tank that was eventually reduced to 1-2 tadpoles/ L following forelimb emergence. Daily feeding began five days post-fertilization with Sera micron algae growth food (ANIDIS, Cat # A-4200720). Following tail regression, this diet was switched to Nasco Frog Brittle for Post-Metamorphic *Xenopus* (Cat # SB29028(LM)MX) and eventually to larger Nasco Frog Brittle for Adult *Xenopus* (Cat # SA05960(LM)MX) after 6 months. Females were fed 3 pellets each while males were fed 2. After tadpoles were metamorphosed into froglets (Nieuwkoop and Faber, stage 66),

pieces of floating plastic square mesh and PVC pipes were placed into tanks as enrichment to encourage natural behaviours. Sexually mature adults of the same age (one year) were selected for this study. On average, *S. tropicalis* reaches maturity after 6 months of age with some variability across individuals that are dependent on factors such as density and nutrition. Reproductive maturity in this species is marked by dark nuptial pads on the ventral forelimbs of males and enlarged cloacae on females.

4.2.1.2. Breeding and injection set up

Chemicals used in this experiment were the GnRH agonist (GnRH-A) (Des-Gly^{10}, D-Ala^{6}, Pro-NHEt^{9})-LHRH acetate salt (BACHEM, Item #4012028.0025, CAT #H-4070.0025), and the selective dopamine receptor D2 antagonist metoclopramide hydrochloride (MET) (Sigma-Aldrich, CAT #M0763-25G). Hormones were weighed using a microbalance (Sartorius M2P) and diluted in 0.7 % NaCl on the day of injection. Doses for this study were selected based on a series of breeding trials in which spawning response to various times, doses and routes of administration was examined. The results of these preliminary experiments are summarized in Table A1.1 in Appendix 1. These trials were conducted to determine the dose at which spawning was successful, here characterized by the production of fertilized eggs, and simultaneously, at which a potential dopaminergic effect could be detected. This is especially important in a very distantly-related species to *Lithobates pipiens* with little or almost no information regarding its reproductive physiology. Increasing doses ranging from 0.025 μg/g to 4 μg/g were tested for these trials, with GnRH-A priming in certain cases. The final dose of GnRH-A chosen for this spawning experiment was 4 μg/g which generated fertilized eggs and a potential dopaminergic effect was observed. In Australian frogs, GnRH-A doses ranging from 2 μg/g to 5 μg/g have been shown to successfully induce spawning (Byrne and Silla, 2010; Silla, 2011), supporting the

choice of 4 µg/g GnRH-A. Following these preliminary investigations, the resulting four treatment groups were as follows: Control (0.7 % NaCl), GnRH-A (4 µg/g), MET (10 µg/g) and the mixture GnRH-A (4 µg/g) + MET (10 µg/g). In each group, 24 couples were injected I.P. (5 µL/g BWt). In the first trials, hCG was used as a positive control to confirm sexual maturity and responsiveness in these frogs.

Injections were performed in a temperature and humidity-controlled room between 8:00 and 10:30. Air temperature ranged from 27.1-28.8 °C and humidity from 40-45 % through the course of the experiment. The pH of dechlorinated water (24.3-24.8 °C) was adjusted to 5.80-6.00 to mimic rainfall water which is a known stimulator of spawning in tropical species. This experimental design was adapted from the Grainger Lab mating protocol for *S. tropicalis* (http://faculty.virginia.edu/xtropicalis/husbandry/mating.html). Two centimeters of water was added to each 8 L breeding tank, enough to allow females access to air while being clasped by males during amplexus. Body weights were recorded and the calculated dose was administered IP (5 µL/g BWt) using a 0.3 CC x 8 mm insulin syringe. Human chorionic gonadotropin is typically injected into the dorsal lymph sac due to rapid circulation of the lymphatic system. One minor trial (Trial 4 and 5; see Table A1.1) showed equal potency in GnRH-A and GnRH-A + MET between IP and DLS injections, therefore IP was chosen to be consistent with *L. pipiens*. All females were injected and placed in the breeding tanks before introducing injected males approximately one hour later. One couple was placed in each tank. A black plastic garbage bag covered ¾ of the tank to minimize visual disturbances. After injections, frogs were allowed to recover for at least one week before subsequent injections. Data in goldfish injected with salmon GnRH and its analogue sGnRH-A indicate that although this agonist is more resistant to degradation in the body, it has a relativeness short half-life nevertheless of 73 minutes (Huang et

al., 1991). More strikingly, this time was notably reduced to 12 minutes in goldfish injected with mammalian GnRH, demonstrating a rapid clearance rate from the body (Sherwood and Harvey, 1986).

4.2.1.3. Spawning survey

Frogs were surveyed at hourly intervals post-injection, a reasonable time frame that would reduce the risk of disturbing reproductive activities. The spawning parameters quantified included: the number of amplexus attempts in each tank, the number of couples in amplexus and the presence of eggs. The time to amplexus and oviposition from injection were also assessed. Oviposition was marked by the first release of eggs in the water. At 12 hours post-injection, any egg masses released were collected from tanks using a plastic transfer pipette and were cleaned with 2 % L-cysteine. This time point was chosen to reduce damage to egg masses. *S. tropicalis* are reportedly known to consume their own eggs and rapid movements in tanks by the couple may disrupt the eggs. Therefore, 12 hours was a reasonable time point to reduce these effects and provide a more accurate estimate of reproductive success. Eggs were allowed to develop overnight in a petri dish and were screened for fertilization and egg numbers the next morning (24 hours). The total number of eggs was counted under a dissecting microscope followed by the number of fertilized eggs.

4.2.1.4. Statistical analysis

Data were obtained from four different couples in each treatment and averaged for six days (n=6). Data were not normally distributed and were analyzed using Kruskall-Wallis one-way ANOVA on ranks followed by Dunn's post-hoc to test for multiple pairwise comparisons ($p < 0.05$). Statistical analyses and graphs were generated using the statistical software SigmaPlot (version 11) and GraphPad Prism (version 6).

4.2.2. Results

4.2.2.1. Spawning activity following injection of 4 µg/g GnRH-A and 10 µg/g MET

Mean (± SD) body weights for females and males at the time of injection were 17.40 g (± 1.96 g) and 9.93 g (± 0.89 g), respectively. Injection with GnRH-A alone or in combination with MET induced enlarged and pink cloacae in all females. Additional breeding behaviours observed in these two treatments groups were heightened male activity such as increased swimming and attempts at amplexus. No sexual activity was observed in control and metoclopramide-injected groups. Out of 24 couples that were injected in each treatment group, 2/24 and 4/24 released egg masses in the GnRH-A and GnRH-A + MET groups, respectively. Interestingly, partial ovulation was observed in females in these groups. From the six egg masses that were released, the total number of eggs and average fertilization rates (%) were greater in groups injected with both GnRH-A and MET (2549, 62.99 %) than GnRH-A alone (637, 28.23 %). There was a significant difference in the number of amplexus attempts across treatments [$H(3) = 17.282$, $p < 0.05$]. Post-hoc pairwise comparisons revealed a statistically significant increase in amplexus attempts in couples injected with GnRH-A compared to control ($p < 0.05$) and compared to MET ($p < 0.05$). An overall significant effect of treatment was detected on the number of couples in amplexus [$H(3) = 9.804$, $p < 0.05$]. Although post-hoc analyses did not reveal any statistically significant differences between those groups, the data illustrate that the majority of couples entering amplexus (10/24) were injected with GnRH-A. These data are in parallel with the previously described data for amplexus attempts. In the group injected with GnRH-A + MET, 4/24 couples went into amplexus. Similarly, a significant difference was present for the time to amplexus [$H(3) = 10.065$, $p , < 0.05$], however with no pairwise differences. No significant differences were detected for fertilization [$H(3) = 4.616$, $p > 0.05$], egg mass number [$H(3) = 4.625$, $p >$

0.05], number of eggs per mass [H(3) = 4.616, $p > 0.05$] and time to oviposition [H(3) = 6.261, $p > 0.05$] (Table 4.1).

	Amplexus Attempts	Couples in Amplexus	Number of Egg Masses	Number of Eggs / Mass	Fertilization (%)	Time to Amplexus (h)	Time to Oviposition (h)
Control	0.00[a] (0.00 – 0.00)	0.00 (0.00 – 0.00)	0.00 (0.00 – 0.00)	0.00 (0.00 – 0.00)	0.00 (0.00 – 0.00)	24.00 (24.00 – 24.00)	24.00 (24.00 – 24.00)
GnRH-A	5.50[b] (0.00 – 12.00)	0.50 (0.00 – 0.75)	0.00 (0.00 – 0.25)	0.00 (0.00 – 86.00)	0.00 (0.00 – 7.94)	14.25 (12.5 – 24.00)	24.00 (20.25 – 24.00)
MET	0.00[a] (0.00 – 0.00)	0.00 (0.00 – 0.00)	0.00 (0.00 – 0.00)	0.00 (0.00 – 0.00)	0.00 (0.00 – 0.00)	24.00 (24.00 – 24.00)	24.00 (24.00 – 24.00)
GnRH-A + MET	2.63[a] (0.75 – 6.25)	0.00 (0.00 – 0.50)	0.00 (0.00 – 0.50)	0.00 (0.00 – 366.00)	0.00 (0.00 – 37.73)	24.00 (13.00 – 24.00)	24.00 (14.00 – 24.00)

Table 4.1 Spawning activity in *Silurana tropicalis* following intraperitoneal injection (5 µL/ g BWt) of GnRH-A (4 µg/g) and MET (10 µg/g) alone and in combination. Data are expressed as median values with minimum to maximum range (n = 6). Statistical significance ($p < 0.05$) is represented by different superscript letters. When amplexus did not occur and/ or no eggs were obtained, the maximum duration of the experiment (24 hours) was used for analysis.

4.3. Effects of GnRH-A and MET injections on pituitary gene expression

4.3.1. Materials and methods

4.3.1.1. Animal care and husbandry

All animal care and husbandry protocols are described in section 4.2.1.1.

4.3.1.2. Injections and sampling

As previously described, chemicals used in this experiment were GnRH-A and MET. Hormones were weighed using a microbalance and diluted in 0.7 % NaCl on the day of injection. Based on previous findings in the spawning experiment, the treatments were as follows: Control (0.7 % NaCl), GnRH-A (4 μg/g), MET (10 μg/g) and GnRH-A (4 μg/g) + MET (10 μg/g). Spawning conditions and injection methods were previously described in section 4.2.1.2. At 12 hours post-injection, females were euthanized in a water bath containing 2 g/ L of MS-222 buffered with 2 g/ L sodium bicarbonate. Three pituitaries were pooled into one RNase-free safelock tube (Eppendorf, CAT #022600044) and preserved on dry ice and for long term storage in -80 °C until molecular analysis. Dissection tools were rinsed with 0.3 % hydrogen peroxide, followed by two washes of 0.1 % DEPC water to inhibit RNAses. Sampling methods were approved by the University of Ottawa Animal Care and Veterinary Service.

4.3.1.3. Total RNA isolation and cDNA synthesis

Total RNA from adult female pituitaries was extracted using TRIzol Reagent (Ambion, Life Technologies, CAT #15596-026). This extraction method followed the manufacturer's protocol (Life Technologies). The extracted RNA was re-suspended in 14 μL of RNase-free water and concentrations of total RNA, the ratio of absorbance at 260 nm and 280 nm and the ratio of absorbance at 260 and 230 nm were measured using a spectrophotometer (NanoDrop 2000, Thermo Scientific). Samples were stored at -80 °C. The integrity of RNA samples was verified by a 1 % (w/v) agarose electrophoresis gel. Two distinct bands representing 18S and 28S

were present and RNA quality was confirmed in these samples. Total cDNA of pituitary samples was synthesized from 1 ug of total RNA using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific, CAT #K1642). The thermal cycling parameters were as follows: an initial cycle at 25 °C for 10 minutes, followed by 50 °C for 30 minutes and a final cycle at 85 °C for 5 minutes. Each 20 uL cDNA reaction was stored at -20 °C. All cDNA for each experiment was synthesized at the same time. Protocols for RNA extraction and cDNA synthesis followed the manufacturer's guidelines.

4.3.1.4. Primer design and gene expression analysis by quantitative real-time PCR

To examine changes in mRNA levels in response to these treatments, qPCR was performed where specific primers for *S. tropicalis* were designed using PRIMER 3 for the same genes measured in *L. pipiens*: luteinizing hormone beta subunit (*LHβ*), follicle-stimulating hormone beta subunit (*FSHβ*), glycoprotein alpha subunit (*α-GSU)*, dopamine receptor D2 (*DRD2*), gonadotropin-releasing hormone receptor 1 (*GnRHR1*) and the reference gene ribosomal protein l8 (*rpl8*). The genome of *S. tropicalis* has been sequenced in its entirety and therefore, gene sequences were obtained via Xenbase and NCBI. Sequence and primer specificity were verified with the BLAST. Primers were supplied by Integrated DNA Technologies. Optimal primer annealing temperatures for qPCR was determined by a thermal gradient (55-65 °C) using cDNA products diluted 1:10. Primer sequences, amplicon location, size and annealing temperatures for each gene are presented in Table 4.2. Thermal gradient qPCR products were run on a 2 % (w/v) agarose electrophoresis gel for 45 minutes. Bands were stained with SYBR Safe (Thermo Fisher Scientific, CAT #S33102) where single products represented a specific primer pair. Bands were excised under UV light and DNA was extracted and purified using the NucleoSpin Gel and PCR Clean-up kit (Machery-Nagel CAT

#740609.50). Purified PCR products were additionally ligated into a pGEM®-T Easy Vector System I containing T7 and SP6 promoter regions (Promega, CAT #A1360). Ligated products were transformed into JM109 high-efficiency competent cells (Promega, CAT #L2004). This procedure followed the manufacturer's guidelines. The DNA from inoculated colonies was purified using the protocol "Plasmid DNA Purification Using the Qiaprep Spin Miniprep Kit and a Microcentrifuge" (Qiagen, CAT #27104). The identity of the amplicon was confirmed by sequencing purified PCR products at StemCore Laboratories, Ottawa Hospital Research Institute. These sequence analyses were used to confirm the specificity of these products. Primer specificity was also verified by a single distinct melt peak in qPCR analysis.

Quantitative real-time PCR was performed using Maxima SYBR Green qPCR Master Mix (Thermo Scientific, CAT #K0252) following the manufacturer's protocol. Each sample was run in duplicates on a 96-well plate, each plate representing one gene. No template and reverse transcriptase controls were included for each plate to confirm the absence of genomic contamination and the specificity of target cDNA amplification. Each 25 uL reaction consisted of 5 uL of cDNA template and 20 uL of Master Mix containing 100 nM specific primers, 1X SYBR Green qPCR Master Mix and nuclease-free water. Thermal cycling parameters using the Bio-Rad CFX96 qPCR system were: an initial cycle of Taq activation at 95 °C for 3 minutes, followed by 40 amplification cycles of denaturation at 95 °C for 10 seconds and primer annealing for 30 seconds at 57 to 64 °C . Denaturing occurred at 95 °C for 10 seconds and a melt curve was generated at 65 °C to 95 °C for 30 seconds, increasing 0.5 °C every 5 seconds. Standard curves for each gene were generated from serial dilutions of pooled cDNA from all samples (2-fold dilutions for *LHβ, DRD2* and *GnRHR1*, and 5-fold dilutions for *FSHβ*, *α-GSU*

and *rpl8*). The efficiency for all standard curves was 90-110 % with an r^2 value of 0.980 or greater (Taylor et al., 2010).

4.3.1.5. Statistical analysis

Relative mRNA levels were calculated using the relative standard curve method based on Cq values and data were normalized using the algorithm NORMA-Gene (Heckmann et al., 2011). Fold change of mRNA levels relative to control was calculated for each sample. Mean fold change in mRNA levels was the average of biological replicates. Normality and homogeneity of variance were verified for each data set using Shapiro-Wilk and Levene's tests, respectively ($p > 0.05$). A one-way ANOVA followed by Tukey's HSD post-hoc test for pairwise comparisons were performed. Gene expression data that did not meet parametric assumptions were analyzed using a non-parametric Kruskall-Wallis test, also known as a one-way ANOVA on ranks. To determine significant changes between specific pairwise treatments, Dunn's post-hoc test was performed ($p < 0.05$). Data were expressed as median values presented in Tukey's box and whisker plots, where boxes extend from the 25th to 75th percentiles, whiskers show the minimum and maximum values, and single points represent outliers. Statistical significance was denoted by a p-value < 0.05. Statistical analyses and graphs were generated using the statistical software SigmaPlot (version 11) and GraphPad Prism (version 6).

4.3.2. Results

Gene	Element	Primer Sequence (5'-3')	Amplicon location and size (bp)	Annealing (°C)
LHβ	Forward	GCCGCAACCTTACTCACTC	518-709 (192)	63
	Reverse	CCCTCCAGTTCTTTCGCTGT		
FSHβ	Forward	GCCAAGAAAAGGAAGAACTGG	392-608 (217)	63
	Reverse	GAGTGTGGGTAAGCAGAGGAA		
α-GSU	Forward	CCAGGTTCATACAAGAGAGAG	37-141 (105)	59
	Reverse	CTGGAAAGGAATGGAGCACA		
DRD2	Forward	GCCTTCACTTGGCTTGGTTATG	1821-2028 (208)	64
	Reverse	AGCACCATTTTCACCAGTCCA		
GnRHR1	Forward	CTCCACCGCCGCAAAGATAC	136-335 (200)	63
	Reverse	TGACATTCCACACAGCGTCC		
rpl8	Forward	CCCTCAACCATCAGGAGAGA	682-769 (88)	57
	Reverse	TCTTTGTACCACGCAGACGA		

Table 4.2 Quantitative real-time PCR primer sets and reaction conditions for *Silurana tropicalis*.

4.3.2.1. Intraperitoneal injections of GnRH-A and MET do not have major effects on pituitary *LHβ, FSHβ, α-GSU, DRD2* and *GnRHR1* expression in *Silurana tropicalis* after 12 hours

The mRNA levels of *LHβ* were significantly different across treatment groups [H(3) = 8.880, $p < 0.05$], where a significant decrease was detected in females injected with GnRH-A compared to control ($p < 0.05$) (Figure 4.1a). A significant treatment effect was found for the mRNA levels of *FSHβ* [F(3) = 7.990, $p < 0.05$]. Post-hoc analysis revealed statistically significant differences between MET and GnRH-A ($p < 0.05$) and MET and GnRH-A + MET ($p < 0.05$) where mRNA levels in the MET group were greater in both cases (Figure 4.1b). A statistically significant effect of treatment was found for *α-GSU* [F(3) = 4.113, $p < 0.05$], particularly an increase in females injected with GnRH-A + MET compared to GnRH-A alone ($p < 0.05$) (Figure 4.1c). In these cases, responses were variable and of low magnitude. No statistically significant differences were present between treatments with regards to the mRNA levels of the receptors *DRD2* [H(3) = 5.807, $p > 0.05$] and *GnRHR1* [H(3) = 3.633, $p > 0.05$] (Figure 4.1d-e).

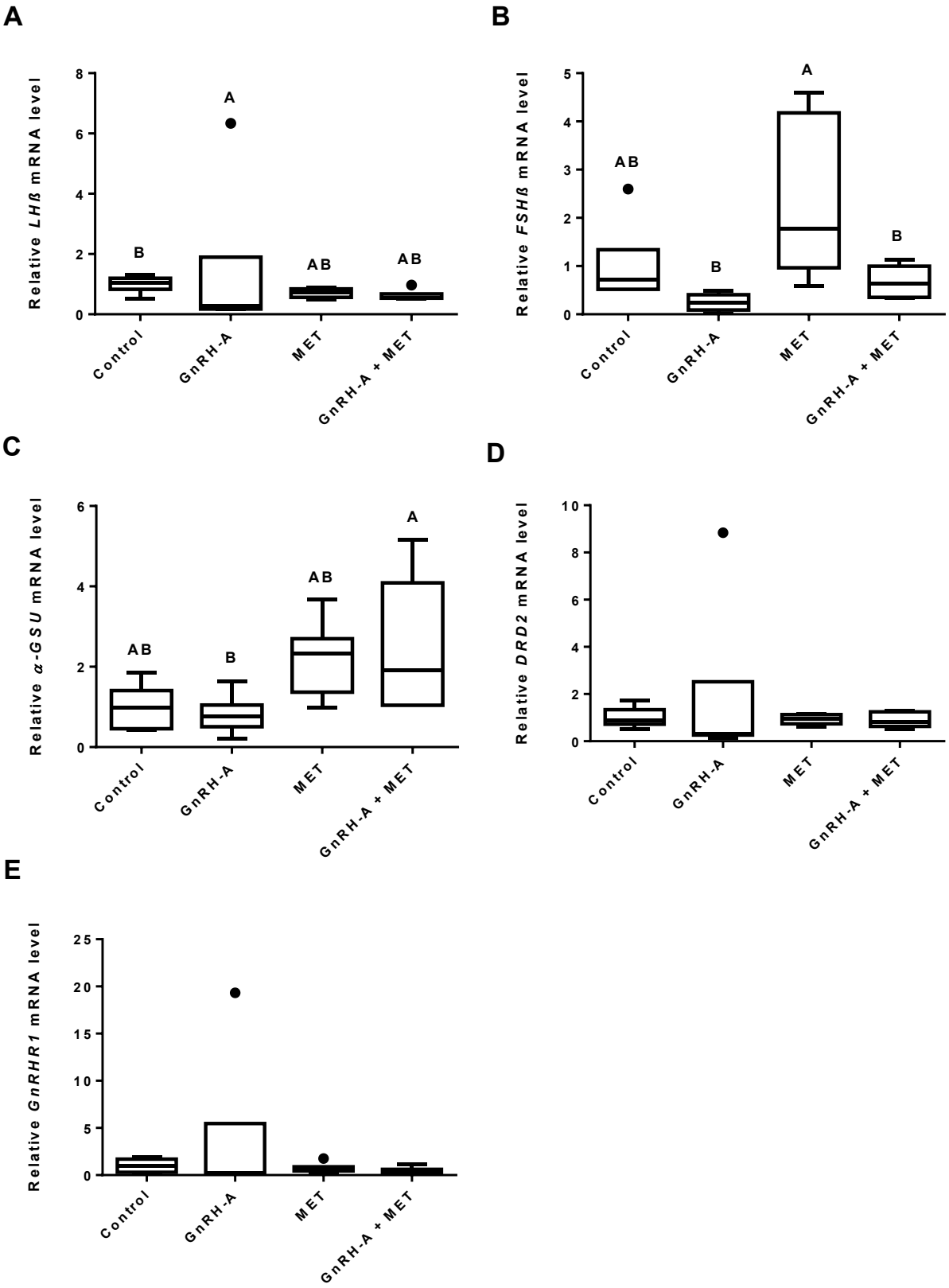

A
Relative LHß mRNA level
8
6
4
2
0
B
A
AB
AB
Control
GnRH-A
MET
GnRH-A + MET
B
Relative FSHß mRNA level
5
4
3
2
1
0
AB
B
A
B
Control
GnRH-A
MET
GnRH-A + MET
C
Relative α-GSU mRNA level
6
4
2
0
AB
B
AB
A
Control
GnRH-A
MET
GnRH-A + MET
D
Relative DRD2 mRNA level
10
8
6
4
2
0
Control
GnRH-A
MET
GnRH-A + MET
E
Relative GnRHR1 mRNA level
25
20
15
10
5
0
Control
GnRH-A
MET
GnRH-A + MET

Figure 4.1 Quantitative real-time PCR analyses illustrating the effects of GnRH-A (4 μg/g) and MET (10 μg/g) delivered separately and in combination on the mRNA levels of pituitary reproductive genes in *Silurana tropicalis*. Females were injected intraperitoneally and the mRNA levels of *LHβ* (A), *FSHβ* (B), *α-GSU* (C), *DRD2* (D) and *GnRHR1* (E) were measured after 12 hours. Data are presented as fold change relative to control. Median values (n = 6) are presented in box and whisker plots, where boxes extend from the 25th to 75th percentiles, whiskers show the minimum and maximum values, and single points represent outliers. Different letters indicate statistically significant differences ($p < 0.05$) between groups.

4.4. Discussion

4.4.1. The effects of GnRH-A and MET injections on spawning

4.4.1.1. Comparison to human chorionic gonadotropin

The major findings from these spawning studies indicate that the standard hCG protocol is a more effective spawning method in the Western clawed frog in comparison to GnRH-A (4 μg/g) alone or in combination with MET (10 μg/g). The early preliminary spawning experiments, particularly trials 1 and 2 (Table A1.1), indicate that pituitary sensitivity decreases with age. Here, older frogs did not enter amplexus or release eggs when injected with GnRH-A and MET alone and in combination, however successfully released fertilized eggs when injected with hCG, demonstrating that the testes and ovaries are responsive to this gonadotropin. Subsequent experiments consistently demonstrated that *S. tropicalis* did not respond well to GnRH-A and MET compared to hCG. This was observed in the lower frequency of frogs entering amplexus and releasing eggs. The reported spawning success rate over the past couple of years, measured in terms of egg mass release, using the hCG protocol in the Trudeau lab is 88 % (64/73 couples) with a failure rate of 12 % (9/73 couples). This is in large contrast to what was observed following the administration of 4 μg/g GnRH-A. Only 2/24 egg masses were released from couples injected with GnRH-A alone, and 4/24 egg masses were released from couples injected with GnRH-A and MET in combination. Interestingly, it appears that the doses of GnRH-A and MET chosen for this study may have stimulated adequate LH release and ovulation in only a few

individuals in this experiment. Spawning using this method did not lead to exceptionally large egg masses where the number of eggs released in these experiments is at the lower limit of the average range for this species. It has been reported that an average female will release between 1000 to 9000 eggs per spawn (Hirsch et al., 2002; Kashiwagi et al., 2010). Comparably, females in this experiment released 293 to 1097 eggs. Partial ovulation has been previously demonstrated in *Xenopus* spp. that were induced with alternative spawning strategies. In 2011, a new method for stimulating ovulation in *Xenopus laevis* was described through the addition of progesterone to the aquatic environment. Although successful, the reported frequency of ovulation, total egg number and fertilization rate using this novel technique were notably lower compared to the hCG protocol (Ogawa et al., 2011). These findings are consistent with the current results from this study which demonstrate that hCG is a more effective spawning method in the Western clawed frog.

It is difficult to currently determine why reproductively mature *S. tropicalis* with gonads highly responsive to hCG failed to spawn. However, *S. tropicalis* has been in captivity for many generations over several decades. It is possible that we have genetic selection for hCG responsiveness in the colony, such that descendant frogs are only obtained from parents that were previously injected with hCG. Indeed, couples that do not respond to hCG are sacrificed and thus eliminated from the colony. Artificial selection for endocrine traits has been previously described. For example, lines have been selected for both high and low juvenile hormone esterase activity in *Gryllus assimilis* over nine generations (Zera, 2006), while pre-selection of broodstock has been applied in aquaculture to improve spawning in species such as the European eel (*Anguilla anguilla*) (Burgerhout et al., 2016). Manipulation of reproductive traits has been widely employed across breeding management programs for livestock in particular, where

genetic variations for reproductive potentials were selected for under optimal environmental stimuli and nutrition. In sheep, litter sizes are greater in individuals that carry both copies of the *FecB* gene (Baird and Campbell, 1998; Notter, 2012), providing opportunities for genetic control over ovulation rate. Since ovulation occurs only once a day in the majority of hens raised under a 24 hour light:dark cycle, selection for persistent ovulators has been intensively sought in the poultry literature to overcome this problem. This has been effectively achieved through exposure of domestic hens to continuous or shorter light cycles to promote positive ovarian functions including increased ovarian follicular maturation and egg production, and shorter oviposition intervals (Gow et al., 1985; Gow et al., 1986; Hocking, 2014; Johnson et al., 2015). Genetic selection for gonadal hCG responsiveness may therefore be a possible explanation to the low pituitary responsiveness of *S. tropicalis* to GnRH-A in this study.

4.4.1.2. The potency of GnRH-A in *Silurana tropicalis* compared to *Lithobates pipiens*

The present findings suggest that GnRH-A may lack potency in the Western clawed frog at these doses. The dose of GnRH-A that elicited male sexual behavior and the release of fertilized eggs in this experiment (4 μg/g) was ten times greater than the one originally reported in the AMPHIPLEX method (0.4 μg/g) (Trudeau et al., 2013; Trudeau et al., 2010). With what has been recently been discovered in the investigations in Chapter 3, the dose of GnRH-A in *S. tropicalis* is 40 times greater than what is actually required in the Northern leopard frog to effectively induce spawning (0.1 μg/g). These variations in GnRH-A potencies were expected in these two distantly-related species. For example, in Australia, GnRH-A doses ranging from 2 to 5 μg/g have been applied to successfully induce sperm and egg release in the Southern Corroboree frog (*Pseudophryne corroboree*) and Günther's toadlet (*Pseudophyrne guentheri*) (Byrne and Silla, 2010; Silla, 2011). It is suspected that these evolutionary differences may be

attributed to the sensitivities and affinities of GnRH receptors in the pituitaries of *S. tropicalis* and *L. pipiens*. The overall amino acid sequence identity for type 1 GnRH receptors in *Lithobates cateisbeianus* (accession number AAG42575.1) and *Silurana tropicalis* (accession number NP_001107549.1) is 56 % of the 410 amino acid sequence length. Sequence comparisons indicate that the 7-transmembrane domains are highly similar, as expected. However, both the ~90 amino acid N-terminal ligand binding domain and the ~90 amino acid C-terminal intracellular signaling domain are less well-conserved. Therefore, sufficiently different ligand-binding and intracellular domains may exist between these two species. Consequently, the GnRH agonist used in these experiments may bind less efficiently to GnRH receptors in the pituitary of *S. tropicalis*. This agonist was selected based on GnRH receptor characterizations in the only known studies conducted with *Lithobates catesbeianus* (Acharjee et al., 2002; Millar et al., 2004; Wang et al., 2001). Future research should concentrate on GnRH receptor binding analysis with the specific goal of identifying superactive agonists for a range of distantly-related amphibian species, including *S. tropicalis*. The GnRH-A dose response trials conducted provided a first step towards examining reproductive physiology *in vivo*. Preliminary results show that 4 μg/g of GnRH-A can induce partial ovulation and some male sexual behavior in the Western clawed frog that are reflected in the increased number of amplexus attempts and fertilization rates.

4.4.1.3. Spawning challenges and future directions

There are multiple confounding factors that may limit successful spawning in this species that should be considered. To control for selective mate choice in future studies, the sex ratio in each tank can be altered from 1:1 to include more males as in the experiments with Northern leopard frogs. Spawning success was possibly hindered by suboptimal environmental conditions

in these experiments. Despite being raised under laboratory conditions, there may be seasonal trends in gonadal development in this species. Although the Western clawed frog can be induced to spawn all year using hCG, seasonality in the pituitary responsiveness to GnRH-A may be an important factor to consider in future experiments. One major limitation in these spawning trials was the inability to obtain fertilized eggs, perhaps due to reproductive dysfunctions in males. Synchronizing egg and sperm release is a major challenge in captive breeding, therefore the time of injection of males and females should be optimized. Intraperitoneal injections were selected in this study to be consistent with studies in the Northern leopard frog, however future studies should explore alternative routes of administration such as the dorsal lymph sac that is classically used for hCG.

4.4.1.4. Novel applications to captive breeding

It is clear that the effectiveness of assisted reproductive technologies varies greatly between species. The lack of response at these doses of GnRH-A and MET may limit the use of this hormone mixture in sustaining captive colonies for *S. tropicalis*. Nevertheless, this was the first study to quantify a GnRH-A dose-response on spawning and therefore, provided the first insights into the GnRH peptide system in this species. Applications of hormone treatments can vary greatly across breeding programs. These current findings represent an initial step towards the development and refinement of spawning induction methods using GnRH-A in *S. tropicalis* and perhaps other members of the family Pipidae. The results from this study will contribute towards the augmentation of captive breeding techniques for amphibians.

4.4.2. The effects of GnRH-A and MET injections on the expression of reproductive genes in the pituitary

To my knowledge, this was the first study to develop gene expression methods for the pituitary of *S. tropicalis*. The opportunity for experimentation was limited by the space and time required to raise large amounts of adults. The results from this study demonstrated no major changes in the expression of gonadotropin subunits, GnRH receptor 1 and dopamine receptor D2 genes in the pituitary 12 hours following injection. Consequently, the time of gonadotropin synthesis and release in response to GnRH remains unknown. As demonstrated in the Northern leopard frog, time of sampling following injection is critical to determine the effectiveness of hormone treatments. Future studies are required in which additional time points are analyzed. There was also considerable individual variation in gene expression levels. This is consistent with what was observed in the spawning trials, where the time to amplexus and oviposition were highly variable among couples. The current results provide data on female pituitary response, however these molecular mechanisms should be further examined in males.

4.5. Conclusion

The most effective dose of GnRH-A to stimulate oviposition, male sexual behavior and fertilization under the conditions of these experiments was determined to be 4 μg/g. However, the overall success was very low at 8 to 25 %. There were no significant changes in the mRNA levels of genes for gonadotropin subunits, GnRH receptor 1 and dopamine receptor D2 12 hours following injection. Therefore, it is difficult to conclude about the role of GnRH or DA in the control of reproductive processes in this species. These preliminary results lay the foundation for future studies in which new doses, analogues and time points may be explored. The postulated dichotomy in response between this species and the Northern leopard frog may be attributed to

differences in GnRH receptor binding affinities and functional protein domains. The current data indicate that captive breeding is more successful through the application of hCG in comparison to GnRH-A and MET in the Western clawed frog.

CHAPTER 5

General Discussion

5.1. Thesis result summary

The research presented in this thesis aimed to elucidate the inhibitory role of dopamine in the neuroendocrine control of reproduction in two amphibian species: the Northern leopard frog (*Lithobates pipiens*) and the Western clawed frog (*Silurana tropicalis*). Firstly, a detailed review of the literature on the neuroendocrine control of spawning in amphibians was conducted (Chapter 2). It was clear from this that very little is known about the hormonal control of spawning, and especially the potential role of dopamine in this vertebrate class. Multiple approaches in the field and laboratory were therefore undertaken where the dopamine receptor D2 antagonist metoclopramide was administered in the presence and absence of a gonadotropin-releasing hormone agonist. The first objective of this study explored the effects of these injections on spawning outcome. Two experiments were conducted during the spring breeding season where Northern leopard frogs were injected with these two hormones in several combinations. The results (Chapter 3) demonstrated that from the time of injection, amplexus peaked at 24 to 48 hours followed by egg release at 72 to 96 hours. In the first season, these treatments evoked a rather rapid response due to the unexpected increase in temperature, therefore amplexus and oviposition peaked distinctly at 24 and 48 hours, respectively. These data did not reveal any interaction between GnRH-A and MET on the average incidences of amplexus per day, egg mass weight and fertilization rate in both years. Although not detected in the data

from 2015, there was a significant interaction between GnRH-A and MET on the average number of egg masses released per female and the number of eggs per mass in 2016. This suggests that dopamine is potentially involved in the inhibition of reproduction under the conditions of these spawning experiments. Spawning results in the Western clawed frog (Chapter 4) demonstrated no significant treatment effects on the number of couples in amplexus, the number of egg masses released, number of eggs per mass, fertilization, time to amplexus and time to oviposition. However, there was a significant increase in the number of amplexus attempts observed in frogs injected with GnRH-A compared to control. Consistently in both species, metoclopramide alone did not induce any spawning activity.

These spawning studies in the Northern leopard frog were complemented by targeted gene expression analyses in the laboratory in the second objective of this research. A series of time- and dose-related studies uncovered the potentiating effects of MET on the mRNA levels of pituitary gonadotropins in female Northern leopard frogs. At 36 hours following injection, the mRNA levels of the gonadotropin subunits *LHβ* and *α-GSU* were significantly increased in females injected with 0.4 μg/g of GnRH-A relative to control. However, the mRNA levels of these two genes in addition to those of *FSHβ* were significantly greater when 0.4 μg/g of GnRH-A was injected in combination with 10 μg/g of MET. These data suggest that an inhibitory action of dopamine exists in this species. The postulated mechanism of action behind these potentiating effects of MET is through the upregulation of GnRH receptor 1 and the downregulation of dopamine receptor D2. Across all time points, the expression *DRD2* decreased with treatment, potentially due to an autoregulatory effect from MET on this receptor. Results at 12 and 36 hours post-injection demonstrated a significant increase in the mRNA levels of *GnRHR1* in females injected with GnRH-A alone and in combination with MET. Interestingly, at 12 hours post-

injection, the mRNA levels of all gonadotropin subunits were significantly decreased in metoclopramide-injected females compared to control, suggesting a biphasic response in the first hours after injection. The current results lend partial support to the dopaminergic inhibition hypothesis proposed in this thesis. Although there is weak evidence suggesting dopaminergic inhibition on amplexus and egg mass release in Northern leopard frogs, the results did not reach statistical significance. In contrast, there are strong data supporting the inhibitory role of dopamine on the expression of reproductive genes in the pituitary. It is speculated that the reason for this dichotomy is due to the presence of a male during spawning but not for the gene expression studies on females. It may be that the presence of a sexually mature male further augmented luteinizing hormone release in females injected with GnRH-A or the GnRH-A plus MET combination. In the absence of amplexus, perhaps the subtle effects of DA inhibition were observed at the level of gene expression in the female leopard frog pituitary. This suggests the presence of such inhibitory actions governed by the dopaminergic system.

5.2. Implications for understanding basic neuroendocrine mechanisms of reproduction

It is evident that knowledge regarding the neuroendocrine control of reproduction in amphibians is lagging considerably compared to other vertebrate models. Specifically, the factors governing gonadotropin synthesis and release remain unclear (Chapter 2). Several studies by Licht and his colleagues in the 1980's indicated a stimulatory role of GnRH on the release of gonadotropins in bullfrogs (Daniels and Licht, 1980; McCreery and Licht, 1983b) and Northern leopard frogs (Stamper and Licht, 1990, 1993a, b). However, this thesis revealed the existence of clear species sensitivities in this GnRH peptide system. This was primarily reflected through dose-response analyses on spawning in the Northern leopard frog and Western clawed frog using a superactive GnRH agonist. It was determined that a dose as low as 0.1 μg/g was sufficient to

induce spawning in the Northern leopard frog. This dose was equally effective at stimulating the complete release of fertilized egg masses in this frog species as 0.4 μg/g reported for the AMPHIPLEX method. This is in parallel with data in goldfish where 0.1 μg/g was used in combination with 10 μg/g of the dopamine antagonist pimozide to successfully induce spawning. It was revealed that DA antagonism plays an essential role in potentiating the release of serum gonadotropins and inducing ovulation such that these effects were stronger when pimozide was injected together with a GnRH agonist (Chang and Peter, 1983; Chang et al., 1984; Peter et al., 1987). Dose-response experiments in the Western clawed frog demonstrated that 4 μg/g of GnRH-A was required to induce partial ovulation, some male sexual activity and fertilization. This represents a dose that is ten times greater than that reported in the AMPHIPLEX method and forty times greater than the effective dose for the Northern leopard frog determined in Chapter 3. Differences in the times to amplexus and oviposition were also observed in these distant species. For instance, egg release following a single injection of GnRH-A alone or in combination with MET resulted in oviposition after 72 to 96 hours in the Northern leopard frog. In marked contrast, eggs were released from the Western clawed frog after 4 to 6 hours. Therefore, the release of LH appears to be occurring at a faster rate in this species compared to the Northern leopard frog.

5.3. Applications to amphibian conservation

The global decline of amphibians was initially acknowledged at the First World Congress of Herpetology in 1989. In 2004, the Global Amphibian Assessment (GAA) was established to provide a comprehensive survey for the status of all amphibians, the findings of which were then incorporated into the IUCN Red List of Threatened Species. In their most recent update in 2008, 32.4 % of the 6260 identified amphibian species were categorized as threatened, ranking

amphibians as the most endangered vertebrate class. Amphibians are characterized by their permeable skin and biphasic life cycle in which they rely on both aquatic and terrestrial habitats for life completion. Consequently, some of the leading contributors to their decline are linked to habitat loss, climate change, environmental contaminants and UVB radiation (Bishop et al., 2012; Vredenburg and Wake, 2007; Wake and Vredenburg, 2008). Recently, large population losses have been attributed to emerging lethal diseases such as *Batrachochytrium dendrobatidis* (Bd) and *Batrachochytrium salamandrivorans* (Bsal) (Berger et al., 1998; Blaustein et al., 2012; Daszak et al., 1999).

Assisted reproductive technologies have been recently applied to amphibians to facilitate the progression of captive breeding programs (Clulow et al., 2014). Hormonal spawning induction methods such as those in the present study are a useful tool towards not only unravelling the mechanisms of reproductive control, but are valuable towards achieving long term conservation goals. Multiple approaches to breed amphibians in captivity have been adopted over the years. However, the success of most studies is dependent on educated guesses and simply report methods that work. The refinement of such programs is highly dependent on novel studies like the present one in which the effective dose, timing and interactions of superactive agonists and antagonists are thoroughly investigated. Although the spawning results for the Northern leopard frog do not demonstrate any statistically significant interactions between GnRH-A and MET, there are biological implications that are meaningful towards captive breeding. It is now known that a single injection of 0.1 μg/g GnRH-A could induce the release of thousands of eggs from one female. The data illustrate clear increases in egg mass release in animals injected with both GnRH-A and MET, representing thousands more eggs produced. The Northern leopard frog is a species that is peripherally declining within its range,

therefore their propagation would prosper from this method. Importantly, this species represents one member of a larger family, therefore these physiological principles could be extrapolated to other ranids in a comparative approach. Likewise, understanding these mechanisms in a distant species such as the Western clawed frog provides the possibility to extend this knowledge to non-native anurans.

5.4. Limitations and future directions

The activities of the GnRH and DA systems in the regulation of gonadotropin synthesis and spawning in amphibians are open areas for future exploration (Vu and Trudeau, 2016). Evidently, there are clear differences in pituitary GnRH-responsiveness between anurans, representing only one out of three amphibian orders. It will be important to test the effectiveness of a range of synthetic GnRH agonists in future studies. To successfully unravel the role of dopamine on spawning, future studies should concentrate on expanding the current dose-response analyses. Lower doses of GnRH-A with other dopamine receptor antagonists should be tested in the Northern leopard frog. These tests would be directed towards designing effective and optimal drug combinations for induced spawning. A better understanding of seasonal spawning cycles and natural variations in pituitary gonadotropin production in these animals would be useful towards targeting times during the year to induce spawning and perform future gene expression analyses. In the Western clawed frog, egg and sperm release can be synchronized to improve fertilization by priming or by staggering injections between males and females. It is clear that experimentation with sampling time was crucial to bring to light the actions of dopamine in the gene expression analysis for the Northern leopard frog. To further trace the timing profile of gonadotropin subunit genes, time points such as 48 hours should be explored to see when these gonadotropins peak. Importantly, new GnRH and DA agonists and

antagonists can be applied. This especially relevant to the Western clawed frog in which the lack of response was potentially attributed to poor receptor-ligand binding affinities with this particular GnRH agonist. Novel hormones with emerging stimulatory roles such as kisspeptins (Chapter 2, section 2.4.3) should also be explored. Finally, given the lack of pituitary hormone assays for most amphibian species, the advancement of these studies would benefit from the development of recombinant gonadotropins, specific antibodies and sensitive radioimmunoassays.

5.5. Concluding remarks

In the face of rapid population declines, reproductive technologies for amphibians are now critical more than ever. The progression of captive breeding programs for the propagation of threatened species is highly reliant on understanding basic endocrine mechanisms and can therefore be impeded by such lack of knowledge. The present study was the first to examine the effects of GnRH and MET administration alone and in combination in several time- and dose-related experiments. Studying these systems in a distant species provides the possibility to apply these principles to other anurans in a comparative approach. Several avenues remain to be explored, but will be necessary to design and refine current captive breeding programs. The majority of amphibian species have never been kept, much less bred, in captivity. There is still much to learn and the success of future applications will require pooling ideas and resources from different fields of research.

Appendix 1. Dose- and time-related breeding trials for *Silurana tropicalis*

Trial	Date of Birth	Injection Site	Rationale	Treatments	Amplexus	Oviposition	Conclusions
1	Before 2013 Before 2013	DLS DLS	Original AMPHIPLEX method dose	Control 0.4 µg/g GnRH-A + 10 µg/g MET	- -	- -	No response to original AMPHIPLEX doses in old frogs.
2	Before 2013 Before 2013 Before 2013	IP DLS DLS	Compare hCG protocol; determine if age is a factor	Control 0.4 µg/g GnRH-A + 10 µg/g MET hCG protocol	- - 5/5	- - 5/5	Frogs respond to hCG. Pituitary sensitivity decreases with age.
3	Before 2013 Before 2013 Before 2013 2014 2014	IP IP DLS IP IP	Determine if AMPHIPLEX method requires priming	Control 0.4 µg/g GnRH-A + 10 µg/g MET hCG protocol 0.4 µg/g GnRH-A + 10 µg/g MET 0.4 µg/g GnRH-A + 10 µg/g MET with priming	- 1/3 3/3 1/3 1/3	- - 3/3 unfertilized - -	Older frogs require priming in AMPHIPLEX to at least induce amplexus.
4	2015 2015 2015 2015	DLS DLS DLS DLS	Try AMPHIPLEX method in younger frogs and new injection site	Control 0.4 µg/g GnRH-A 10 µg/g MET 0.4 µg/g GnRH-A + 10 µg/g MET	- 2/2 - 2/2	- 2/2 unfertilized - 2/2 unfertilized	AMPHIPLEX can stimulate with both injection methods. Use I.P. to be consistent with *Lithobates pipiens.*
5	2015 2015	IP IP	Try AMPHIPLEX in younger frogs and new injection site	0.4 µg/g GnRH-A 0.4 µg/g GnRH-A + 10 µg/g MET	1/1 1/1	1/1 unfertilized 1/1 fertilized	
6	2014, 2015 2014, 2015 2014, 2015	IP IP IP	GnRH-A dose response. Do not want to induce 100 % spawning with GnRH-A alone.	Control 0.025 µg/g GnRH-A 0.1 µg/g GnRH-A	- - -	- - -	No response at these low doses of GnRH-A.

7	2013 2013 2013	IP IP IP	GnRH-A dose response	Control 0.2 µg/g GnRH-A 0.2 µg/g GnRH-A + 10 µg/g MET	- 1/3 3/3	- - 1/3 unfertilized	Beginning to see some dopaminergic effects.
8	2013 2013 2013	IP IP IP	Priming (0.02 µg/g GnRH-A) 24 h prior full ovulatory dose to obtain fertilized eggs	Control GnRH-A (0.2 µg/g) + priming 0.2 µg/g GnRH-A + 10 µg/g MET with priming	- 1/3 1/3	- 1/3 unfertilized -	Priming is not effective / improving spawning success.
9	2015 2015 2015 2015	IP IP IP IP	Data in fish suggest a shorter priming window (12 h) and higher priming dose (0.2 µg/g GnRH-A) (Chang and Peter, 1983; Chang et al., 1984; Peter, 1980).	Control GnRH-A (0.2 µg/g) + priming 0.2 µg/g GnRH-A + 10 µg/g MET with priming	- 2/4 - 1/4	- - - -	Lost dopaminergic effects; potentially overstimulating the system with this new priming method.
10	2015 2015 2015 2015	IP IP IP IP	Original AMPHIPLEX method dose (Trudeau et al., 2013; Trudeau et al., 2010)	Control 0.4 µg/g GnRH-A 10 µg/g MET 0.4 µg/g GnRH-A + 10 µg/g MET	- 2/4 - 1/4	- 1/4 unfertilized - 1/4 unfertilized	This original dose will not guarantee fertilized eggs.
11	2015 2015 2015 2015	IP IP IP IP	GnRH-A dose in Australian frogs range from 2-5 µg/g (Byrne and Silla, 2010; Silla, 2011).	Control 1 µg/g GnRH-A 10 µg/g MET 1 µg/g GnRH-A + 10 µg/g MET	- 2/4 - 1/4	- 2/4; 1/4 fertliz. - 1/4	Starting to see some fertilization at this dose. Could increase higher.

Table A1.1 Spawning response in the Western clawed frog (*Silurana tropicalis*) to a gonadotropin-releasing hormone agonist (GnRH-A) and the dopamine receptor D2 antagonist metoclopramide (MET) administered at various doses. No activity is represented by a dash (-), whereas successful amplexus or egg release is specified by a fraction to the number of couples per treatment. The experimental body sites of injections were intraperitoneal (IP) and dorsal lymph sac (DLS).

Appendix 2. Other contributions to research

a. Publications

Navarro-Martín, L., **Vu, M.**, Hamilton, C.K., Bulaeva, E., Pauli, B. and Trudeau, V.L. (2017). Can sex steroids administered orally completely sex reverse frogs? (In progress).

Vu, M. and Trudeau, V.L. (2016). Neuroendocrine control of spawning in amphibians and its practical applications. *General and Comparative Endocrinology*, 234: 28-39.

Ward, T.D., Algera, D.A., Gallagher, A.J., Hawkins, E., Horodysky, A., Jørgensen, C., Killen, S.S., McKenzie, D.J., Metcalfe, J.D., Peck, M.A., **Vu, M.** and Cooke, S.J. (2016). Understanding the individual to implement ecosystem approach to fisheries management. *Conservation Physiology*, 4(1), doi:10.1093/ conphys/cow005.

Chapman, J.M., Algera, D., Dick, M., Hawkins, E.E., Lawrence, M.J., Lennox, R.J., Rous, A.M., Souliere, C.M., Stemberger, H.L.J., Struthers, D.P., **Vu, M.**, Ward, T.D., Zolderdo, A.J. and Cooke, S.J. (2015). Being relevant: Practical guidance for early career researchers interested in solving conservation problems. *Global Ecology and Conservation*, 4:334-348.

Vu, M., Navarro-Martín, L., Gutierrez-Villagomez, J.M. and Trudeau, V.L. (2015). Development of an *in vitro* ovary culture system to evaluate endocrine disruption in wood frog tadpoles. *Journal of Toxicology and Environmental Health, Part A: Current Issues*, 78 (18): 1137-1141.

Clulow, J., Trudeau, V.L. and Kouba, A.J. (2014). Amphibian declines in the 21st century: why we need assisted reproductive technologies. In the special issue of "Reproductive Sciences in Animal Conservation – Progress and Prospects" (ed. W.V. Holt, J.L. Brown and P. Comizzoli). *Adv Exp Med Biol.,* 753:275-316. (acknowledged for editing).

b. Conference proceedings (*Presenting author)

***Vu, M.** and Trudeau, V.L. Hormonal induction of spawning and its applications to amphibian conservation. Canadian Herpetological Society Annual Conference, Toronto, ON, Sept. 16-19, 2016.

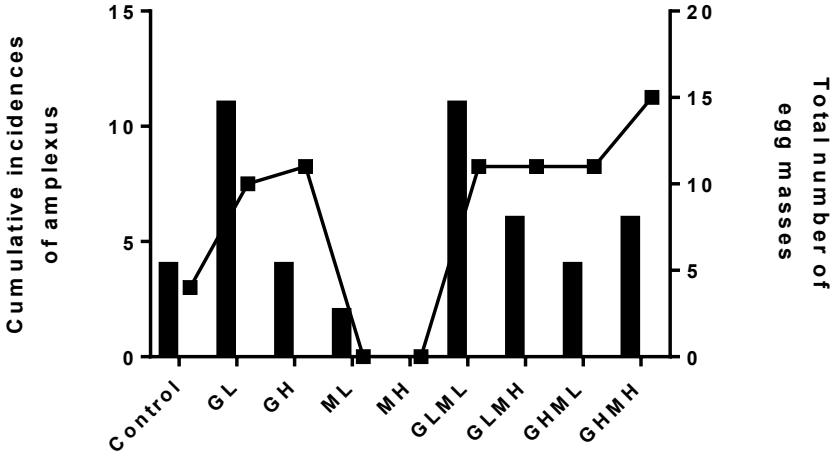
*Trudeau, V.L., **Vu, M.**, Xia, X., Thoney, D., McGinnity, D. and Dancosse, J. Applying the principle of evolutionary endocrinology to develop spawning induction methods in amphibians. 3rd Biennial Conference for The North American Society for Comparative Endocrinology, Ottawa, ON, June 21-25, 2015.

*Gutierrez-Villagomez, J.M., ***Vu, M.**, Gan, A., *Horsman, N. and Trudeau, V.L. Amphibian model for ecotoxicological analyses. Second China-Canada Fish Physiology and Developmental Biology Symposium, University of Ottawa, Oct. 14, 2014.

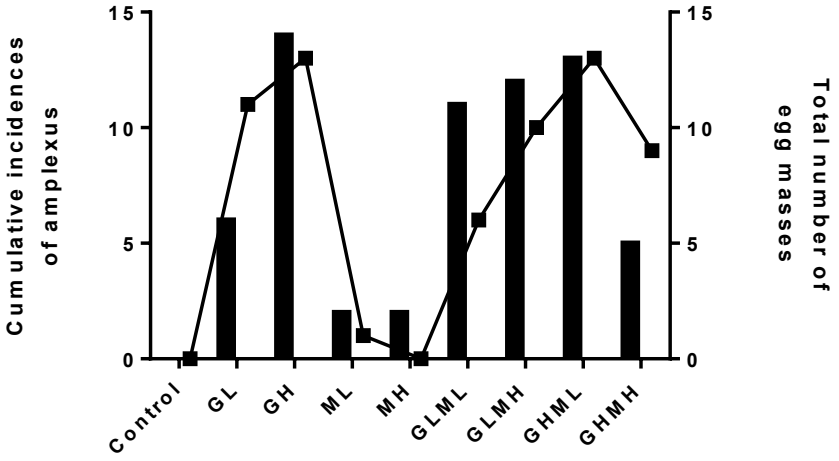
***Vu, M.**, Navarro-Martín, L., Gutierrez-Villagomez, J.M. and Trudeau, V.L. Development of an *in vitro* culture system to test the potential endocrine-disrupting effects of naphthenic acids on wood frog (*Lithobates sylvaticus*) tadpole ovarian development. 41st Aquatic Toxicity Workshop, Ottawa, ON, Sept. 28-Oct 1, 2014.

***Vu, M.**, Navarro-Martín, L., Gutierrez-Villagomez, J.M. and Trudeau, V.L. Sex in a dish: exploring endocrine disruption of gonadal development *in vitro* in wood frogs (*Lithobates sylvaticus*). Comparative Physiology and Biochemistry Workshop, Elmirst's Resort, Keene, ON, Jan. 31-Feb. 2, 2014.

Supplementary Material



Supplementary Figure 3.1 Cumulative incidences of amplexus (solid bars) and total number of egg masses released (lines) in *Lithobates pipiens* four days following injection in May 2015. A GnRH agonist (G) at two doses [High (H) = 0.4 µg/g, Low (L) = 0.2 µg/g)] was administered alone and in combination with two doses of the dopamine antagonist metoclopramide (M) [High (H) = 10 µg/g, Low (L) = 5 µg/g].



Supplementary Figure 3.2 Cumulative incidences of amplexus (solid bars) and total number of egg masses released (lines) in *Lithobates pipiens* six days following injection in May 2016. A GnRH agonist (G) at two doses [High (H) = 0.4 µg/g, Low (L) = 0.1 µg/g)] was administered alone and in combination with two doses of the dopamine antagonist metoclopramide (M) [High (H) = 10 µg/g, Low (L) = 5 µg/g].

Treatment	Number of Egg Masses	p-value
Control (0.7 % NaCl)	4/15	-
GnRH-A Low (0.2 μg/g)	10/15	0.0656
GnRH-A High (0.4 μg/g)	11/15	0.0268*
MET Low (5 μg/g)	0/15	0.0996
MET High (10 μg/g)	0/15	0.0996
GnRH-A Low + MET Low	11/15	0.0268*
GnRH-A Low + MET High	11/15	0.0268*
GnRH-A High + MET Low	11/15	0.0268*
GnRH-A High + MET High	15/15	< 0.0001*

Supplementary Table 3.1 Total number of egg masses released from *Lithobates pipiens* four days following injection in May 2015. A GnRH agonist (G) at two doses [High (H) = 0.4 μg/g, Low (L) = 0.2 μg/g)] was administered alone and in combination with two doses of the dopamine antagonist metoclopramide (M) [High (H) = 10 μg/g, Low (L) = 5 μg/g]. Data were compared to control using Fisher's two-tailed exact test (* $p < 0.05$).

Treatment	Number of Egg Masses	p-value
Control (0.7 % NaCl)	0/15	-
GnRH-A Low (0.1 μg/g)	11/15	< 0.0001*
GnRH-A High (0.4 μg/g)	13/15	< 0.0001*
MET Low (5 μg/g)	1/15	-
MET High (10 μg/g)	0/15	-
GnRH-A Low + MET Low	6/15	0.0169*
GnRH-A Low + MET High	10/15	0.0002*
GnRH-A High + MET Low	13/15	< 0.0001*
GnRH-A High + MET High	9/15	0.0007*

Supplementary Table 3.2 Total number of egg masses released from *Lithobates pipiens* six days following injection in May 2016. A GnRH agonist (G) at two doses [High (H) = 0.4 μg/g, Low (L) = 0.1 μg/g)] was administered alone and in combination with two doses of the dopamine antagonist metoclopramide (M) [High (H) = 10 μg/g, Low (L) = 5 μg/g]. Data were compared to control using Fisher's two-tailed exact test (* $p < 0.05$).

References

Acharjee, S., Maiti, K., Soh, J.M., Im, W.-B., Seong, J.Y., Kwon, H.B., 2002. Differential desensitization and internalization of three different bullfrog gonadotropin-releasing hormone receptors. Mol. Cells 14, 101-107.

Ahn, R.S., Yoo, M.S., Kwon, H.B., 1999. Evidence for two-cell model of steroidogenesis in four species of amphibian. J. Exp. Zool. 284, 91-99.

Alonso-Bedate, M., Carballada, R., Delgado, M.J., 1990. Effects of melatonin on gonadal steroids and glucose plasma levels in frogs (*Rana perezi* and *Rana temporaria*). J. Pineal Res. 8, 79-89.

Alroy, J., 2015. Current extinction rates of reptiles and amphibians. Proc. Natl. Acad. Sci. U.S.A. 112, 13003-13008.

Amoss, M., Burgus, R., Blackwell, R., Vale, W., Fellows, R., Guillemin, R., 1971. Purification, amino acid composition and N-terminus of the hypothalamic luteinizing hormone releasing factor (LRF) of ovine origin. Biochem. Biophys. Res. Commun. 44, 205-210.

Baird, D.T., Campbell, B.K., 1998. Follicle selection in sheep with breed differences in ovulation rate. Mol. Cell. Endocrinol. 145, 89-95.

Ball, J.N., 1981. Hypothalamic control of the pars distalis in fishes, amphibians, and reptiles. Gen. Comp. Endocrinol. 44, 135-170.

Bentley, G.E., Perfito, N., Ukena, K., Tsutsui, K., Wingfield, J.C., 2003. Gonadotropin-inhibitory peptide in song sparrows (*Melospiza melodia*) in different reproductive conditions, and in house sparrows (*Passer domesticus*) relative to chicken-gonadotropin-releasing hormone. J. Neuroendocrinol. 15, 794-802.

Berger, L., Speare, R., Daszak, P., Green, D.E., Cunningham, A.A., Goggin, C.L., Slocombe, R., Ragan, M.A., Hyatt, A.D., McDonald, K.R., Hines, H.B., Lipsi, K.R., Marantelli, G., Parkes, H., 1998. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. Proc. Natl. Acad. Sci. 95, 9031-9036.

Bertrand, F., Thiery, J., Picard, S., Malpaux, B., 1999. Implication of D2-like dopaminergic receptors in the median eminence during the establishment of long-day inhibition of LH secretion in the ewe. J. Endocrinol. 163, 243-254.

Bickford, D., Iskandar, D., Barlian, A., 2008. A lungless frog discovered on Borneo. Curr. Biol. 18, R374-375.

Biju, S.D., Bossuyt, F., 2003. New frog family from India reveals an ancient biogeographical link with the Seychelles. Nature 425, 711-714.

Bishop, P.J., Angulo, A., Lewis, J.P., Moore, R.D., Rabb, G.B., Garcia Moreno, J., 2012. The amphibian extinction crisis - what will it take to put the action into the Amphibian Conservation Action Plan? Surveys and Perspectives Integrating Environment and Society (S.A.P.I.EN.S.) 5.2.

Blaustein, A.R., Gervasi, S.S., Johnson, P.T., Hoverman, J.T., Belden, L.K., Bradley, P.W., Xie, G.Y., 2012. Ecophysiology meets conservation: understanding the role of disease in amphibian population declines. Philos. Trans. R. Soc. Lond. B Biol. Sci. 367, 1688-1707.

Boyd, S.K., 1992. Sexual differences in hormonal control of release calls in bullfrogs. Horm. Behav. 26, 522-535.

Browne, R.K., Li, H., Seratt, J., Kouba, A., 2006. Progesterone improves the number and quality of hormone induced Fowler toad (*Bufo fowleri*) oocytes. Reprod. Biol. Endocrinol. 4, 1-7.

Burgerhout, E., Minegishi, Y., Brittijn, S.A., de Wijze, D.L., Henkel, C.V., Jansen, H.J., Spaink, H.P., Dirks, R.P., van den Thillart, G.E., 2016. Changes in ovarian gene expression profiles and plasma hormone levels in maturing European eel (*Anguilla anguilla*); Biomarkers for broodstock selection. Gen. Comp. Endocrinol. 225, 185-196.

Burmeister, S.S., Wilczynski, W., 2005. Social signals regulate gonadotropin-releasing hormone neurons in the green tree frog. Brain Behav. Evol. 65, 26-32.

Byrne, P.G., Silla, A.J., 2010. Hormonal induction of gamete release, and *in-vitro* fertilisation, in the critically endangered southern corroboree frog, *Pseudophryne corroboree*. Reprod. Biol. Endocrinol. 8, 144.

Carnevali, O., Mosconi, G., Yamamoto, K., Kobayashi, T., Kikuyama, S., Polzonetti-Magni, A.M., 1993. *In-vitro* effects of mammalian and amphibian prolactins on hepatic vitellogenin synthesis in *Rana esculenta*. J. Endocrinol. 137, 383-389.

Cesa, R., Guastalla, A., Cottone, E., Mackie, K., Beltramo, M., Franzoni, M.F., 2002. Relationships between CB1 cannabinoid receptors and pituitary endocrine cells in *Xenopus laevis*: an immunohistochemical study. Gen. Comp. Endocrinol. 125, 17-24.

Chakraborty, M., Burmeister, S.S., 2009. Estradiol induces sexual behavior in female túngara frogs. Horm. Behav. 55, 106-112.

Chakraborty, M., Burmeister, S.S., 2015. Effects of estradiol on neural responses to social signals in female túngara frogs. J. Exp. Biol. 218, 3671-3677.

Chang, J.P., Peter, R.E., 1983. Effects of pimozide and des Gly 10,[D-Ala 6] luteinizing hormone-releasing hormone ethylamide on serum gonadotropin concentrations, germinal vesicle migration, and ovulation in female goldfish, *Carassius auratus*. Gen. Comp. Endocrinol. 52, 30-37.

Chang, J.P., Peter, R.E., Nahorniak, C.S., Sokolowska, M., 1984. Effects of catecholaminergic agonists and antagonists on serum gonadotropin concentrations and ovulation in goldfish: evidence for specificity of dopamine inhibition of gonadotropin secretion. Gen. Comp. Endocrinol. 55, 351-360.

Chen, Y.N., Fan, H.F., Hsieh, S.L., Kuo, C.M., 2003. Physiological involvement of DA in ovarian development of the freshwater giant prawn, *Macrobrachium rosenbergii*. Aquaculture 228, 383-395.

Cheon, M., Park, D., Kim, K., Dai Park, S., Ryu, K., 1999. Homologous upregulation of GnRH receptor mRNA by continuous GnRH in cultured rat pituitary cells. Endocrine 11, 49-55.

Chianese, R., Ciaramella, V., Fasano, S., Pierantoni, R., Meccariello, R., 2013. Kisspeptin receptor, GPR54, as a candidate for the regulation of testicular activity in the frog *Rana esculenta*. Biol. Reprod. 88, 73.

Chow, M.M., Kight, K.E., Gothilf, Y., Alok, D., Stubblefield, J., Zohar, Y., 1998. Multiple GnRHs present in a teleost species are encoded by separate genes: analysis of the sbGnRH and cGnRH-II genes from the striped bass, *Morone saxatilis*. J. Mol. Endocrinol. 21, 277-289.

Chu, J., Wilczynski, W., 2002. Androgen effects on tyrosine hydroxylase cells in the northern leopard frog, *Rana pipiens*. Neuroendocrinology 76, 18-27.

Clulow, J., Trudeau, V.L., Kouba, A.J., 2014. Amphibian declines in the twenty-first century: why we need assisted reproductive technologies. Adv. Exp. Med. Biol. 753, 275-316.

Coddington, E.J., Cree, A., 1995. Effect of acute captivity stress on plasma concentrations of corticosterone and sex steroids in female whistling frogs, *Litoria ewingi*. Gen. Comp. Endocrinol. 100, 33-38.

Collin, F., Chartrel, N., Fasolo, A., Conlon, J.M., Vandesande, F., Vaudry, H., 1995. Distribution of two molecular forms of gonadotropin-releasing hormone (GnRH) in the central nervous system of the frog *Rana ridibunda*. Brain Res. 703, 111-128.

Cooper, N., Bielby, J., Thomas, G.H., Purvis, A., 2008. Macroecology and extinction risk correlates of frogs. Glob. Ecol. Biogeogr. 17, 211-221.

Corn, P.S., Fogleman, J.C., 1984. Extinction of montane populations of the northern leopard frog (*Rana pipiens*) in Colorado. J. Herpetol., 147-152.

Costa, C.S., Trudeau, V.L., Ronco, A.E., Natale, G.S., 2015. Exploring antipredator mechanisms: new findings in ceratophryid tadpoles. J. Herpetol.

Cottone, E., Guastalla, A., Mackie, K., Franzoni, M.F., 2008. Endocannabinoids affect the reproductive functions in teleosts and amphibians. Mol. Cell. Endocrinol. 286, S41-S45.

Cottone, E., Pomatto, V., Bovolin, P., 2013. Role of the endocannabinoid system in the central regulation of nonmammalian vertebrate reproduction. Int. J. Endocrinol. 2013, 1-8.

Cottone, E., Salio, C., Conrath, M., Franzoni, M.F., 2003. *Xenopus laevis* CB1 cannabinoid receptor: molecular cloning and mRNA distribution in the central nervous system. J. Comp. Neurol. 464, 487-496.

Creighton, A., Satterfield, D., Chu, J., 2013. Effects of dopamine agonists on calling behavior in the green tree frog, *Hyla cinerea*. Physiol. Behav. 116, 54-59.

Creighton, A.E., Wilczynski, W., 2014. Influence of dopamine D2-type receptors on motor behaviors in the green tree frog, *Hyla cinerea*. Physiol. Behav. 127, 71-80.

Cynthia, P.d.A., Uetanabaro, M., Haddad, C.F., 2005. Breeding activity patterns, reproductive modes, and habitat use by anurans (Amphibia) in a seasonal environment in the Pantanal, Brazil. Amphibia-Reptilia 26, 211-221.

d'Istria, M., Palmiero, C., Serino, I., Izzo, G., Minucci, S., 2003. Inhibition of the basal and oestradiol-stimulated mitotic activity of primary spermatogonia by melatonin in the testis of the frog, *Rana esculenta*, *in vivo* and *in vitro*. Reproduction 126, 83-90.

d'Istria, M., Serino, I., Izzo, G., Ferrara, D., De Rienzo, G., Minucci, S., 2004. Effects of melatonin treatment on Leydig cell activity in the testis of the frog *Rana esculenta*. Zygote 12, 293-299.

Dailey, R.A., Tsou, R.C., Tindall, G.T., Neill, J.D., 1978. Direct hypophysial inhibition of luteinizing hormone release by dopamine in the rabbit. Life Sci. 22, 1491-1497.

Daniels, E., Licht, P., 1980. Effects of gonadotropin-releasing hormone on the levels of plasma gonadotrophins (FSH and LH) in the bullfrog, *Rana catesbeiana*. Gen. Comp. Endocrinol. 42, 455-463.

Daniels, E.L., Licht, P., Farmer, S.W., Papkoff, H., 1977. Immunochemical studies on the pituitary gonadotropins (FSH and LH) from the bullfrog, *Rana catesbeiana*. Gen. Comp. Endocrinol. 32, 146-157.

Daszak, P., Berger, L., Cunningham, A.A., Hyatt, A.D., Green, D.E., Speare, R., 1999. Emerging infectious diseases and amphibian population declines. Emerg. Infect. Dis. 5, 735-748.

Davis, A., Abraham, E., McEvoy, E., Sonnenfeld, S., Lewis, C., Hubbard, C.S., Dolence, E.K., Rose, J.D., Coddington, E., 2015. Corticosterone suppresses vasotocin-enhanced clasping behavior in male rough-skinned newts by novel mechanisms interfering with V1a receptor availability and receptor-mediated endocytosis. Horm. Behav. 69, 39-49.

Delgado, M.J., Vivien-Roels, B., 1989. Effect of environmental temperature and photoperiod on the melatonin levels in the pineal, lateral eye, and plasma of the frog, *Rana perezi*: importance of ocular melatonin. Gen. Comp. Endocrinol. 75, 46-53.

Della Togna, G., Trudeau, V.L., Gratwicke, B., Evans, M., Augustine, L., Chia, H., Bronikowski, E.J., Murphy, J.B., Comizzoli, P., 2017. Effects of hormonal stimulation on the concentration and quality of excreted spermatozoa in the critically endangered Panamanian golden frog (*Atelopus zeteki*). Theriogenology 91, 27-35.

Deng, J., Carbajal, L., Evaul, K., Rasar, M., Jamnongjit, M., Hammes, S.R., 2009. Nongenomic steroid-triggered oocyte maturation: of mice and frogs. Steroids 74, 595-601.

Deviche, P., Moore, F.L., 1988. Steroidal control of sexual behavior in the rough-skinned newt (*Taricha granulosa*): effects of testosterone, estradiol, and dihydrotestosterone. Horm. Behav. 22, 26-34.

Donnelly, M.A., Guyer, C., 1994. Patterns of reproduction and habitat use in an assemblage of Neotropical hylid frogs. Oecologia 98, 291-302.

Duarte-Guterman, P., Langlois, V.S., Pauli, B.D., Trudeau, V.L., 2010. Expression and T3 regulation of thyroid hormone-and sex steroid-related genes during *Silurana (Xenopus) tropicalis* early development. Gen. Comp. Endocrinol. 166, 428-435.

Duellman, W.E., Trueb, L., 1986. Biology of amphibians. McGraw-Hill Publishing Company, New York.

Dufour, S., Weltzien, F.A., Sebert, M.E., Le Belle, N., Vidal, B., Vernier, P., Pasqualini, C., 2005. Dopaminergic inhibition of reproduction in teleost fishes: ecophysiological and evolutionary implications. Ann. N. Y. Acad. Sci. 1040, 9-21.

Farmer, S.W., Licht, P., Papkoff, H., Daniels, E.L., 1977. Purification of gonadotropins in the leopard frog (*Rana pipiens*). Gen. Comp. Endocrinol. 32, 158-162.

Ghosh, P.K., Ghosh, A.K., Biswas, N.M., 1984. Effect of cadmium chloride on steroidogenic enzymes in the Bidder's organ of the toad (*Bufo melanostictus*). Cell. Mol. Life Sci. 40, 91-92.

Gibbs, E.L., Nace, G.W., Emmons, M.B., 1971. The live frog is almost dead. Bioscience 21, 1027-1034.

Gonzalez, A., Smeets, W.J., 1991. Comparative analysis of dopamine and tyrosine hydroxylase immunoreactivities in the brain of two amphibians, the anuran *Rana ridibunda* and the urodele *Pleurodeles waltlii*. J. Comp. Neurol. 303, 457-477.

Gordon, N.M., Gerhardt, H.C., 2009. Hormonal modulation of phonotaxis and advertisement-call preferences in the gray treefrog (*Hyla versicolor*). Horm. Behav. 55, 121-127.

Gow, C., Sharp, P., Carter, N., Scaramuzzi, R., Sheldon, B., Yoo, B., Talbot, R., 1985. Effects of selection for reduced oviposition interval on plasma concentrations of luteinising hormone during the ovulatory cycle in hens on a 24 h lighting cycle. Br. Poult. Sci. 26, 441-451.

Gow, C., Sharp, P., Carter, N., Sheldon, B., Scaramuzzi, R., Yoo, B., 1986. Plasma concentrations of luteinising hormone during the ovulatory cycle in hens selected for reduced oviposition interval and maintained in continuous light or a 24 h light: dark cycle. Br. Poult. Sci. 27, 137-146.

Gracia-Navarro, F., Lamacz, M., Tonon, M.C., Vaudry, H., 1992. Pituitary adenylate cyclase-activating polypeptide stimulates calcium mobilization in amphibian pituitary cells. Endocrinology 131, 1069-1074.

Gracia-Navarro, F., Licht, P., 1987. Subcellular localization of gonadotrophic hormones LH and FSH in frog adenohypophysis using double-staining immunocytochemistry. J. Histochem. Cytochem. 35, 763-769.

Grey, C.L., Chang, J.P., 2013. Differential modulation of ghrelin-induced GH and LH release by PACAP and dopamine in goldfish pituitary cells. Gen. Comp. Endocrinol. 191, 215-224.

Griffiths, R.A., Pavajeau, L., 2008. Captive breeding, reintroduction, and the conservation of amphibians. Conserv. Biol. 22, 852-861.

Gurdon, J.B., Hopwood, N., 2003. The introduction of *Xenopus laevis* into developmental biology: of empire, pregnancy testing and ribosomal genes. Int. J. Dev. Biol. 44, 43-50.

Haddad, C.F., Prado, C.P., 2005. Reproductive modes in frogs and their unexpected diversity in the Atlantic Forest of Brazil. Bioscience 55, 207-217.

Halvorson, L.M., 2014. PACAP modulates GnRH signaling in gonadotropes. Mol. Cell. Endocrinol. 385, 45-55.

Haraguchi, S., Koyama, T., Hasunuma, I., Vaudry, H., Tsutsui, K., 2010. Prolactin increases the synthesis of 7alpha-hydroxypregnenolone, a key factor for induction of locomotor activity, in breeding male newts. Endocrinology 151, 2211-2222.

Harding, G., Griffiths, R.A., Pavajeau, L., 2015. Developments in amphibian captive breeding and reintroduction programs. Conserv. Biol.

Hayes, M.P., Jennings, M.R., 1986. Decline of ranid frog species in western North America: are bullfrogs (*Rana catesbeiana*) responsible? J. Herpetol., 490-509.

Heckmann, L.-H., Sørensen, P.B., Krogh, P.H., Sørensen, J.G., 2011. NORMA-Gene: A simple and robust method for qPCR normalization based on target gene data. BMC Bioinformatics 12, 250.

Hellsten, U., Harland, R.M., Gilchrist, M.J., Hendrix, D., Jurka, J., Kapitonov, V., Ovcharenko, I., Putnam, N.H., Shu, S., Taher, L., 2010. The genome of the Western clawed frog *Xenopus tropicalis*. Science 328, 633-636.

Herbison, A.E., Porteous, R., Pape, J.-R., Mora, J.M., Hurst, P.R., 2008. Gonadotropin-releasing hormone neuron requirements for puberty, ovulation, and fertility. Endocrinology 149, 597-604.

Hill, J.E., Kilgore, K.H., Pouder, D.B., Powell, J.F.F., Watson, C.A., Yanong, R.P.E., 2009. Survey of ovaprim use as a spawning aid in ornamental fishes in the United States as administered through the University of Florida tropical aquaculture laboratory. N. Am. J. Aquac. 71, 206-209.

Hirsch, N., Zimmerman, L.B., Grainger, R.M., 2002. *Xenopus*, the next generation: *X. tropicalis* genetics and genomics. Dev. Dyn. 225, 422-433.

Hocking, P.M., 2014. Unexpected consequences of genetic selection in broilers and turkeys: problems and solutions. Br. Poult. Sci. 55, 1-12.

Hödl, W., 1990. Reproductive diversity in Amazonian lowland frogs. Fortschr. Zool. 38, 41-60.

Horseman, N.D., Smith, C.A., Culley Jr, D.D., 1978. Effects of age and photoperiod on ovary size and condition in bullfrogs (*Rana catesbeiana* Shaw)(Amphibia, Anura, Ranidae). J. Herpetol. 12, 287-290.

Houlahan, J.E., Findlay, C.S., Schmidt, B.R., Meyer, A.H., Kuzmin, S.L., 2000. Quantitative evidence for global amphibian population declines. Nature 404, 752-755.

Howard, C.M., Lutterschmidt, D.I., 2015. The effects of melatonin on brain arginine vasotocin: relationship to sex and seasonal differences in MT1 in green treefrogs (*Hyla cinerea*). J. Neuroendocrinol. 27, 670-679.

Huang, Y., Peng, C., Peter, R., 1991. Metabolism of gonadotropin-releasing hormone in goldfish: Serum clearance and tissue uptake studies. Gen. Comp. Endocrinol. 84, 67-75.

Huseman, C.A., Kugler, J.A., Schneider, I.G., 1980. Mechanism of dopaminergic suppression of gonadotropin secretion in men*. J. Clin. Endocrinol. Metab. 51, 209-214.

Iela, L., D'Aniello, B., Di Meglio, M., Rastogi, R.K., 1994. Influence of gonadectomy and steroid hormone replacement therapy on the gonadotropin-releasing hormone neuronal system in the anterior preoptic area of the frog (*Rana esculenta*) brain. Gen. Comp. Endocrinol. 95, 422-431.

Ishii, S., Itoh, M., 1992. Amplexus induces surge of luteinizing hormone in male toads, *Bufo japonicus*. Gen. Comp. Endocrinol. 86, 34-41.

Isorna, E., Delgado, M.J., Guijarro, A.I., López-Patiño, M.A., Alonso-Bedate, M., Alonso-Gómez, A.L., 2004. 2-[125I]-Melatonin binding sites in the central nervous system and neural retina of the frog *Rana perezi*: regulation by light and temperature. Gen. Comp. Endocrinol. 139, 95-102.

Itoh, M., Inoue, M., Ishii, S., 1990. Annual cycle of pituitary and plasma gonadotropins and plasma sex steroids in a wild population of the toad, *Bufo japonicus*. Gen. Comp. Endocrinol. 78, 242-253.

Iwata, T., Toyoda, F., Yamamoto, K., Kikuyama, S., 2000. Hormonal control of urodele reproductive behavior. Biochem. Physiol. Biochem. Mol. Biol. 126, 221-229.

Janssenswillen, S., Vandebergh, W., Treer, D., Willaert, B., Maex, M., Van Bocxlaer, I., Bossuyt, F., 2014. Origin and diversification of a salamander sex pheromone system. Mol. Biol. Evol. 32, 472-480.

Johnson, P., Stephens, C., Giles, J., 2015. The domestic chicken: causes and consequences of an egg a day. Poult. Sci. 94, 816-820.

Johnson, P.T., McKenzie, V.J., Peterson, A.C., Kerby, J.L., Brown, J., Blaustein, A.R., Jackson, T., 2011. Regional decline of an iconic amphibian associated with elevation, land-use change, and invasive species. Conserv. Biol. 25, 556-566.

Kashiwagi, K., Kashiwagi, A., Kurabayashi, A., Hanada, H., Nakajima, K., Okada, M., Takase, M., Yaoita, Y., 2010. *Xenopus tropicalis*: an ideal experimental animal in amphibia. Exp. Anim. 59, 395-405.

Kelley, D.B., Pfaff, D.W., 1976. Hormone effects on male sex behavior in adult South African clawed frogs, *Xenopus laevis*. Horm. Behav. 7, 159-182.

Khokha, M.K., Chung, C., Bustamante, E.L., Gaw, L.W., Trott, K.A., Yeh, J., Lim, N., Lin, J.C., Taverner, N., Amaya, E., 2002. Techniques and probes for the study of *Xenopus tropicalis* development. Dev. Dyn. 225, 499-510.

Kikuyama, S., Hasunuma, I., Toyoda, F., Haraguchi, S., Tsutsui, K., 2009. Hormone-mediated reproductive behavior in the red-bellied newt. Ann. N. Y. Acad. Sci. 1163, 179-186.

King, J.A., Millar, R.P., 1986. Identification of His 5, Trp 7, Tyr 8-GnRH (chicken GnRH II) in amphibian brain. Peptides 7, 827-834.

Kirkpatrick, B.L., Esquivel, E., Moss, G.E., Hamernik, D.L., Wise, M.E., 1998. Estradiol and gonadotropin-releasing hormone (GnRH) interact to increase GnRH receptor expression in ovariectomized ewes after hypothalamic-pituitary disconnection. Endocrine 8, 225-229.

Koda, A., Ukena, K., Teranishi, H., Ohta, S., Yamamoto, K., Kikuyama, S., Tsutsui, K., 2002. A novel amphibian hypothalamic neuropeptide: isolation, localization, and biological activity. Endocrinology 143, 411-419.

Köhler, J., Vieites, D.R., Bonett, R.M., Garcia, F.H., Glaw, F., Steinke, D., Vences, M., 2005. Boost in species discoveries in a highly endangered vertebrate group: new amphibians and global conservation. Bioscience 55, 693-696.

Kotani, M., Detheux, M., Vandenbogaerde, A., Communi, D., Vanderwinden, J.-M., Le Poul, E., Brézillon, S., Tyldesley, R., Suarez-Huerta, N., Vandeput, F., 2001. The metastasis suppressor gene KiSS-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54. J. Biol. Chem. 276, 34631-34636.

Kouba, A., Vance, C., Calatayud, N., Rowlison, T., Langhorne, C., Willard, S., 2012. Assisted Reproductive Technologies (ART) for Amphibians. Amphibian Husbandry Resource Guide 60.

Kubokawa, K., Ishii, S., 1980. Follicle-stimulating hormone (FSH) receptors in the testis of the newt, *Cynops pyrrhogaster*, and comparison of temperature dependency of the receptors with those of the other vertebrates. Gen. Comp. Endocrinol. 40, 425-433.

Kubokawa, K., Ishii, S., 1987. Receptors for native gonadotropins in amphibian liver. Gen. Comp. Endocrinol. 68, 260-270.

Kwon, H.B., Ahn, R.S., 1994. Relative roles of theca and granulosa cells in ovarian follicular steroidogenesis in the amphibian, *Rana nigromaculata*. Gen. Comp. Endocrinol. 94, 207-214.

Langlois, V.S., Duarte-Guterman, P., Ing, S., Pauli, B.D., Cooke, G.M., Trudeau, V.L., 2010. Fadrozole and finasteride exposures modulate sex steroid-and thyroid hormone-related gene expression in *Silurana* (*Xenopus*) *tropicalis* early larval development. Gen. Comp. Endocrinol. 166, 417-427.

Lee, Y.R., Tsunekawa, K., Moon, M.J., Um, H.N., Hwang, J.I., Osugi, T., Otaki, N., Sunakawa, Y., Kim, K., Vaudry, H., Kwon, H.B., Seong, J.Y., Tsutsui, K., 2009. Molecular evolution of multiple forms of kisspeptins and GPR54 receptors in vertebrates. Endocrinology 150, 2837-2846.

Leonard, W.P., McAllister, K.R., Friesz, R.C., 1999. Survey and assessment of northern leopard frog (*Rana pipiens*) populations in Washington State. Northwest Nat., 51-60.

Levavi-Sivan, B., Bogerd, J., Mañanós, E.L., Gómez, A., Lareyre, J.J., 2010. Perspectives on fish gonadotropins and their receptors. Gen. Comp. Endocrinol. 165, 412-437.

Licht, P., 1979. Reproductive endocrinology of reptiles and amphibians: Gonadotropins. Ann. Rev. Physiol. 41, 337-351.

Licht, P., 1995. Conservation of endangered species in captivity: an interdisciplinary approach, in: Gibbons Jr., E.F., Durrant, B.S., Demarest, J. (Eds.), Reproductive physiology and reptiles and amphibians. State University of New York Press, New York, USA, pp. 169-186.

Licht, P., McCreery, B.R., Barnes, R., Pang, R., 1983. Seasonal and stress related changes in plasma gonadotropins, sex steroids, and corticosterone in the bullfrog, *Rana catesbeiana*. Gen. Comp. Endocrinol. 50, 124-145.

Licht, P., Papkoff, H., 1974. Separation of two distinct gonadotropins from the pituitary gland of the bullfrog *Rana catesbeiana*. Endocrinology 94, 1587-1594.

Licht, P., Tsai, P.S., Sotowska-Brochocka, J., 1994. The nature and distribution of gonadotropin-releasing hormones in brains and plasma of ranid frogs. Gen. Comp. Endocrinol. 94, 186-198.

Lin, X.-W., Lin, H.-R., Peter, R.E., 1994. Seasonal variations in gonadotropin responsiveness, self-priming, and desensitization to GnRH peptides in the common carp pituitary *in vitro*. Gen. Comp. Endocrinol. 93, 275-287.

Lopez, J.M., Morona, R., Gonzalez, A., 2010. Immunohistochemical localization of DARPP-32 in the brain and spinal cord of anuran amphibians and its relation with the catecholaminergic system. J. Chem. Neuroanat. 40, 325-338.

Lutterschmidt, D.I., Wilczynski, W., 2012. Sexually dimorphic effects of melatonin on brain arginine vasotocin immunoreactivity in green treefrogs (*Hyla cinerea*). Brain Behav. Evol. 80, 222-232.

Lynch, K.S., Wilczynski, W., 2006. Social regulation of plasma estradiol concentration in a female anuran. Horm. Behav. 50, 101-106.

Martin, D., Hong, H., 1991. The use of Bactine® in the treatment of open wounds and other lesions in captive anurans. Herpetol. Rev. 22, 21.

Matsunaga, M., Ukena, K., Baulieu, E.E., Tsutsui, K., 2004. 7alpha-hydroxypregnenolone acts as a neuronal activator to stimulate locomotor activity of breeding newts by means of the dopaminergic system. Proc. Natl. Acad. Sci. U.S.A. 101, 17282-17287.

Matsuo, H., Baba, Y., Nair, R.G., Arimura, A., Schally, A.V., 1971. Structure of the porcine LH- and FSH-releasing hormone. I. The proposed amino acid sequence. Biochem. Biophys. Res. Commun. 43, 1334-1339.

McCallum, M.L., 2007. Amphibian decline or extinction? Current declines dwarf background extinction rate. J. Herpetol. 41, 483-491.

McClelland, B.E., Wilczynski, W., 1989. Release call characteristics of male and female *Rana pipiens*. Copeia 4, 1045-1049.

McCreery, B.R., Licht, P., 1983a. Induced ovulation and changes in pituitary responsiveness to continuous infusion of gonadotropin-releasing hormone during the ovarian cycle in the bullfrog, *Rana catesbeiana*. Biol. Reproduc. 29, 863-871.

McCreery, B.R., Licht, P., 1983b. Pituitary and gonadal responses to continuous infusion of gonadotropin releasing hormone in the male bullfrog, *Rana catesbeiana*. Biol. Reproduc. 29, 129-136.

McCreery, B.R., Licht, P., 1984. Effects of gonadectomy and sex steroids on pituitary gonadotrophin release and response to gonadotrophin-releasing hormone (GnRH) agonist in the bullfrog, *Rana catesbeiana*. Gen. Comp. Endocrinol. 54, 283-296.

McCreery, B.R., Licht, P., Barnes, R., Rivier, J.E., Vale, W.W., 1982. Actions of agonistic and antagonistic analogs of gonadotropin releasing hormone (Gn-RH) in the bullfrog *Rana catesbeiana*. Gen. Comp. Endocrinol. 46, 511-520.

Meccariello, R., Chianese, R., Cobellis, G., Pierantoni, R., Fasano, S., 2007. Cloning of type 1 cannabinoid receptor in *Rana esculenta* reveals differences between genomic sequence and cDNA. FEBS J. 274, 2909-2920.

Meccariello, R., Chianese, R., Fasano, S., Pierantoni, R., 2013. Endocannabinoids and kisspeptins: two modulators in fight for the regulation of GnRH activity, in: Vizcarra, J. (Ed.), Gonadotropins. InTech, Rijeka, Croatia, pp. 57-88.

Meccariello, R., Franzoni, M.F., Chianese, R., Cottone, E., Scarpa, D., Donna, D., Cobellis, G., Guastalla, A., Pierantoni, R., Fasano, S., 2008. Interplay between the endocannabinoid system and GnRH-I in the forebrain of the anuran amphibian *Rana esculenta*. Endocrinology 149, 2149-2158.

Mendonça, M.T., Licht, P., Ryan, M.J., Barnes, R., 1985. Changes in hormone levels in relation to breeding behavior in male bullfrogs (*Rana catesbeiana*) at the individual and population levels. Gen. Comp. Endocrinol. 58, 270-279.

Michael, S.F., Buckley, C., Toro, E., Estrada, A.R., Vincent, S., 2004. Induced ovulation and egg deposition in the direct developing anuran *Eleutherodactylus coqui*. Reprod. Biol. Endocrinol. 2, 6.

Millar, R.P., Lu, Z.L., Pawson, A.J., Flanagan, C.A., Morgan, K., Maudsley, S.R., 2004. Gonadotropin-releasing hormone receptors. Endocr. Rev. 25, 235-275.

Moon, J.S., Lee, Y.R., Oh, D.Y., Hwang, J.I., Lee, J.Y., Kim, J.I., Vaudry, H., Kwon, H.B., Seong, J.Y., 2009. Molecular cloning of the bullfrog kisspeptin receptor GPR54 with high sensitivity to *Xenopus* kisspeptin. Peptides 30, 171-179.

Moore, F.L., 1978. Differential effects of testosterone plus dihydrotestosterone on male courtship of castrated newts, *Taricha granulosa*. Horm. Behav. 11, 202-208.

Moore, F.L., 1987. Reproductive endocrinology of amphibians, in: Chester-Jones, I., Ingleton, P.M., Phillips, J.G. (Eds.), Fundamentals of Comparative Vertebrate Endocrinology. Springer, US, pp. 207-221.

Moore, F.L., Boyd, S.K., Kelley, D.B., 2005. Historical perspective: hormonal regulation of behaviors in amphibians. Horm. Behav. 48, 373-383.

Moore, F.L., Miller, L.J., 1984. Stress-induced inhibition of sexual behavior: corticosterone inhibits courtship behaviors of a male amphibian (*Taricha granulosa*). Horm. Behav. 18, 400-410.

Moore, F.L., Zoeller, R.T., 1985. Stress-induced inhibition of reproduction: evidence of suppressed secretion of LH-RH in an amphibian. Gen. Comp. Endocrinol. 60, 252-258.

Nakano, M., Hasunuma, I., Okada, R., Yamamoto, K., Kikuyama, S., Machida, T., Kobayashi, T., 2010a. Molecular cloning of bullfrog D2 dopamine receptor cDNA: tissue distribution of three isoforms of D2 dopamine receptor mRNA. Gen. Comp. Endocrinol. 168, 143-148.

Nakano, M., Minagawa, A., Hasunuma, I., Okada, R., Tonon, M.C., Vaudry, H., Yamamoto, K., Kikuyama, S., Machida, T., Kobayashi, T., 2010b. D2 Dopamine receptor subtype mediates the inhibitory effect of dopamine on TRH-induced prolactin release from the bullfrog pituitary. Gen. Comp. Endocrinol. 168, 287-292.

Nieuwkoop, P.D., Faber, J., 1994. Normal table of *Xenopus laevis* (Daudin): a systematical and chronological survey of the development from the fertilized egg till the end of metamorphosis. Garland Publishing Inc, New York.

Notter, D., 2012. Genetic improvement of reproductive efficiency of sheep and goats. Anim. Reprod. Sci. 130, 147-151.

O'Connell, L.A., Matthews, B.J., Ryan, M.J., Hofmann, H.A., 2010. Characterization of the dopamine system in the brain of the tungara frog, *Physalaemus pustulosus*. Brain Behav. Evol. 76, 211-225.

Ogawa, A., Dake, J., Iwashina, Y.-k., Tokumoto, T., 2011. Induction of ovulation in *Xenopus* without hCG injection: the effect of adding steroids into the aquatic environment. Reprod. Biol. Endocrinol. 9, 11.

Ogielska, M., 2009. Reproduction of amphibians. Science Publishers, Enfield, New Hampshire.

Oguchi, A., Tanaka, S., Aida, T., Yamamoto, K., Kikuyama, S., 1997. Enhancement by prolactin of the GnRH-induced release of LH from dispersed anterior pituitary cells of the bullfrog (*Rana catesbeiana*). Gen. Comp. Endocrinol. 107, 128-135.

Orchinik, M., Licht, P., Crews, D., 1988. Plasma steroid concentrations change in response to sexual behavior in *Bufo marinus*. Horm. Behav. 22, 338-350.

Osugi, T., Ukena, K., Bentley, G.E., O'Brien, S., Moore, I.T., Wingfield, J.C., Tsutsui, K., 2004. Gonadotropin-inhibitory hormone in Gambel's white-crowned sparrow (*Zonotrichia leucophrys gambelii*): cDNA identification, transcript localization and functional effects in laboratory and field experiments. J. Endocrinol. 182, 33-42.

Pagotto, U., Marsicano, G., Cota, D., Lutz, B., Pasquali, R., 2006. The emerging role of the endocannabinoid system in endocrine regulation and energy balance. Endocr. Rev. 27, 73-100.

Pancak-Roessler, M.K., Norris, D.O., 1991. The effects of orchidectomy and gonadotropins on steroidogenesis and oogenesis in Bidder's organs of the toad *Bufo woodhousii*. J. Exp. Zool. 260, 323-336.

Papkoff, H., Farmer, S.W., Licht, P., 1976. Isolation and characterization of luteinizing hormone from amphibian (*Rana catesbeiana*) pituitaries. Life Sci. 18, 245-250.

Pavgi, S., Licht, P., 1989. Effects of gonadectomy and steroids on pituitary gonadotropin secretion in a frog, *Rana pipiens*. Biol. Reprod. 41, 40-48.

Perry, G., Wallace, M.C., Perry, D., Curzer, H., Muhlberger, P., 2011. Toe Clipping of Amphibians and Reptiles: Science, Ethics, and the Law. J. Herpetol. 45, 547-555.

Peter, R.E., 1980. Serum gonadotropin levels in mature male goldfish in response to luteinizing hormone--releasing hormone (LH-RH) and des-Gly10-[D-Ala6]-LH-RH ethylamide. Can. J. Zool. 58, 1100-1104.

Peter, R.E., Lin, H.R., Van Der Kraak, G., 1988. Induced ovulation and spawning of cultured freshwater fish in China: advances in application of GnRH analogues and dopamine antagonists. Aquaculture 74, 1-10.

Peter, R.E., Sokolowska, M., Nahorniak, C.S., Rivier, J.E., Vale, W.W., 1987. Comparison of [d-Arg6, Trp7, Leu8, Pro9 N Et]-luteinizing hormone-releasing hormone (sGnRH-A), and [d-Ala6, Pro9 N Et]-luteinizing hormone-releasing hormone (LHRH-A), in combination with pimozide, in stimulating gonadotropin release and ovulation in the goldfish, *Carassius auratus*. Can. J. Zool. 65, 987-991.

Phillott, A.D., Skerratt, L.F., McDonald, K.R., Lemckert, F.L., Hines, H.B., Clarke, J.M., Alford, R.A., Speare, R., 2007. Toe-clipping as an acceptable method of identifying individual anurans in mark recapture studies. Herpetol. Rev. 38, 305-308.

Pinelli, C., Jadhao, A.G., Biswas, S.P., Tsutsui, K., D"Aniello, B., 2015. Neuroanatomical organization of the brain gonadotropin-inhibitory hormone and gonadotropin-releasing hormone systems in the frog *Pelophylax esculentus*. Brain Behav. Evol. 85, 15-28.

Polzonetti-Magni, A.M., Mosconi, G., Carnevali, O., Yamamoto, K., Hanaoka, Y., Kikuyama, S., 1998. Gonadotropins and reproductive function in the anuran amphibian, *Rana esculenta*. Biol. Reprod. 58, 88-93.

Popesku, J.T., Martyniuk, C.J., Mennigen, J., Xiong, H., Zhang, D., Xia, X., Cossins, A.R., Trudeau, V.L., 2008. The goldfish (*Carassius auratus*) as a model for neuroendocrine signaling. Mol. Cell. Endocrinol. 293, 43-56.

Porter, D.A., Licht, P., 1985. Pituitary responsiveness to superfused GnRH in two species of ranid frogs. Gen. Comp. Endocrinol. 59, 308-315.

Rao, P.P., Hartwig, H.G., 1974. Monoaminergic tracts of the diencephalon and innervation of the pars intermedia in *Rana temporaria*. Cell. Tissue Res. 151, 1-26.

Rasar, M.A., Hammes, S.R., 2006. The physiology of the *Xenopus laevis* ovary, in: Liu, X.J. (Ed.), Xenopus Protocols: Cell Biology and Signal Transduction. Humana Press, Totowa, New Jersey, pp. 17-30.

Ribeiro, L.F., Bornschein, M.R., Belmonte-Lopes, R., Firkowski, C.R., Morato, S.A., Pie, M.R., 2015. Seven new microendemic species of *Brachycephalus* (Anura: Brachycephalidae) from southern Brazil. PeerJ 3, e1011.

Rivier, J., Rivier, C., Branton, D., Millar, R., Spiess, J., Vale, W., 1981. HPLC purification of ovine CRF, rat extra hypothalamic brain somatostatin and frog brain GnRH, Peptides: Synthesis-structure-function. Pierce Chemical Company Rockford, IL, pp. 771-776.

Roa, J., Aguilar, E., Dieguez, C., Pinilla, L., Tena-Sempere, M., 2008. New frontiers in kisspeptin/GPR54 physiology as fundamental gatekeepers of reproductive function. Front. Neuroendocrinol. 29, 48-69.

Rogers, S.D., Peacock, M.M., 2012. The disappearing northern leopard frog (*Lithobates pipiens*): conservation genetics and implications for remnant populations in western Nevada. Ecol. Evol. 2, 2040-2056.

Saligaut, C., Linard, B., Breton, B., Anglade, I., Bailhache, T., Kah, O., Jego, P., 1999. Brain aminergic systems in salmonids and other teleosts in relation to steroid feedback and gonadotropin release. Aquaculture 177, 13-20.

Sassone, A.G., Regueira, E., Scaia, M.F., Volonteri, M.C., Ceballos, N.R., 2015. Development and steroidogenic properties of the Bidder's organ of the tadpole of *Rhinella arenarum* (Amphibia, Anura). J. Exp. Zool. A Ecol. Genet. Physiol. 323, 137-145.

Scaia, M.F., Czuchlej, S.C., Cervino, N., Ceballos, N.R., 2015. Apoptosis, proliferation and presence of estradiol receptors in the testes and Bidder's organ of the toad *Rhinella arenarum* (Amphibia, Anura). J. Morphol.

Scaia, M.F., Regueira, E., Sassone, A.G., Volonteri, M.C., Ceballos, N.R., 2011. The Bidder's organ of the toad *Rhinella arenarum* (Amphibia, Anura). Presence of steroidogenic enzymes. J. Exp. Zool. A Ecol. Genet. Physiol. 315, 439-446.

Schally, A.V., 1978. Aspects of hypothalamic regulation of the pituitary gland. Science 202, 18-28.

Seong, J.Y., Wang, L., Oh, D.Y., Yun, O., Maiti, K., Li, J.H., Soh, J.M., Choi, H.S., Kim, K., Vaudry, H., 2003. Ala/Thr201 in extracellular loop 2 and Leu/Phe290 in transmembrane domain 6 of type 1 frog gonadotropin-releasing hormone receptor confer differential ligand sensitivity and signal transduction. Endocrinology 144, 454-466.

Sharp, P.J., Talbot, R.T., Macnamee, M.C., 1989. Evidence for the involvement of dopamine and 5-hydroxytryptamine in the regulation of the preovulatory release of luteinizing hormone in the domestic hen. Gen. Comp. Endocrinol. 76, 205-213.

Sherwood, N.M., Harvey, B., 1986. Topical absorption of gonadotropin-releasing hormone (GnRH) in goldfish. Gen. Comp. Endocrinol. 61, 13-19.

Sherwood, N.M., Zoeller, R.T., Moore, F.L., 1986. Multiple forms of gonadotropin-releasing hormone in amphibian brains. Gen. Comp. Endocrinol. 61, 313-322.

Silla, A.J., 2011. Effect of priming injections of luteinizing hormone-releasing hormone on spermiation and ovulation in Gupsilonnther's toadlet, *Pseudophryne guentheri*. Reprod. Biol. Endocrinol. 9, 68.

Skinner, D.C., Robinson, J.E., 1997. Luteinising hormone secretion from the perifused ovine *pars tuberalis* and *pars distalis*: effects of gonadotropin-releasing hormone and melatonin. Neuroendocrinology 66, 263-270.

Soderstrom, K., Leid, M., Moore, F.L., Murray, T.F., 2000. Behavioral, pharmacological, and molecular characterization of an amphibian cannabinoid receptor. J. Neurochem. 75, 413-423.

Sokolowska, M., Peter, R.E., Nahorniak, C.S., Chang, J.P., 1985. Seasonal effects of pimozide and des Gly 10 [D-Ala 6] LH-RH ethylamide on gonadotrophin secretion in goldfish. Gen. Comp. Endocrinol. 57, 472-479.

Sotowska-Brochocka, J., 1988. The stimulatory and inhibitory role of the hypothalamus in the regulation of ovulation in grass frog, *Rana temporaria* L. Gen. Comp. Endocrinol. 70, 83-90.

Sotowska-Brochocka, J., Licht, P., 1992. Effect of infundibular lesions on GnRH and LH release in the frog, *Rana temporaria*, during hibernation. Gen. Comp. Endocrinol. 85, 43-54.

Sotowska-Brochocka, J., Martyńska, L., Licht, P., 1994. Dopaminergic inhibition of gonadotropic release in hibernating frogs, *Rana temporaria*. Gen. Comp. Endocrinol. 93, 192-196.

Stamper, D.L., Licht, P., 1990. Effect of gonadotropin-releasing hormone on gonadotropin biosynthesis in pituitaries of the frog, *Rana pipiens*. Biol. Reprod. 43, 420-426.

Stamper, D.L., Licht, P., 1991. Time-dependent changes in gonadotropin synthesis following gonadectomy in the frog, *Rana pipiens*. Biol. Reprod. 44, 798-805.

Stamper, D.L., Licht, P., 1993a. Further studies on the influence of GnRH on the biosynthesis of gonadotropins in female frogs (*Rana pipiens*). Gen. Comp. Endocrinol. 92, 104-112.

Stamper, D.L., Licht, P., 1993b. *In vitro* exposure of frog (*Rana pipiens*) pituitaries to gonadotropin-releasing hormone results in a persistent stimulation of gonadotropin biosynthesis. J. Exp. Zool. 265, 646-652.

Stamper, D.L., Licht, P., 1994. Influence of androgen on the GnRH-stimulated secretion and biosynthesis of gonadotropins in the pituitary of juvenile female bullfrogs, *Rana catesbeiana*. Gen. Comp. Endocrinol. 93, 93-102.

Tam, J.K., Lee, L.T., Cheng, C.H., Chow, B.K., 2011. Discovery of a new reproductive hormone in teleosts: pituitary adenylate cyclase-activating polypeptide-related peptide (PRP). Gen. Comp. Endocrinol. 173, 405-410.

Tang, H., Liu, Y., Luo, D., Ogawa, S., Yin, Y., Li, S., Zhang, Y., Hu, W., Parhar, I.S., Lin, H., 2015. The kiss/kissr systems are dispensable for zebrafish reproduction: evidence from gene knockout studies. Endocrinology 156, 589-599.

Taylor, S., Wakem, M., Dijkman, G., Alsarraj, M., Nguyen, M., 2010. A practical approach to RT-qPCR—publishing data that conform to the MIQE guidelines. Methods 50, S1-S5.

Tena-Sempere, M., Felip, A., Gómez, A., Zanuy, S., Carrillo, M., 2012. Comparative insights of the kisspeptin/kisspeptin receptor system: lessons from non-mammalian vertebrates. Gen. Comp. Endocrinol. 175, 234-243.

Tischkau, S.A., Howell, R.E., Hickok, J.R., Krager, S.L., Bahr, J.M., 2011. The luteinizing hormone surge regulates circadian clock gene expression in the chicken ovary. Chronobiol. Int. 28, 10-20.

Toyoda, F., Hasunuma, I., Nakada, T., Haraguchi, S., Tsutsui, K., Kikuyama, S., 2012. Involvement of the neurosteroid 7alpha-hydroxypregnenolone in the courtship behavior of the male newt *Cynops pyrrhogaster*. Horm. Behav. 62, 375-380.

Toyoda, F., Hasunuma, I., Nakada, T., Haraguchi, S., Tsutsui, K., Kikuyama, S., 2015. Possible hormonal interaction for eliciting courtship behavior in the male newt, *Cynops pyrrhogaster*. Gen. Comp. Endocrinol. 224, 96-103.

Toyoda, F., Ito, M., Tanaka, S., Kikuyama, S., 1993. Hormonal induction of male courtship behavior in the Japanese newt, *Cynops pyrrhogaster*. Horm. Behav. 27, 511-522.

Toyoda, F., Matsuda, K., Yamamoto, K., Kikuyama, S., 1996. Involvement of endogenous prolactin in the expression of courtship behavior in the newt, *Cynops pyrrhogaster*. Gen. Comp. Endocrinol. 102, 191-196.

Trudeau, V.L., 1997. Neuroendocrine regulation of gonadotrophin II release and gonadal growth in the goldfish, *Carassius auratus*. Rev. Reprod. 2, 55-68.

Trudeau, V.L., 2015. Kiss and tell: deletion of kisspeptins and receptors reveal surprising results. Endocrinology 156, 769-771.

Trudeau, V.L., Schueler, F.W., Navarro-Martin, L., Hamilton, C.K., Bulaeva, E., Bennett, A., Fletcher, W., Taylor, L., 2013. Efficient induction of spawning of northern leopard frogs (*Lithobates pipiens*) during and outside the natural breeding season. Reprod. Biol. Endocrinol. 11, 14.

Trudeau, V.L., Somoza, G.M., Natale, G.S., Pauli, B., Wignall, J., Jackman, P., Doe, K., Schueler, F.W., 2010. Hormonal induction of spawning in 4 species of frogs by coinjection with a gonadotropin-releasing hormone agonist and a dopamine antagonist. Reprod. Biol. Endocrinol. 8, 36.

Tsai, P.-S., Jones, J.T., 2005. Steroid-induced changes in the morphology of GnRH neurons in the male leopard frog, *Rana pipiens*: correlation with plasma gonadotropin and gonadal size. Gen. Comp. Endocrinol. 141, 152-160.

Tsai, P.-S., Kessler, A.E., Jones, J.T., Wahr, K.B., 2005. Alteration of the hypothalamic-pituitary-gonadal axis in estrogen-and androgen-treated adult male leopard frog, *Rana pipiens*. Reprod. Biol. Endocrinol. 3.

Tsutsui, K., Bentley, G.E., Kriegsfeld, L.J., Osugi, T., Seong, J.Y., Vaudry, H., 2010a. Discovery and evolutionary history of gonadotrophin-inhibitory hormone and kisspeptin: new key neuropeptides controlling reproduction. J. Neuroendocrinol. 22, 716-727.

Tsutsui, K., Haraguchi, S., Matsunaga, M., Koyama, T., Do Rego, J.L., Vaudry, H., 2010b. Identification of 7alpha-hydroxypregnenolone, a novel bioactive amphibian neurosteroid stimulating locomotor activity, and its physiological roles in the regulation of locomotion. Gen. Comp. Endocrinol. 168, 275-279.

Tsutsui, K., Saigoh, E., Ukena, K., Teranishi, H., Fujisawa, Y., Kikuchi, M., Ishii, S., Sharp, P.J., 2000. A novel avian hypothalamic peptide inhibiting gonadotropin release. Biochem. Biophys. Res. Commun. 275, 661-667.

Ubuka, T., Ukena, K., Sharp, P.J., Bentley, G.E., Tsutsui, K., 2006. Gonadotropin-inhibitory hormone inhibits gonadal development and maintenance by decreasing gonadotropin synthesis and release in male quail. Endocrinology 147, 1187-1194.

Vanecek, J., 1999. Inhibitory effect of melatonin on GnRH-induced LH release. Rev. Reprod. 4, 67-72.

Vredenburg, V.T., Wake, D.B., 2007. Global declines of amphibians. Encyclopedia of Biodiversity, 1-9.

Vu, M., Trudeau, V.L., 2016. Neuroendocrine control of spawning in amphibians and its practical applications. Gen. Comp. Endocrinol. 234, 28-39.

Waggener, W.L., Carroll, E.J., 1998. A method for hormonal induction of sperm release in anurans (eight species) and *in vitro* fertilization in *Lepidobatrachus* species. Dev. Growth Differ. 40, 19-25.

Wake, D.B., Vredenburg, V.T., 2008. Are we in the midst of the sixth mass extinction? A view from the world of amphibians. Proc. Natl. Acad. Sci. 105, 11466-11473.

Wang, L., Ahn, R.S., Park, J.-Y., Seong, J.Y., Kwon, H.B., 2003. Differential G protein coupling preference of mammalian and nonmammalian gonadotropin-releasing hormone receptors. Mol. Cell. Endocrinol. 205, 89-98.

Wang, L., Bogerd, J., Choi, H.S., Seong, J.Y., Soh, J.M., Chun, S.Y., Blomenrohr, M., Troskie, B.E., Millar, R.P., Yu, W.H., McCann, S.M., Kwon, H.B., 2001. Three distinct types of GnRH receptor characterized in the bullfrog. Proc. Natl. Acad. Sci. U.S.A 98, 361-366.

Waring, H., Landgrebe, F., Neill, R., 1941. Ovulation and oviposition in Anura. J. Exp. Biol. 18, 11-25.

Werner, J.K., 2003. Status of the northern leopard frog (*Rana pipiens*) in western Montana. Northwest Nat., 24-30.

Wetzel, D.M., Kelley, D.B., 1983. Androgen and gonadotropin effects on male mate calls in South African clawed frogs, *Xenopus laevis*. Horm. Behav. 17, 388-404.

Wilczynski, W., Lynch, K.S., 2011. Female sexual arousal in amphibians. Horm. Behav. 59, 630-636.

Wilson, G.A., Fulton, T.L., Kendell, K., Scrimgeour, G., Paszkowski, C.A., Coltman, D.W., 2008. Genetic diversity and structure in Canadian northern leopard frog (*Rana pipiens*) populations: implications for reintroduction programs. Can. J. Zool. 86, 863-874.

Woodley, S., 2015. Chemosignals, hormones, and amphibian reproduction. Horm. Behav. 68, 3-13.

Yamanouchi, H., Ishii, S., 1990. Positive cooperative action of follicle-stimulating hormone on binding of luteinizing hormone to testicular receptors from the bullfrog (*Rana catesbeiana*). Gen. Comp. Endocrinol. 78, 231-241.

Yaron, Z., 1995. Endocrine control of gametogenesis and spawning induction in the carp. Aquaculture 129, 49-73.

Yaron, Z., Gur, G., Melamed, P., Rosenfeld, H., Elizur, A., Levavi-Sivan, B., 2003. Regulation of fish gonadotropins. Int. Rev. Cytol. 225, 131-185.

Zera, A.J., 2006. Evolutionary genetics of juvenile hormone and ecdysteroid regulation in *Gryllus*: a case study in the microevolution of endocrine regulation. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 144, 365-379.

Zhang, L., Kessler, A.E., Tsai, P.-S., 2007. Characterization and steroidal regulation of gonadotropin beta subunits in the male leopard frog, *Rana pipiens*. Gen. Comp. Endocrinol. 150, 66-74.

Zmora, N., Stubblefield, J.D., Wong, T.-T., Levavi-Sivan, B., Millar, R.P., Zohar, Y., 2015. Kisspeptin antagonists reveal kisspeptin 1 and kisspeptin 2 differential regulation of reproduction in the teleost, *Morone saxatilis*. Biol. Reprod., biolreprod-115.