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Gene Regulation in the Hypothalamic-Pituitary-Gonadal Axis of the Green Anole Lizard (Anolis carolinensis).

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Abstract: Studies examining the hypothalamic-pituitary-gonad axis (HPG) in *Anolis carolinensis* provide insight into the regulation of this axis under extremely different reproductive conditions. Molecular studies indicate that kisspeptin proteins, transcribed from *kiss1* and *kiss2* genes, are produced in the hypothalamus where they bind G protein-coupled receptor 54 (GPR54), activating the HPG axis. Gonadotropin inhibitory hormone (GnIH) and its receptors, G protein-coupled receptor 147 and 74 (GPR147 and GPR74), down regulate HPG axis function. In this experiment we examined HPG axis gene expression in the green anole lizard (*Anolis carolinensis)*, a seasonally breeding species with higher steroid hormone levels in the breeding season than non-breeding season, making it ideal for hormone studies. We measured the gene expression of *kiss1*, *kiss2*, and GnIH, as well as GPRs 54, 147 and 74 using reverse transcriptase quantitative polymerase chain reaction (qPCR) analysis. This was done by collecting brain tissue from breeding and non-breeding lizards, extracting RNA and reverse transcribing it to cDNA, developing primers for genes of interest, and measuring mRNA via qPCR analysis. We hypothesized an increase of *kiss1*, *kiss2*, and GPR54 mRNAs in the breeding season, and a decrease of GnIH, GPR147, and GPR74 in the breeding season. Our statistics show no significant differences within sex, season, or an interaction. Our results are most likely due to factors such as stress on anoles during transport and whole brain homogenizations. Though we did not find significant differences in our mRNA measures, it is important to note that this is the first isolation of *kiss1* in the anole genome which could provide insight for evolutionary studies on the HPG axis.

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Introduction

The HPG axis:

Steroid hormones and the regulation of the hypothalamic-pituitary-gonad axis (HPG) are important in controlling sexual development and reproduction in many organisms (Forni & Wray, 2015). In the HPG axis, neurons in the hypothalamus secrete gonadotropin releasing hormone (GnRH; Chen et al., 2008). GnRH binds to GnRH receptor (GnRHR) on gonadotropic cells in the anterior pituitary to release luteinizing hormone (LH) and follicle stimulating hormone (FSH) into the blood. LH and FSH travel through the blood to stimulate the gonads, testes or ovaries, to produce steroid hormones such as testosterone (T) and estradiol (E2; Semaan et al., 2012). More specifically, a study by Fortune & Eppig (1979) showed that, in females, LH binds its receptor (LHR) on theca cells in the follicles of ovaries, leading to the production of androstenedione from cholesterol. FSH binds its receptor (FSHR) on granulosa cells in the follicles of ovaries, leading to the production of progesterone as well as estradiol from androstenedione. Estradiol and progesterone act as positive and negative feedback mechanisms on the hypothalamus and pituitary, respectively. In males, LH binds LHR on leydig cells in the testes to produce testosterone. FSH binds FSHR on sertoli cells in the testes to stimulate spermatogenesis as well as the production of inhibin. Testosterone and inhibin work on the hypothalamus and pituitary to inhibit the production of LH and FSH (Fortune & Eppig, 1979).

Hormones in the HPG axis are highly conserved across both mammalian and non-mammalian vertebrates. The universality of this system is evident through many studies done in humans, mice, frogs, fish, and lizards (Reviewed in Tsutsui et al., 2010). The

conservation of the HPG system is likely a byproduct of two rounds of whole genome duplication, which has been said to have occurred early in vertebrate evolution (Yun et al., 2015). A whole genome duplication event creates an organism with additional copies of its entire genome. Over evolutionary time, through fractionation events, duplicate genes are lost returning the species to a diploid state. The end result is a genome where the overall structure has changed, with the retention of certain gene families (Yun et al, 2015).

Kisspeptin and GPR54:

Expanding on evolutionary concepts and the origins of peptides in the HPG system, Yun et al. (2015) studied kisspeptin receptors and other similar neuropeptides comparing them with non-vertebrate, chordate, and protostomian neuropeptides. From these classifications, the study suggests that the diversity of these peptide families came about through a whole genome duplication event prior to the emergence of vertebrates (Yun et al., 2015). This suggests that kisspeptin emerged very early in evolution, providing a foundation for a conserved HPG system.

The gene for kisspeptin proteins in mammals, *kiss1*, was first identified in 1996 as a malignant melanoma metastasis suppressor (Lee et al., 1996), but also as a HPG axis regulator. Kisspeptin is a 145 amino acid peptide belonging to the RF-amide family, which are known for their Arg-Phe-Nh2 C-terminal characteristic (Messager et al., 2005). This peptide undergoes post-translational cleavage as it is processed into various C-terminal fragments including kisspeptin 54, 14, 13, and 10 (Messager et al., 2005). Different forms of kisspeptin exist depending on the species. For example, mammals

have one form of kisspeptin, whereas fish and lizards have two forms of kisspeptin (Tsutsui et al., 2010).

First discovered in the rat brain (Lee et al., 1999), the G-protein coupled receptor 54 (GPR54) gene codes for a 398 amino acid protein with seven hydrophobic trans-membrane domains. As kisspeptin binds GPR54 it activates the phosphatidylinositol 4, 5-bisphosphate (PIP_2), inositol trisphosphate (IP_3), diacylglycerol (DAG) pathway resulting in the release of GnRH (Fig. 1; Pinilla et al., 2012). GnRH release, in turn, results in surges of LH and FSH into the bloodstream and an increase in T and E2.

GnIH, GPR147, and GPR74:

In 2000, gonadotropin inhibitory hormone (GnIH) was found to be the inhibitory agent of the HPG axis in the quail (*Coturnix japonica*) hypothalamus (Tsutsui et al., 2000), down regulating GnRH release in the hypothalamus, as well as directly inhibiting LH and FSH release from the pituitary (Fig. 1). Two GnIH receptors have been identified, G-protein coupled receptor 147 (GPR147) and G-protein coupled receptor 74 (GPR74) which inhibit gonadal development and maintenance in birds and rodents (Zhao et al., 2010). As GnIH binds GPR147 and GPR74 it inhibits the cyclic adenosine monophosphate (cAMP), protein kinase A (PKA), mitogen activated protein kinase 3/extracellular signal regulated kinase 1 (MAPK3/ERK1) pathway, resulting in the downregulation of GnRH (Son et al., 2012). Since its discovery GnIH orthologs have been discovered in other vertebrates from fish to humans, indicating a conserved role for this neuropeptide across species (Tsutsui et al., 2010). In mammals GnIH is commonly

called RFamide-related peptide (RFRP) due to the Arg-Phe-NH_2 motif on its C-terminal domain (Findeisen et al., 2011).

The HPG axis in mammals:

Kisspeptin has been shown to activate the HPG system, while GnIH has been shown to inhibit the system. Studies on localizations, functionalities, and receptor mutations of the HPG axis have provided insight into the effects these peptides have on breeding behavior, puberty, and fertility.

Kisspeptin and GPR54 are distributed in many regions of the mammalian brain. Kisspeptins have been found in the arcuate nucleus (ARC), preoptic area (POA), median eminence (ME), bed nucleus of the stria terminalis (BNST), rostral periventricular region of the third ventricle (RP3V), dorsal medial hypothalamus, medial amygdala (MeA), and ventromedial hypothalamus (VMH; Reviewed in Nabi et al., 2015; Reviewed in Lehman et al., 2010). GPR54 has also been found in many areas of the mammalian brain, including the dentate gyrus (DG), septum, periaqueductal grey, pontine nuclei, dorsal cochlear nucleus, medial basal hypothalamus (MBH), POA, ARC, MeA, locus coeruleus, substantia nigra, cingulate gyrus, and anterior pituitary (Reviewed in Nabi et al., 2015; Reviewed in Lehman et al., 2010; Herbison et al., 2010). GPR54 is often co-localized with GnRH in the hypothalamus and pituitary, supporting its role in the release of GnRH (Messager et al., 2005). Evidence for this was demonstrated by Messager and colleagues (2005) with the direct administration of kisspeptin intracerebroventricularly in sheep (*Ovis aries*) which resulted in the release of GnRH and LH, indicating the direct role of kisspeptin on GPR54 co-localized with GnRH neurons.

In addition, GnIH, GPR147, and GPR74 have been localized in many areas of the mammalian brain. GnIH has been found in the DMH, with projections to areas where GnRH can be found, including the POA, medial septum, diagonal band of Broca, and anterior hypothalamus (Kriegsfeld et al., 2006). In addition, data reveals that GnIH can act at the level of GnRH and RP3V kisspeptin neurons. GPR147 mRNA, but not 74, has been found to be more prevalent in the RP3V, but the cells that express these receptors remain to be characterized in mammals (Rizwan et al., 2012).

Kisspeptin has been shown to activate the HPG axis in many mammalian species. For example, the Syrian hamster (*Mesocricetus auratus*) is a seasonally breeding organism in which photoperiod regulates reproduction to ensure that offspring are produced during summer months. Low levels of kisspeptin are found during short winter days, and high levels during long summer days (Revel et al., 2007). Interestingly, when hamsters are injected with kisspeptin during the non-breeding season, breeding behavior is induced (Revel et al., 2007). GnIH has been show to inhibit the HPG axis in mammals. A study done by Kriegsfeld and colleagues (2006) involving hamsters, rats, and mice showed that the administration of GnIH inhibits LH, therefore inhibiting the stimulation of gonads and the HPG system as a whole. In addition, GnIH neurons express estrogen receptor α and immediate early gene expression after gonadal stimulation. This puts the role of GnIH in the center of the negative feedback system due to increased estrogen levels, and the inhibition of GnRH release, in turn downregulating the production of LH. This suggests direct regulation of GnRH via GnIH (Kriegsfeld et al., 2006)

In addition to the above mentioned role of kisspeptins and GnIH ligands, the role of their receptors have also been characterized. Seminara et al. (2003) identified multiple mutations in GPR54 in patients with idiopathic hypogonadotropic hypogonadism (IHH), which results in reduced or absent puberty, low libido, and infertility. This effect has also been found in mice (Seminara et al., 2003). Regarding GnIH receptors, Rizwan et al. (2012) treated rat brains with a GPR147 antagonist peripherally, which inhibited GnIH's downregulation of LH, resulting in an increase of LH levels in castrated male rats. With the addition of a GnRH antagonist, LH levels did not increase. This suggests that GnIH inhibition of gonadotropes is dependent on the presence of GnRH. In addition to GnIH acting to inhibit GnRH cells, GnIH may also inhibit kisspeptin cells, particularly in the RP3V (Rizwan et al., 2012).

Overall models of the mammalian HPG system show that kisspeptin and GnIH activate and inhibit breeding behavior, puberty, and fertility. GPR54 can be found on GnRH neurons, releasing GnRH and therefore gonadotropins. GPR147 can be found on kisspeptin as well as GnRH neurons to inhibit the system. Though the localization of kisspeptin, GnIH, and associated receptors varies slightly between mammalian species, the functionalities of these genes are universal.

The HPG axis in non-mammals:

Since *kiss1's* discovery, *kiss1* orthologs have been identified in non-mammalian organisms including fish, reptiles, amphibians, and birds. Fish and lizards have two forms of kisspeptin, *kiss1* and *kiss2* as well as associated receptors, whereas frogs possess three forms of kisspeptin, *kiss1a*, *kiss1b*, and *kiss2*, as well as associated receptors (Lee et al.,

2009). Bird kisspeptins have been more difficult to isolate, but recent analyses suggest birds contain a *kiss2-like* gene in the genome of at least three different bird species (Pasquier et al., 2014).

A study by Felip and colleagues (2009) showed the presence of two genes encoding *kiss1* and *kiss2* in the brain and gonads of sea bass (*Dicentrarchus labrax)*, medaka (*Oryzias latipes)* and zebrafish (*Danio rerio*). *Kiss2* was more potent in inducing LH and FSH secretion than was *kiss1* in the seabass. This study was the first to provide evidence that *kiss2* plays a dominant role in gonadotropic release in teleosts (Felip et al., 2009). GnIH has also been localized in teleosts. Sawada and colleagues (2002) showed the expression of GnIH mRNA and neuropeptide in the nucleus posterioris periventricularis of the hypothalamus in goldfish (*Carassius auratus auratus*). This is one of the first localizations of an HPG axis inhibitory peptide in fish (Sawada et al., 2002).

The kisspeptin receptor GPR54, and GnIH have been isolated in amphibian species as well, including the bullfrog (*Rana catesbeiana)*. Moon et al (2009) showed that GPR54 mRNA is expressed in the forebrain, hypothalamus, and pituitary of the bullfrog. Limited studies have been carried out regarding GnIH, but the bullfrog (Koda et al., 2002), green frog (*Pelophylax esculentus*; Chartrel et al., 2002*),* and Japanese newt (*Cynops pyrrhogaster*; Chowdhury et al., 2001) have been studied to identify GnIH orthologs. The localization of GnIH varies among species, and was named frog growth hormone releasing peptide (fGHR) due to its role in releasing growth hormone in frogs in addition to inhibiting GnRH (Koda et al., 2002).

Exogenous kisspeptins have been shown to have physiological effects in birds, but there has been little genomic evidence for kisspeptins in avian species. Comparing twelve bird species, Pasquier et al (2014) found the existence of a *kiss2-like* sequence in the mallard duck (*Anas platyrhynchos)*, zebra finch (*Taeniopygia guttata)* and pigeon (*Columbidae*) with a high sequence identity with other sauropsid (bird and reptile) *kiss2(10)* sequences. Though this study shows the first presence of a *kiss2-like* gene in three bird species, the differences in this peptide when compared to other vertebrates and the absence of the gene in other birds suggests kisspeptin degeneration in avian species (Pasquier et al., 2014). The effects of GnIH have also been discovered in the HPG axis of avian species. Tsutsui et al (2000) localized GnIH to the paraventricular nucleus (PVN) and ME of the quail brain. In addition, GnIH was shown to inhibit gonadotropin release in quail anterior pituitaries (Tsutsui et al., 2000).

Pasquier et al (2014) have studied kisspeptins in many reptilian species. *Kiss1*, *kiss2*, and their receptor have been found in the Indian python (*Python molurus*) Chinese turtle (*Pelodiscus sinensis*), and Painted turtle (*Chrysemys picta*) genomes. In addition, *kiss1* was found in each of the three crocodilian genomes including the American alligator (*Alligator mississippiensis*), saltwater crocodile (*Crocodylus porosus*), and Indian garial (*Gavialis gangeticus*), along with the kisspeptin receptor (Pasquier et al., 2014). Reptilian studies on GnIH are limited, but a study by Ukena et al (2016) localized GnIH mRNA and neuropeptide in the PVN of the red-eared slider turtle (*Trachemys scripta elegans*). This study claims to have provided the first evidence of GnIH in reptiles (Ukena et al., 2016).

Compiling data across species suggests that kisspeptins bind associated receptors, causing neurons to secrete GnRH, which activates the release of LH and FSH, and therefore steroid hormone production. GnIH seems to inhibit this molecular pathway by downregulating kisspeptin or GnRH release in the hypothalamus, or directly inhibiting LH and FSH release form the pituitary. Though the localizations of these genes may differ slightly between species, the HPG system appears to be universal across mammalian and non-mammalian organisms.

Green anole lizards:

In this experiment we studied the expression of mRNA that, when translated, regulates the HPG axis in the green anole lizard, *Anolis carolinensis*. Due to large fluctuations in morphology, behavior, and steroid hormone levels between breeding and non-breeding seasons, the anole offers the opportunity to measure large differences in HPG neural control.

During breeding and non-breeding seasons, changes in hormone levels and reproductive morphology can be observed. Anoles typically show high steroid hormone levels in the breeding season, and low steroid hormone levels in the non-breeding season (Lovern et al., 2001). Females in the breeding season have thick oviducts and yolking follicles, while during the non-breeding season females have small oviducts with no yolking follicles. In addition, breeding season males have large testes and thick milky vasa deferentia, while during the non-breeding season males have small testes and thin clear vasa deferentia (Lovern et al., 2004).

The differences in morphology and circulating steroid hormone levels can be associated with behavioral changes in the anole. Males are much more aggressive in the breeding than non-breeding season, as they perform courtship behaviors and defend their territory with head bobbing displays and extensions of a bright red throat fan, called a dewlap (Lovern et al., 2004). Lesions of the anterior POA caused a decrease in spermatogenesis and abolished male courtship in intact and castrated/androgen-treated anoles (Wheeler and Crews, 1978). These behaviors are also related to soma size and the volume of the POA, which are both generally larger in males than females, as well as in the breeding season compared to the non-breeding season (O'Bryant and Wade, 2002; Beck et al., 2008). In addition, although kisspeptin has been found in many areas of the brain across species, kisspeptin protein was found only in the POA of non-breeding anoles (Dunham et al., 2009). More studies are needed to determine the pattern of expression and the localization of kisspeptins in anoles.

Our study focused on changes in levels of mRNA that when translated regulate the HPG axis. We examined the gene expression of *kiss1*, *kiss2*, GPR54, GnIH, GPR147 and GPR74 through qPCR analysis, which allowed us to determine differences in expression of mRNAs in the brains of breeding and non-breeding lizards. We hypothesized that breeding anoles would have an increase in *kiss1*, *kiss2*, and GPR54, and a decrease in GnIH, GPR147, and GPR74, as compared to non-breeding anoles.

Methods

Tissue harvesting:

10 adult male and 10 adult female green anoles were purchased from Charles Sullivan (Nashville, TN, USA) during May (breeding season) and October (non-breeding season). The lizards were caught in the wild and shipped within a few days. Upon arrival animals were rapidly decapitated and brains were collected. The tissue was instantly frozen in cold methylbutane and stored at -80 °C. Breeding state was confirmed at this time by observing the state of oviducts, follicles, testes, and vasa deferentia. This visual check of breeding state in the anole has been utilized in multiple studies (e.g., Cohen and Wade, 2011).

Primer design:

Primers were designed to bind target genes (β-actin, *kiss1*, *kiss2*, GPR54, GnIH, GPR147 and GPR74). This was done by first establishing the sequences of the genes of interest using the National Center for Biotechnology Information website (NCBI). At the time of this study all genes of interest were annotated in the anole genome, except *kiss1,* therefore orthologs were identified in other species for purposes of primer design. To design primers we identified *Kiss1* in the Western Painted Turtle, *Chrysemys picta bellii* (NCBI: XM_005308323.2) and used this sequence to find a putative *kiss1* sequence in the anole genome using the NCBI BLAST algorithm.

Once the sequences were determined, we used Primer3 v. 0.4.0 (Untergrasser et al., 2012) to design the primers for all genes of interest. Primer3 v.0.4.0 produced 20 forward primers and 20 reverse primers, which were nucleotide-nucleotide blasted to the

Anolis carolinensis nucleotide collection database in NCBI for gene specificity. The most specific single primer sets were then chosen. Primer guidelines also included amplicon lengths of 100-200 bp, primers18-24 bp, primers ending in G or C with 50-60% GC content, and melting temperatures (Tm) 57-63°C. Using OligoAnalyzer 3.1 on the Integrated DNA Technologies website, hairpin loops, self-dimers, and hetero-dimers were analyzed to select primers least likely to form dimers, making them more specific to the genes of interest. The primers were ordered from Integrated DNA Technologies (Table 1).

RNA extraction:

Whole anole brains were homogenized and RNA was extracted from the homogenates using the RNeasy Lipid Tissue Mini Kit (Qiagen), as per manufacturer instructions, and stored at -80°C. RNA was run on a gel to confirm RNA integrity. In addition 260/280 nm RNA absorbance ratios near 2.0 were observed. 1ug/ul of RNA was then reverse transcribed into cDNA using the ProtoScript II First Strand cDNA Synthesis Kit (New England BioLabs, NEB) as per manufacturer instructions and stored at -20°C.

Polymerase chain reaction:

The binding of primers to target genes in the cDNA was confirmed using Polymerase Chain Reaction (PCR). To do this Quick-load Taq 2X master Mix (NEB), forward primers, reverse primers, cDNA, and H_20 were added to a PCR tube. The reaction was denatured at 95°C for 30 seconds and run for 40 cycles of 95°C for 30 seconds, 55°C for 1 minute, and 68°C for 1 minute. A control using RNA instead of cDNA was also conducted to test for gDNA contamination of RNA samples. Primer

dilutions of 1µM, .5µM, and .2µM were carried out to determine correct primer concentrations. In addition, an annealing temperature gradient was conducted between 55°C and 65°C for all genes to confirm sufficient primer binding temperatures. Following the replication of our seven target genes, each PCR band was cut from the gel and sent to Functional Biosciences for sequencing.

Quantitative polymerase chain reaction:

Once the above experimental conditions were established we used qPCR analysis to determine relative levels of the genes of interest. This is a process that replicates cDNA utilizing a fluorophore that fluoresces and quantifies double stranded cDNA at each replication cycle (Alvarado et al., 2013), quantifying the amount of mRNA in each of our cDNA samples. We did this by adding SYBR Green Real-Time PCR Master Mix (Applied Biosystems), forward primers (1µM), reverse primers (1µM), a cDNA template, and H_2O into a 96 well qPCR plate. The reaction was run for 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The melt curve stage was run at 95°C for 15 seconds, 60°C for 1 minute, and 95°C for 15 seconds.

A qPCR efficiency curve was carried out for all genes to ensure efficient binding of primers to target genes, and to determine the appropriate concentration of cDNA. To do this the above contents where loaded into qPCR wells along with .01, .1, 1, 10, and 100ng/µl cDNA templates for each target gene. The output Ct values were used to calculate the slope of the dilutions and the final efficiencies for each gene as follows, primer efficiency = $10^{(-1/slope)}$ (Pfaffl et al., 2001).

After obtaining acceptable efficiencies, experimental qPCRs were run with a 10ng cDNA template for all genes. Three plates were run for each gene. One plate contained two genes of interest and β actin, each sample for each gene was run in replicates of three. Samples for each gene included a no template control, two female BS, two female NBS, two male BS, and two male NBS, as well as a single male NBS sample that was run across all plates for measures of variability between plates.

A melt curve and agarose gel analyses were also carried out for each plate to ensure specificity. Samples that showed multiple melt curve temperatures were run on a gel to confirm single amplicon replication. Lastly, Ct values were gathered for statistical analysis via ANOVA.

Analysis:

For each sample, we used the average Ct value for each gene to calculate the relative expression value, normalized to β-actin using the following equation, Relative Expression = $100*(E\beta\text{-actin}^{Ct\beta\text{-actin}}/(Etarget\ ^{Cttarget})$ (Burmeister et al. 2007). Mean relative expressions were analyzed via ANOVA, comparing sex and seasonal effects on the HPG axis. In addition, one sample was run with all genes across all plates. This sample was used as a baseline for adjustments to genes that showed a significant difference in Ct values across plates via ANOVA. *Kiss1* and β-actin showed a significant difference across plates. To normalize *kiss1*, the original *kiss1* Ct values for all animals in run 1 were subtracted by the across plate animal *kiss1* Ct value in run 1. This was done for *kiss1* runs 2-3 in a similar fashion. The same was carried out to normalize β-actin.

Relative expressions were then calculated with adjusted Ct values and an ANOVA was carried out.

Results

Troubleshooting and efficiencies:

Successful RNA extractions were observed on gels as two large rRNA bands and other RNA fragments (Fig. 2A). The no reverse transcriptase control did not show replicated gene sequences for any of our genes of interest, therefore no gDNA contamination was observed (Fig. 2B). Faint bands were visible at 40-50bp during the no reverse transcriptase control, indicating primer dimer formation, but these dimer formations were less visible when adding a cDNA template. Following the initial replication of each gene via PCR, clear bands were observed just below 200bp, matching the amplicon lengths specified in table 1 (Fig. 2C). Primer dimers were still observed at this time, so primer dilutions were performed. The primer dilution gel shows primer concentrations of 1μM, .5μM, and .2μM for the replication of *kiss2* (Fig. 2D). Faint primer dimers were observed at 1μM and .5μM, therefore a .2μM concentration of primer was used for *kiss2* (Fig. 2D). All other genes required a 1μM concentration of primers. Regarding temperature gradients, β-actin and *kiss1* temperature gradients are shown in Fig. 2E. Primer binding and gene replication was observed to be most effective at 60°C for all genes of interest, with no visible primer dimers. Following successful replication of our genes of interested, sequences received from Functional Biosciences were compared to the anole genomic data available via NCBI BLAST algorithms. The fragments of our sequenced amplicons that matched with NCBI genomic data can be

found in Table 2. Lastly, efficiencies via qPCR fell within an acceptable range around a value of 2.0 efficiency for all genes, indicating an exponential growth following each replication cycle (Table 1).

Kisspeptin:

As described in the methods, *kiss1* was not annotated in the anole genome at the time of this study, so orthologs were identified in other species for comparison. There was a 75% identity between the Western Painted Turtle and Green Anole genomes when comparing a 69bp *kiss1* nucleotide sequence, which was used for primer design. Across species percent identities for *kiss1* nucleotides are shown in Table 3. In addition, the *kiss1* nucleotide sequence was compared across species using NCBI BLAST algorithms to find predicted protein identities. The predicted anole *kiss1* translated protein compared with the *kiss1* translated proteins of arbitrarily chosen species all showed between 71%-73% positives (Table 4).

A separate analysis was conducted for each *kiss* gene to determine whether there was an effect of sex, season, or an interaction between sex and season. We did not detect a significant effect of sex ($F_{(1, 19)} = 0.72$ $p = 0.408$), season ($F_{(1, 19)} = 0.11$, $p = 0.744$), or an interaction between sex and season ($F_{(1, 19)} = 1.64$, $p = 0.216$) on *kiss1* expression (Fig. 3A). There was also no significant effect of sex ($F_{(1, 12)} = 2.23$, $p = 0.161$), season ($F_{(1, 12)} = 0.92$, $p = 0.358$), or an interaction between sex and season ($F_{(1, 12)} = 0.82$, $p = 0.384$) on *kiss2* expression (Fig. 3B). In addition, there was no significant effect of sex ($F_{(1, 12)} = 2.62$, $p = 0.132$), season ($F_{(1, 12)} = 0.70$, $p = 0.420$), or an interaction between sex and

season ($F_{(1, 12)} = 2.15$, $p = 0.168$) on GPR54 expression (Fig. 3C). qPCR graphs for relevant kisspeptin genes can be found in Figures 4-6.

Gonadotropin inhibitory hormone:

A separate analysis was conducted for each GnIH gene, comparing sex, season, and an interaction between sex and season. GnIH relative expression showed no significant differences between sex ($F_{(1, 12)} = 3.79$, $p = 0.075$), season ($F_{(1, 12)} = 0.27$, $p = 0.613$), or an interaction between sex and season ($F_{(1, 12)} = 0.49$, $p = 0.498$; Fig. 7A). GPR147 relative expression showed no significant differences between sex ($F_{(1, 12)} = 0.09$, $p = 0..771$), season ($F_{(1, 12)} = 0.07$, $p = 0.800$), or an interaction between sex and season ($F_{(1, 12)} = 0.00$, $p = 0.993$; Fig. 7B). GPR74 relative expression showed no significant differences between sex ($F_{(1, 12)} = 0.51$, $p = 0.491$), season ($F_{(1, 12)} = 0.64$, $p = 0.438$), or an interaction between sex and season ($F_{(1, 12)} = 0.13$, $p = 0.723$; Fig. 7C). qPCR graphs for relevant GnIH genes can be found in Figures 8-10.

Discussion

In this experiment we examined the gene expression of *kiss1*, *kiss2*, GPR54, GnIH, GPR147 and GPR74 through qPCR analysis, which allowed us to determine differences in expression of mRNAs in the brains of breeding and non-breeding lizards. We hypothesized that breeding anoles would have an increase in *kiss1*, *kiss2*, and GPR54, and a decrease in GnIH, GPR147, and GPR74, as compared to non-breeding anoles. The were no significant differences for our genes of interest, but this project has described *kiss1* for the first time in the green anole. Things to consider when analyzing our qPCR

data include fetal and post-natal development, gonadal stages, and the diversification of gene families.

kiss1, kiss2, and GPR54:

The expression of *kiss1*, *kiss2*, and GPR54 mRNA varies across species depending on a variety of factors including development and sex differentiation, gonadal stages, and the diversification of gene families.

Fetal and postnatal development plays an important role in sex differentiation and how the HPG axis functions within and across species. Knoll et al. (2013) studied the localization and mRNA expression of *kiss1* and GPR54 during development in mice via *In situ* hybridization (ISH) and qPCR. At E17 *Kiss1* in the ARC showed two-fold higher mRNA levels in females than males, lasting until P35, while GPR54 showed no significant differences between males and females (Knoll et al., 2013). Though *kiss1* sex differences were observed, circulating sex steroids often regulate *kiss1* mRNA expression, and circulating sex steroids between males and females are more likely correlated with SRY, SOX9, SF-1, and Wnt4 genes in early development (Knoll et al., 2013). In any case, this study indicates the possible role of *kiss1* in sexual differentiation during development and adulthood. Relating this to our experimental results, we did find that baseline anole *kiss2* mRNA is generally higher in females than in males, though non-significant. Future studies might analyze the role of *kiss1* and *kiss2* in sexual differentiation during development and adulthood in the anole.

Different gonadal stages across species also plays a vital role in the HPG system. For example, Selvaraj et al. (2010) studied the changes of *kiss2* mRNA in the whole brain

of chub mackerel via qPCR (*Scomber japonicas)* during different gonadal stages. Results indicated that whole brain male *kiss2* showed a slight non-significant increase from immature to late spermatogenesis with a significant decline thereafter. Whole brain female *kiss2* showed a non-significant increase during early vitellogenesis, with a significant decline thereafter, similar to males (Selvaraj et al., 2010). This study examines the role of *kiss2* during different gonadal stages where it is generally heightened during pertinent breeding phases. Cross comparing anole breeding and non-breeding seasons with teleost spawning cycles, our experiment does show a slight increase in female anole *kiss2* from nonbreeding to breeding seasons, though statistically non-significant. Future analysis of hypothalamic and pituitary specific *kiss2* may shed more light in this area.

The analysis of gene families also becomes important when considering kisspeptin and its receptors. Pasquier et al. (2012) studied variations in the GPR54 gene family among key phylogenetic positions, and measured the mRNA of these receptors via qPCR. For the first time, three *kissr's* were found in a teleost, the European eel (*Anguilla anguilla*). Mature female eel *kissr-1* expression in the olfactory bulbs, telencephalon, and di/mesencephalon significantly increased as compared to prepubertal controls, while no changes were detected in *kissr-2* or *kissr-3*. *Kissr-1* and *kissr-2* expression in the pituitary significantly decreased in mature eels, while *kissr-3* did not change (Pasquier et al., 2012). This study highlights the importance of development, and takes into consideration the evolutionary perspective that multiple kisspeptin receptors can be differentially expressed within or between species. Our study shows similar GPR54 mRNA expression between female breeding, female nonbreeding, and male nonbreeding anoles, with a non-

significant decreased expression in breeding males. Though our qPCR results are not cross comparable to the above experiment due to whole brain homogenizations, our inability to decipher age of anoles, and the lack of data on multiple *kissr's* in the anole specifically. Thus, Future studies should take into consideration that kisspeptin receptor gene families vary across species, and multiple receptors for the same ligand, or vice versa, may be differentially regulated in the same species (Pasquier et al., 2014). For example, three forms of *kissr's* have been suggested in teleosts and amphibians, and four receptor "clades" between mammals, avian species, amphibians and reptiles, each of which can be differentially regulated within or between species (Pasquier et al., 2012).

In accordance with gene families it is interesting to consider hypotheses regarding vertebrate whole genome duplication events, and the phylogenetic link between teleosts and sauropsids (Yun et al., 2015). Navigating these avenues can give insight into qPCR research in the anole as well as other species, providing an evolutionary guide for what type of gene to look for, where to look, and why expression patterns may vary within or across species. Keeping in mind that *kiss2* is most likely the primary functioning kisspeptin gene in anoles (Pasquier et al., 2014), it is possible that *kiss1* in the anole is not a functional gene, but a remnant of a once functional form of kisspeptin. If so, our isolation and replication of *kiss1* in the anole could be used to compare genetic phylogeny's across species, and give insight into the origin and divergence of kisspeptins. Extending this idea to kisspeptin expression patterns, *kiss2* has been observed to be more potent than *kiss1* in the release of gonadotrops in teleosts (Felip et al., 2009). An across species evolutionary analysis of kisspeptins would suggest a similar *kiss2* potency in the

anole (Pasquier et al., 2014). The analysis of endogenous and exogenous neuropeptides becomes important here. For example, the presence of *kiss2* and the absence of *kiss1* has been observed in avian species, with differing exogenous kisspeptins able to activate kiss receptors in avian lineages (Pasquier et al., 2014). It is possible that exogenous kisspeptins could activate GPR54 in anoles, and future studies should be done to measure potencies of different kisspeptins. It seems likely that there is a link between whole genome duplication events and functional types of *kiss1* and *kiss2* in teleosts and sauropsids. The divergence of these peptides from teleosts to sauropsids and the absence of *kiss1* in many birds suggest *kiss1* degeneration in the anole (Kim et al., 2012; Pasquier et al., 2014). Future studies could gain insight by studying the level of functionality of *kiss1* from an evolutionary point of view, as well as the link between *kiss1* and *kiss2* from a divergence perspective.

It is difficult to compare *kiss1*, *kiss2*, and GPR54 expression patterns from other studies with our experimental results, partly due to different localizations and functions of HPG neruopeptides in fish, amphibians, reptiles, birds, and mammals. It is clear that the HPG system carries out a similar reproductive function across species, yet the intricacies of the system vary between organisms and gene family.

GnIH, GPR147, and GPR74:

The quantification of GnIH, GPR147, and GPR74 mRNA also varies across species depending on a variety of factors including fetal and post-natal development and gonadal stages.

Regarding the effects of gonadal stages on the HPG system, Moussavi et al. (2012) studied GnIH neuropeptide in relation to goldfish pituitary GPR74 mRNA levels via qPCR. After intraperitoneal injections with GnIH neuropeptide during early, mid, mid-late, and late spawning periods GPR74 levels did not change during early and mid, but were significantly reduced during mid-late and late spawning (Moussavi et al., 2012). This indicates that the mechanisms behind GnIH are dependent on gonadal stages, the downregulation of GPR74 being necessary during mid-late and late spawning when the HPG is most active. Our study was able to identify the gonadal stages of breeding and non-breeding anoles. Cross comparing to the above study, we also found a slight decrease of whole brain GPR74 expression in breeding males and females, when the HPG is most active, though our results were non-significant.

The importance of gonadal stages in the regulation of the HPG axis is also pointed out in Li et al. (2012), who investigated the role of GnIH and GPR147 in female virginal Suzhong pigs by determining relative mRNA levels during cyclic reproductive stages via qPCR. In the hypothalamus the highest levels of GnIH mRNA were observed during proestrous, followed by a decrease in estrus, increase in metestrus, and decrease in diestrus. Through qPCR analysis, levels of GnIH mRNA in the pituitary were low during proestrus, estrus, and diestrus but significantly increased during metestrus. GPR147 mRNA was also observed in the hypothalamus and the pituitary. GPR147 mRNA in the hypothalamus was high in proestrus, metestrous, and diestrous, but low during estrus. In the pituitary GPR147 mRNA analyzed via qPCR was low during proestrous, estrus, and diestrous and elevated during metestrous (Li et al., 2012). These results indicate the

importance of gonadal stages on GnIH and GPR147 function, in this case GnIH and GPR147 being least active during estrus, when HPG function is active and ovulation occurs. Cross-comparing the results from our study with the above, we do find the lowest levels of GnIH in breeding females, when HPG function is elevated, and we do not see the same trend for male anoles. Our study also showed very little difference in the levels of GPR147 expression between sex or season in anoles. More specific localizations of GnIH and GPR147 in our qPCR analysis could lead to more similar results to those stated in Li et al. (2012).

Development is another important factor to take into consideration when analyzing HPG inhibitory neuropeptides and receptors. Iwasa et al. (2012) studied GnIH and GPR147 mRNAs in relation to development via qPCR. Male rat hypothalami showed a significant increase in GnIH and GPR147 mRNA from postnatal days 12-16, with a plateau at 35-42. Female rat GnIH mRNA's increased throughout development, while GPR147 mRNA's rose from postnatal day 16-28 (Iwasa et al., 2012). The rat model for mammalian HPG function indicates that there is a slow inhibition of the HPG axis with age. Our study cannot be directly cross compared with the above due to a lack of facilities to house anoles during our experiment. Even so, it is clear that green anoles partake in definitive breeding and nonbreeding cycles every 4-6 months, and regardless of age or development, we do see an increase in GnIH mRNA expression from breeding to non-breeding female anoles, though statistically non-significant. More analysis could be done regarding the effects of age and development on the anole HPG axis.

Similar to kisspeptin and associated receptors, it is difficult to compare the above GnIH, GPR147, and GPR74 expression patterns with our experimental results due to different localizations and functions of HPG neruopeptides in fish, amphibians, reptiles, birds, and mammals. One of many avenues to take in clarifying across species HPG variation is to emphasize within season variations of gonadal stages. For example, monoallochronic ovulation has been observed in the anole, where a single egg is laid alternately from each ovary every 14 days during the breeding season (Hamlett, 1952). It would be beneficial for future anole studies to specify when in the 14 day ovarian cycle organisms are sacrificed, and observe peek hormone levels and breeding behaviors within this cycle (Jones et al., 1983; Crews, 1980) and other species' cycles (Radder et al., 2001; Moussavi et al., 2012; Li et al., 2012).

Other considerations:

Many factors could have affected the results of our experiment including stress on the anoles, the method of RNA extraction, neurokinin B and dynorphin expression, and obstacles in identifying GnRH.

Regarding GnIH and stress, Calisi and colleagues (2008) examined adult female and male house sparrows (*Passer domesticus)* during stressed and non-stressed states, as well as during spring breeding and fall non-breeding seasons. The stressed group were captured and put in ventilated bags for one hour, which has been shown to increase glucocorticoid in many species. During fall non-breeding months GnIH was more abundant than in spring breeding months, and GnIH was up regulated in the spring in accordance with induced stressful environments (Calisi et al., 2008). Stress has been

known to inhibit reproductive physiology across vertebrate species by increasing GnIH expression, inhibiting the HPG axis (Calisi et al., 2008; Revel et al., 2007). In addition, corticosterone can have an inhibitory effect on reproduction depending on circulating plasma levels, social context, and species (Moore et al., 2003). During this experiment, anoles were gathered from the wild and shipped overnight prior to arriving in our lab. Tissue harvesting took place within a few hours of arrival. This is important to take into consideration as the stressful environment in which the anoles were exposed to could have induced the upregulation of GnIH and the inhibition of the HPG axis across breeding and non-breeding groups. Future studies should house anoles for an extended period of time prior to HPG experimentation.

Another point to take into account is our process of RNA extraction. Due to the small size of the anole brain, whole brain homogenizations were carried out to extract RNA from tissue, therefore it is possible that the target gene RNA was diluted in the process. Future studies could correct for this issue via pituitary and hypothalamic micro-punches to isolate the areas of interest. Whole brain homogenizations could also have affected our results if *kiss1*, *kiss2*, GnIH and their receptors play other roles in other areas of the anole brain. With that said, Dunham et al (2009) found through immunohistochemical experiments that kisspeptin is found only in the POA of the hypothalamus in non-breeding anoles, suggesting that kisspeptin may be present and functional only in the anole hypothalamus.

Neurokinin B and dynorphin could have also played a role in our experimental results, acting directly or indirectly on GnRH. The kisspeptin, neurokinin B, dynorphin

neuropeptide group is often abbreviate KNDy, and recent studies suggest that these peptides are conserved from rodents to humans (Reviewed in Lehman et al., 2010). Neurokinin B, and dynorphin were recently co-localized with kisspeptin in the hypothalamus of mammals, playing a major role in episodic GnRH release, neurokinin B acting to stimulate, and dynorphin to inhibit GnRH (Leham et al., 2010). Current studies suggest that GnIH may inhibit kisspeptin, GnRH, or LH/FSH cells, but the data is somewhat inconsistent (Rizwan et al., 2012). It is possible that dynorphin is acting independently of GnIH through its own steroid hormone negative feedback system to inhibit the HPG axis. Functional studies on neurokinin B and dynorphin could provide insights into the gap of knowledge underlying GnIH mechanisms.

There have also been obstacles in identifying GnRH in anoles. Twelve known forms of GnRH have been noted across species (Lakra et al., 2003), two of the more well-known forms being chicken GnRH-I and GnRH-II (cGnRH-I, cGnRH-II). GnRH has not yet been annotated in the anole genome, but the existence of cGnRH-II has been observed in the anole via high performance liquid chromatography (Lescheid et al., 1997). In addition, through immunohistochemistry Rosen et al (1997) found GnRHir cells in the midbrain and hindbrain of anoles, fibers projecting to oculomotor and vestibular nuclei (Rosen et al., 1997). A study successfully isolating and identifying the function of GnRH in the anole hypothalamus would give insight into the behavior of other ligands and receptors in the HPG system.

Conclusion:

At the start of this experiment we hypothesized an increase of *kiss1*, *kiss2*, and GPR54 mRNAs in the breeding season, and a decrease of GnIH, GPR147, and GPR74 in the breeding season, as compared to nonbreeding anoles. Although our results were statistically non-significant, common trends can be observed when comparing our results with other studies on the HPG axis, including mechanisms of sex differentiation, gonadal stages, gene families, and pre/post-natal development.

Kiss1 has been analyzed for its possible role in sexual differentiation during development and adulthood in mice, with females having higher levels than males (Knoll et al., 2013). In our study we did find that anole *kiss2* mRNA is slightly higher in females than males. When analyzing gonadal stages it has also been observed that *kiss2* is elevated during pertinent breeding phases in the chub mackerel (Selvaraj et al., 2010). Our study does show a moderate increase in female anole *kiss2* from nonbreeding to breading, and male *kiss2* levels did not change. Regarding GPR54, studies have touched on the importance of not only gonadal stages and development, but also the role gene families and molecular evolution play in the analysis of deferentially expressed receptors within and between species (Pasquier et al., 2012). Our study shows similar GPR54 mRNA expression between female breeding, female nonbreeding, and male nonbreeding anoles, with a slight decrease in breeding males. This is difficult to compare with other studies, as up to four GPR54-like receptors have been identified, each being differentially expressed within and between species. Future anole studies could benefit from cross

comparing the anole GPR54 sequence with the three other suggested GPR54-like receptors (Pasquier et al., 2014).

GnIH and GPR147 have also been analyzed in relation to development and gonadal stages. A slow inhibition of HPG function in rats has been observed throughout development due to increasing GnIH and GPR147 mRNA's (Iwasa et al., 2012). In addition, GnIH and GPR147 are least active during estrus in pigs, when HPG function is active and ovulation occurs (Li et al., 2012). Our study could not take into account certain age and development factors, but results do indicate low levels of GnIH in breeding females when HPG is most active, and male GnIH did not vary between seasons. GPR147 expression did not vary between sex or season, which could be due to across species variation. GPR74 has also been analyzed in relation to gonadal stages in goldfish, a downregulation observed when the HPG axis is most active (Moussavi et al., 2012). Our results do show a slight decrease of GPR74 mRNA in breeding males and females, when the HPG is most active.

We did not find significant differences between sex and season for our genes of interest, most likely due to experimental considerations outlined in the discussion. The significance of this experiment comes to light when considering the isolation and replication of *kiss1* for the first time in the anole genome. The discovery of a *kiss1-like* sequence in the anole provides another tool for identifying the origins, development, and function of HPG ligands and receptors across species.

Figures and Tables

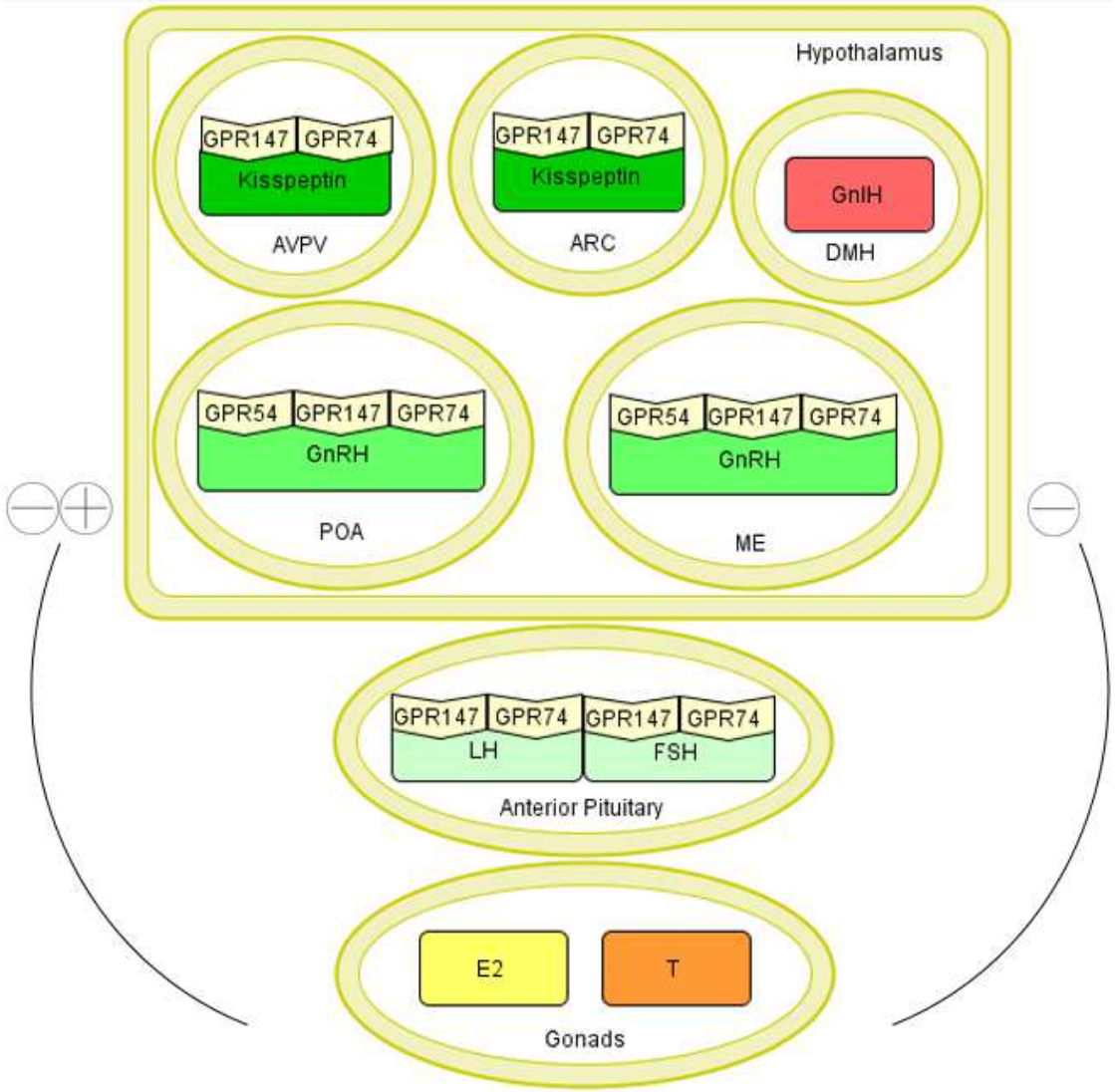


Figure 1: Across species studies of the HPG axis show that neurons in the anteroventral periventricular nucleus (AVPV) and arcuate nucleus (ARC) of the hypothalamus produce *kiss1*. This nueropeptide binds GPR54 in the preoptic area (POA) and median eminence (ME) causing neurons to secrete gonadotropin-releasing hormone (GnRH). GnRH binds to GnRH receptor in the anterior pituitary to release luteinizing hormone (LH) and follicle stimulating hormone (FSH) into the blood. LH and FSH travel through the blood and stimulate the gonads, testes or ovaries, to produce steroid hormones such as testosterone (T) and estradiol (E2). GnIH, found mostly in the dorsal medial hypothalamus (DMH) and its receptors, GPR147 and GPR74 inhibit the HPG axis by down regulating kisspeptin and GnRH in the hypothalamus, as well as directly inhibiting LH and FSH release from the pituitary.

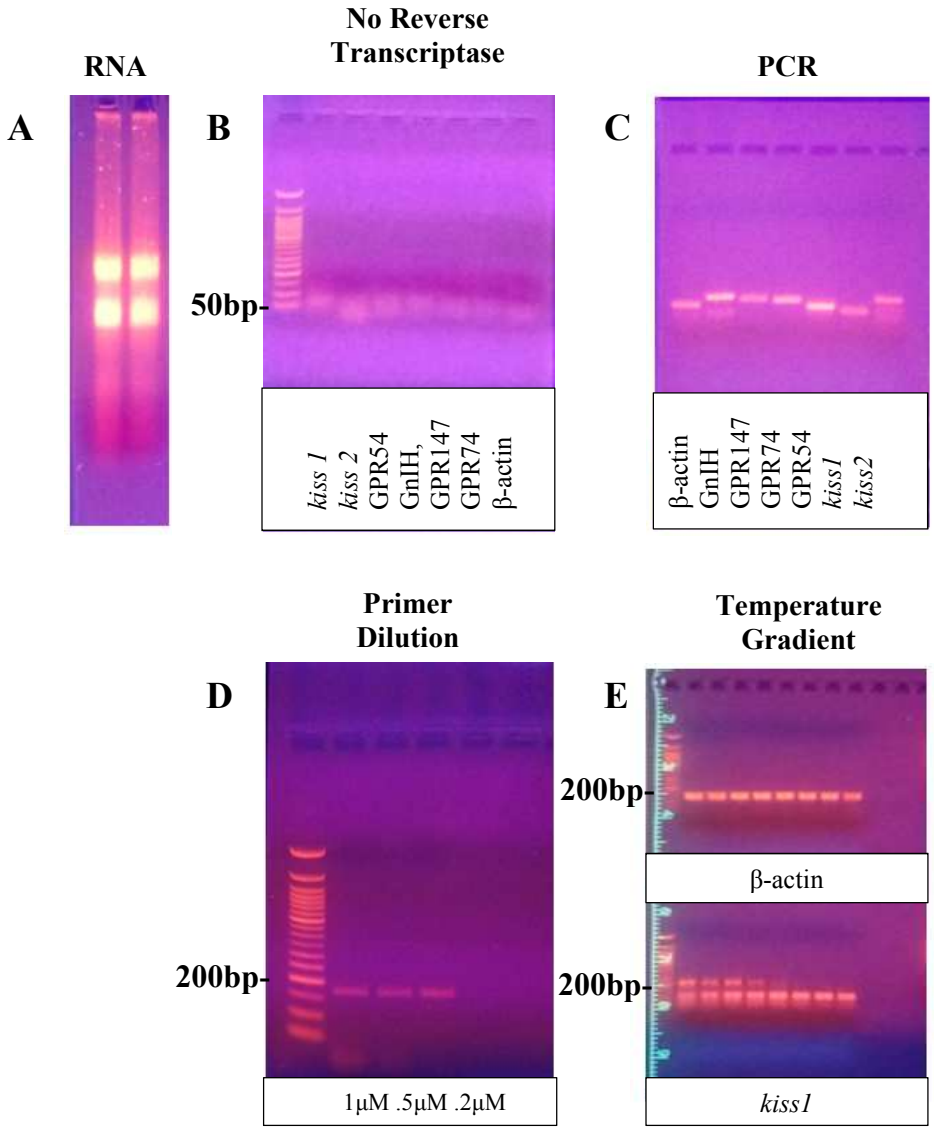


Figure 2: Troubleshooting: A) RNA was run on a gel to confirm effective extraction. B) A no reverse transcriptase experiment was carried out to provide evidence for no gDNA contamination (left to right, ladder, *kiss1*, *kiss2*, GPR54, GnIH, GPR147, GPR74, β-actin). C) A PCR was run to confirm primers binding to target genes (left to right, β-actin, GnIH, GPR147, GPR74, GPR54, *kiss1*, *kiss2*). D) Primer dilutions were set up to indicate the correct concentration for accurate target replication and to prevent primer dimers (left to right *kiss2* primer dilutions, 1μM, .5μM, .2μM). E) A temperature gradient was carried out to decipher which temperatures more effectively replicated our genes of interest (β-actin top, *kiss1* bottom; left to right 55-65°C).

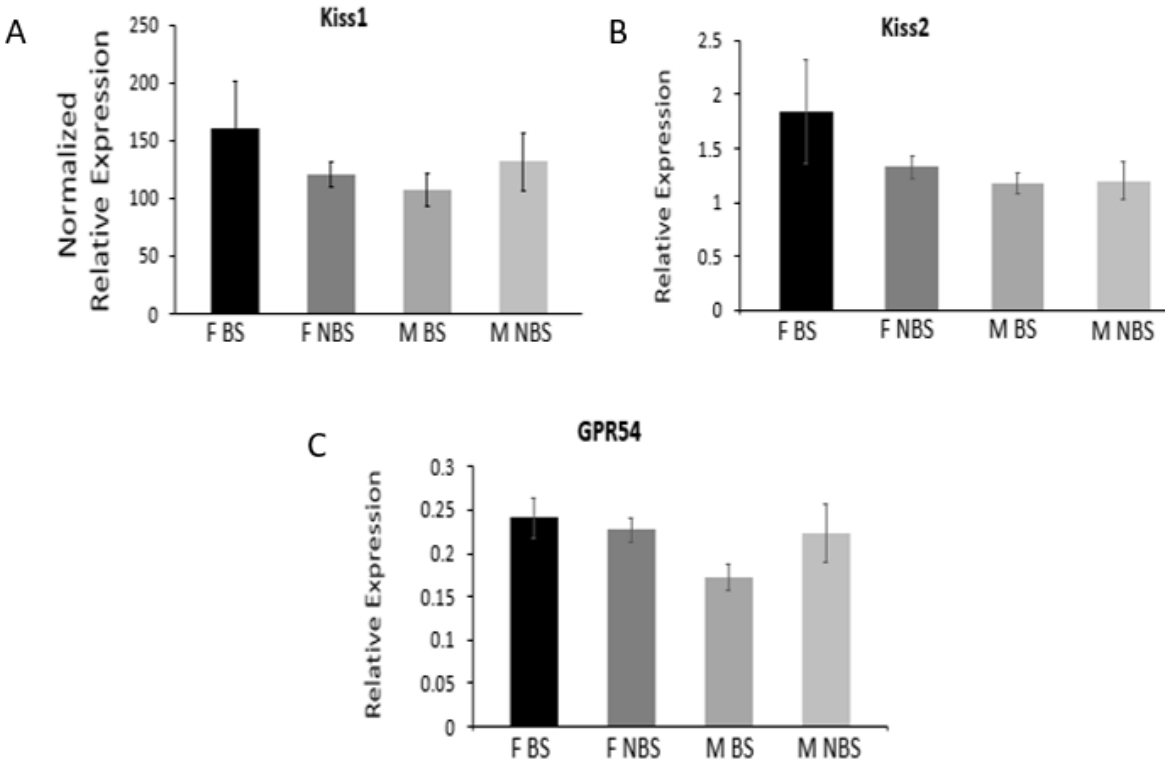


Figure 3: No significant differences were found for *kiss1* (A), *kiss2* (B), or GPR54 (C) between sex, season, or an interaction between sex and season (mean +/- 1 SEM).

Figure 4: Experimental qPCR data for *kiss1* representing fluorescence of SYBR Green (ΔRn) vs. Cycle. An arbitrary 0.2 baseline threshold was set for raw data comparison across genes.

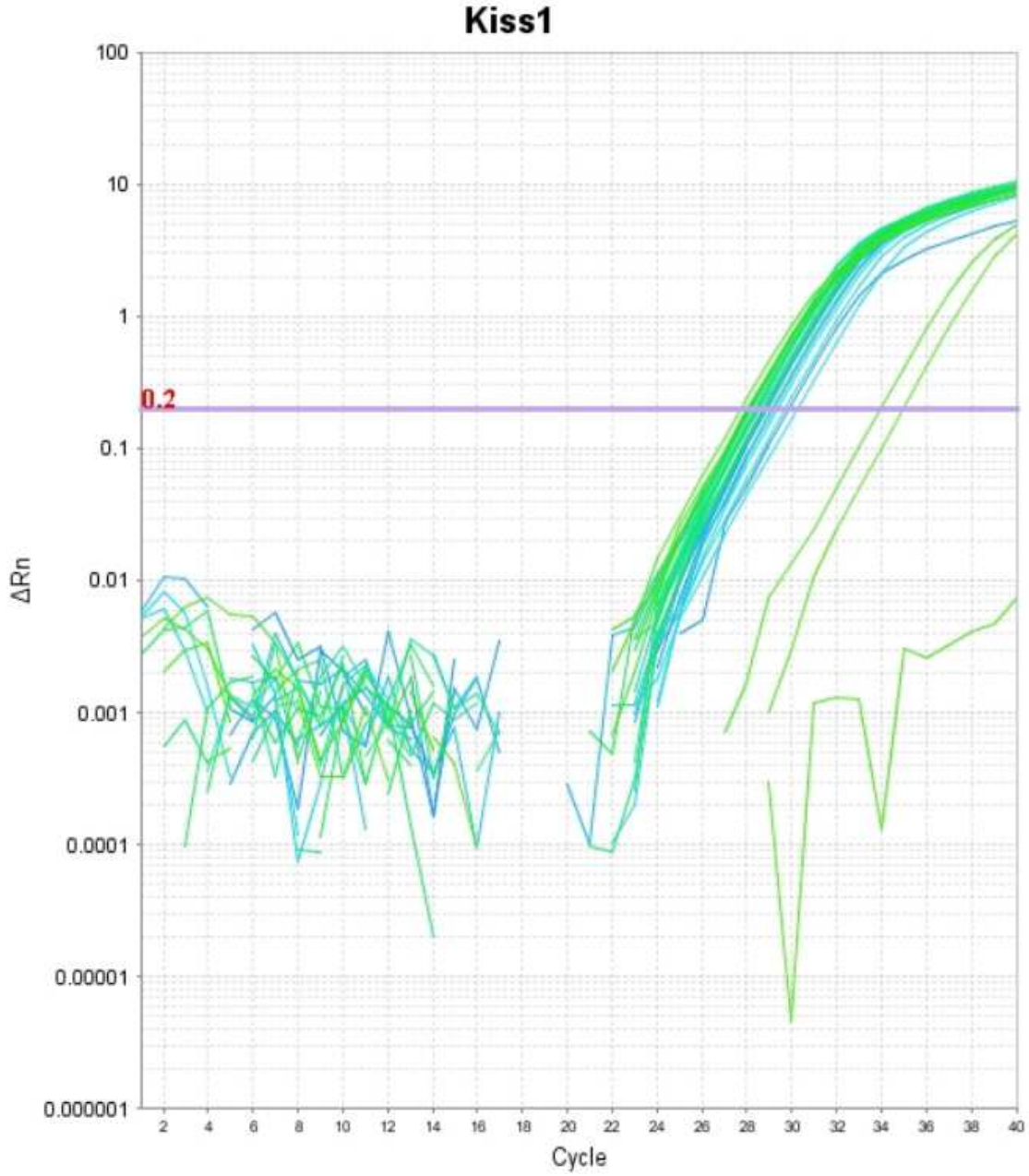


Figure 5: Experimental qPCR data for *kiss2* representing fluorescence of SYBR Green (ΔRn) vs. Cycle. An arbitrary 0.2 baseline threshold was set for raw data comparison across genes.

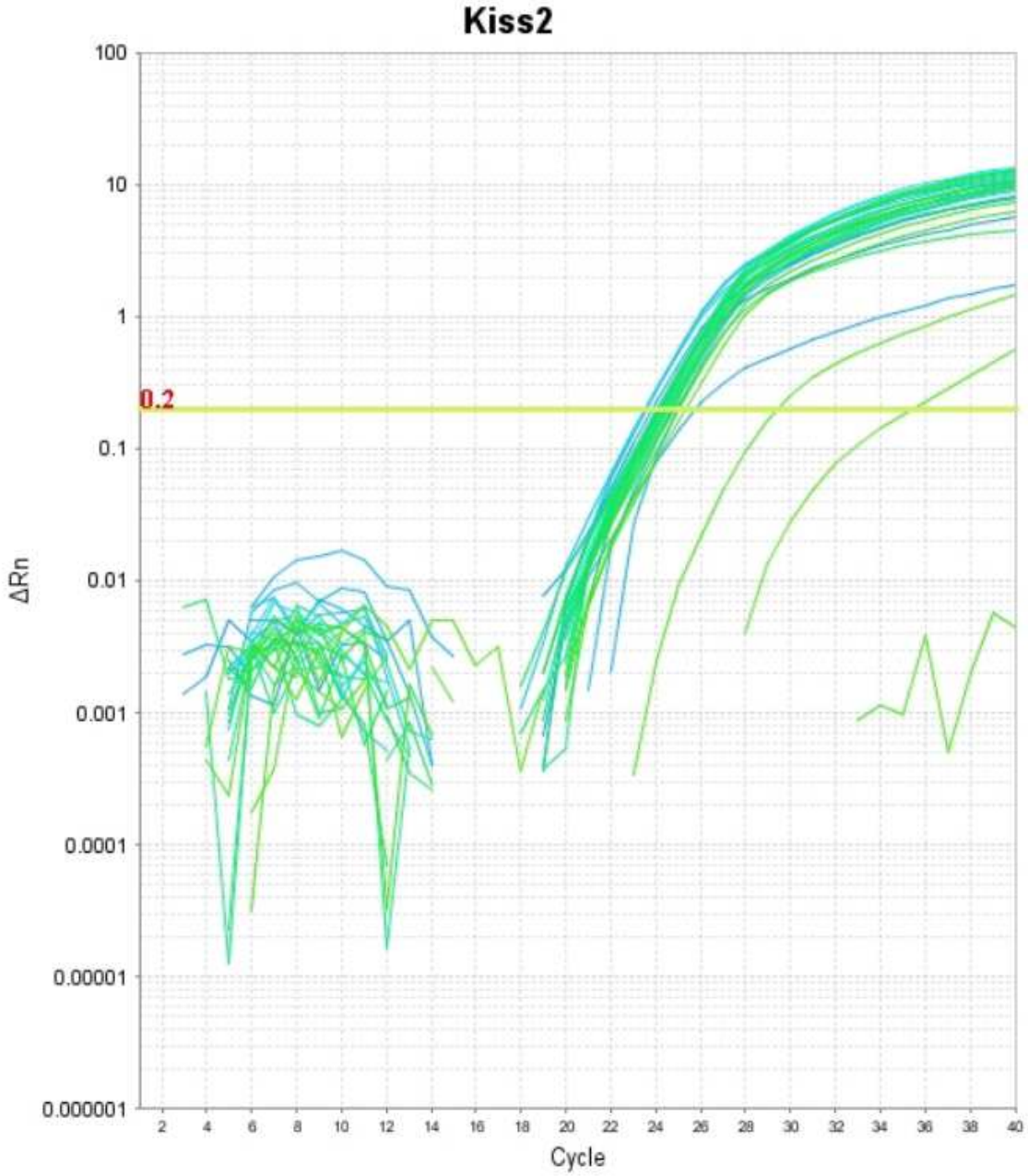
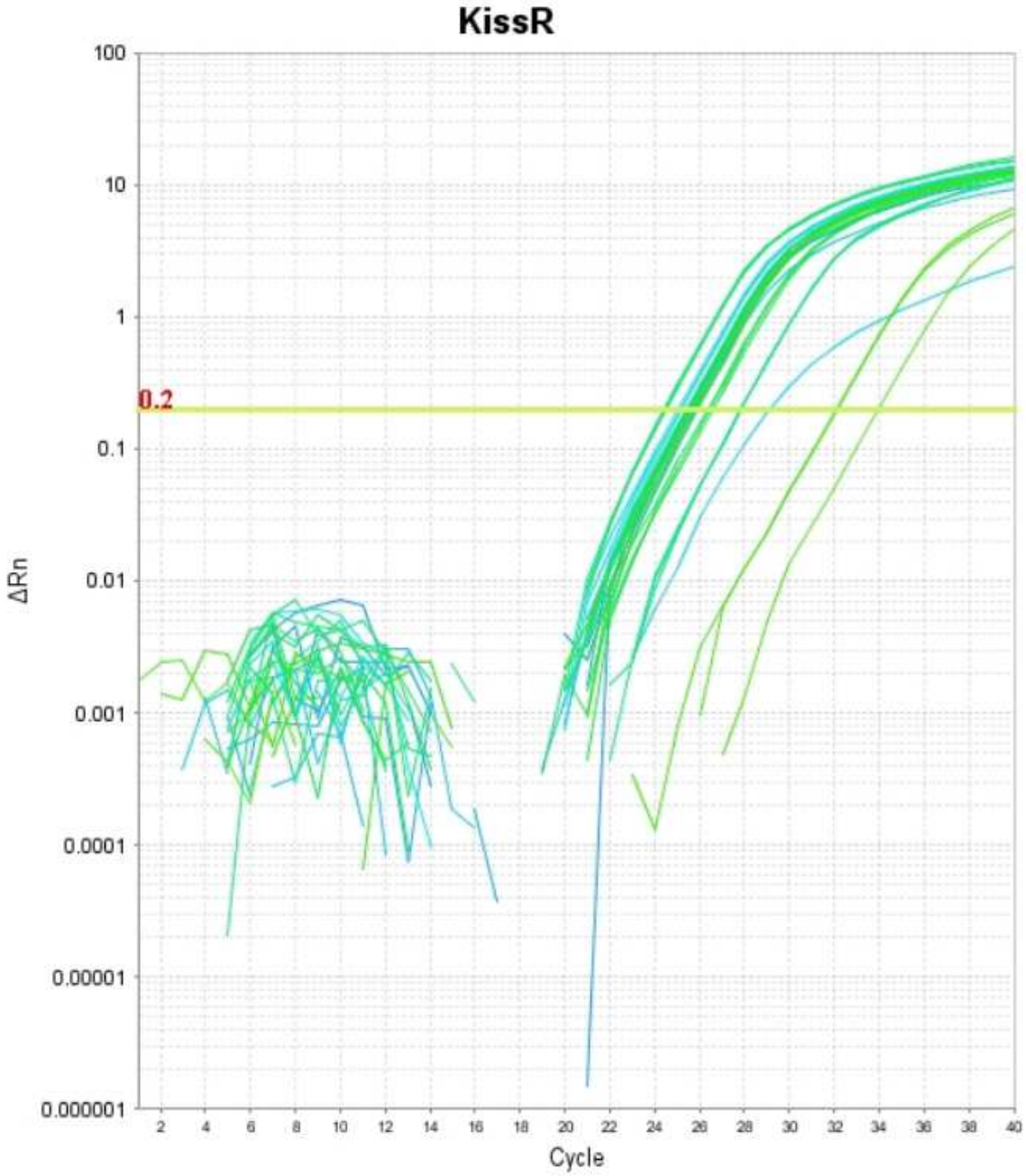
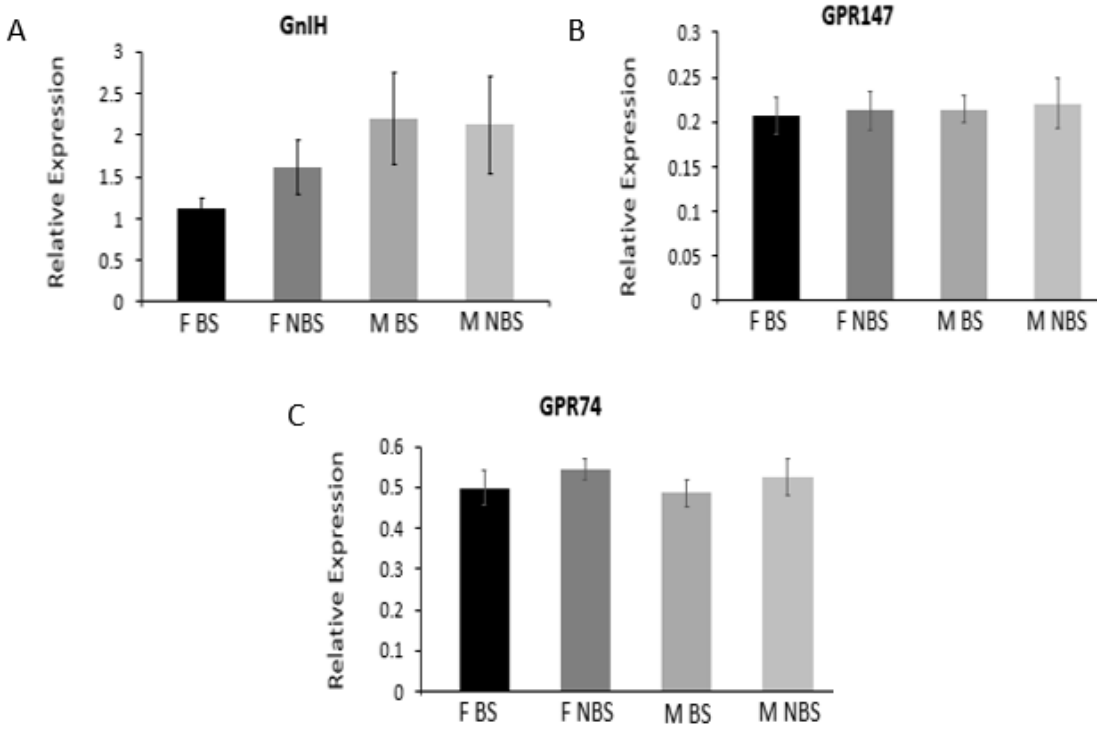


Figure 6: Experimental qPCR data for GPR54 representing fluorescence of SYBR Green (ΔRn) vs. Cycle. An arbitrary 0.2 baseline threshold was set for raw data comparison across genes.





Figures 7: No significant differences were found for GnIH (A), GPR147 (B), or GPR74 (C) between sex, season, or an interaction between sex and season (mean +/- 1 SEM).

Figure 8: Experimental qPCR data for GnIH representing fluorescence of SYBR Green (ΔRn) vs. Cycle. An arbitrary 0.2 baseline threshold was set for raw data comparison across genes.

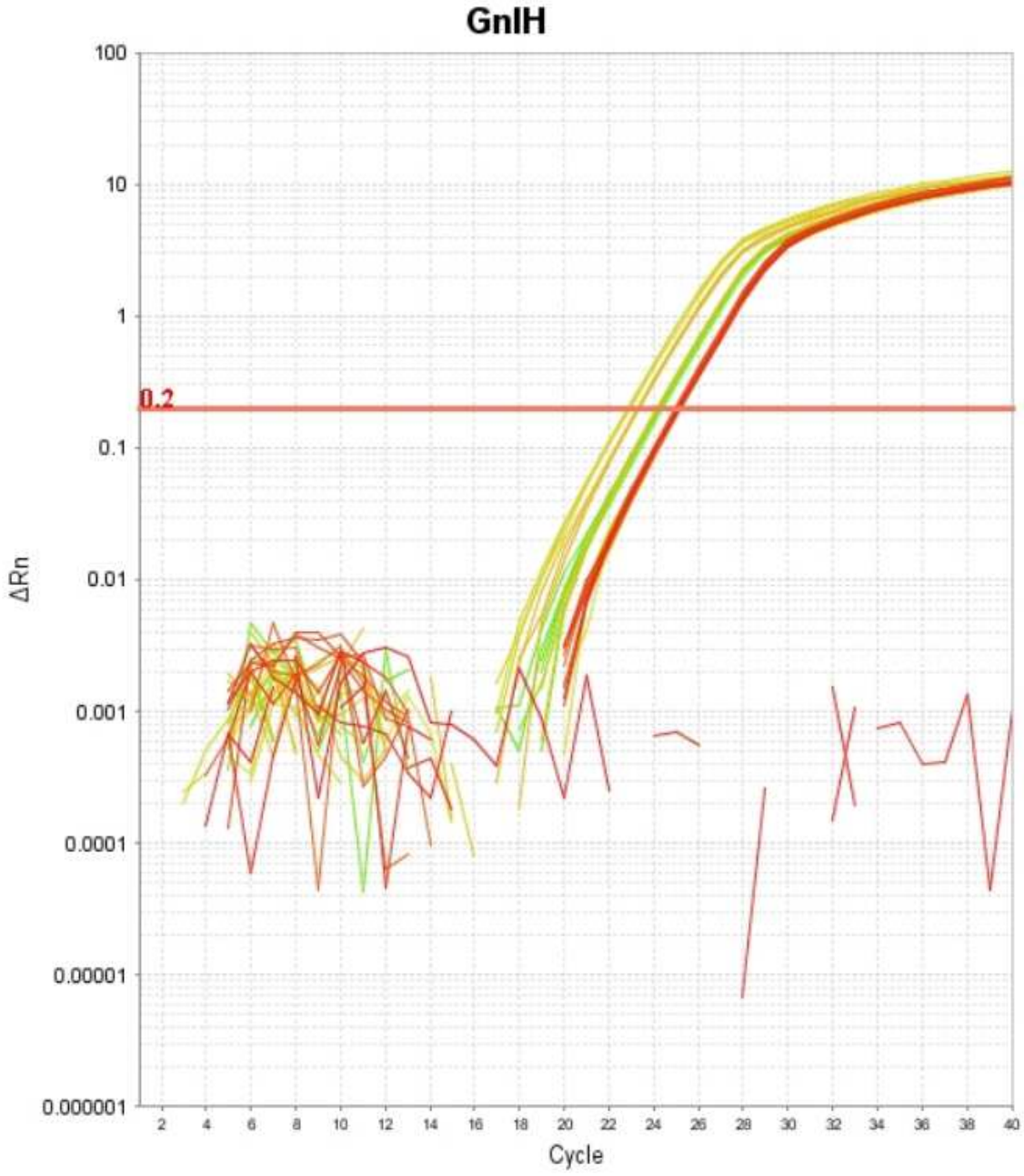


Figure 9: Experimental qPCR data for GPR147 representing fluorescence of SYBR Green (ΔRn) vs. Cycle. An arbitrary 0.2 baseline threshold was set for raw data comparison across genes.

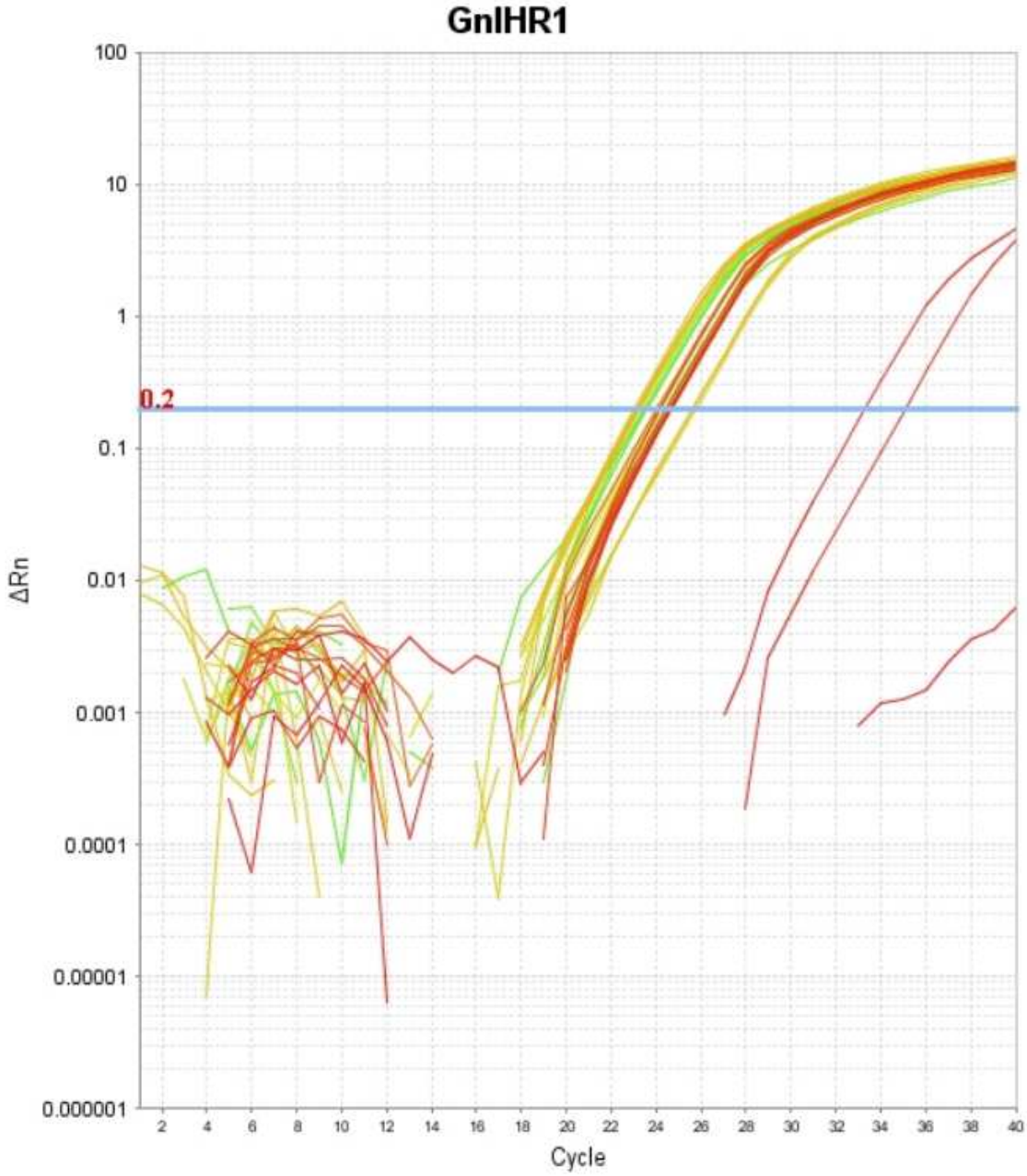


Figure 10: Experimental qPCR data for GPR74 representing fluorescence of SYBR Green (ΔRn) vs. Cycle. An arbitrary 0.2 baseline threshold was set for raw data comparison across genes.

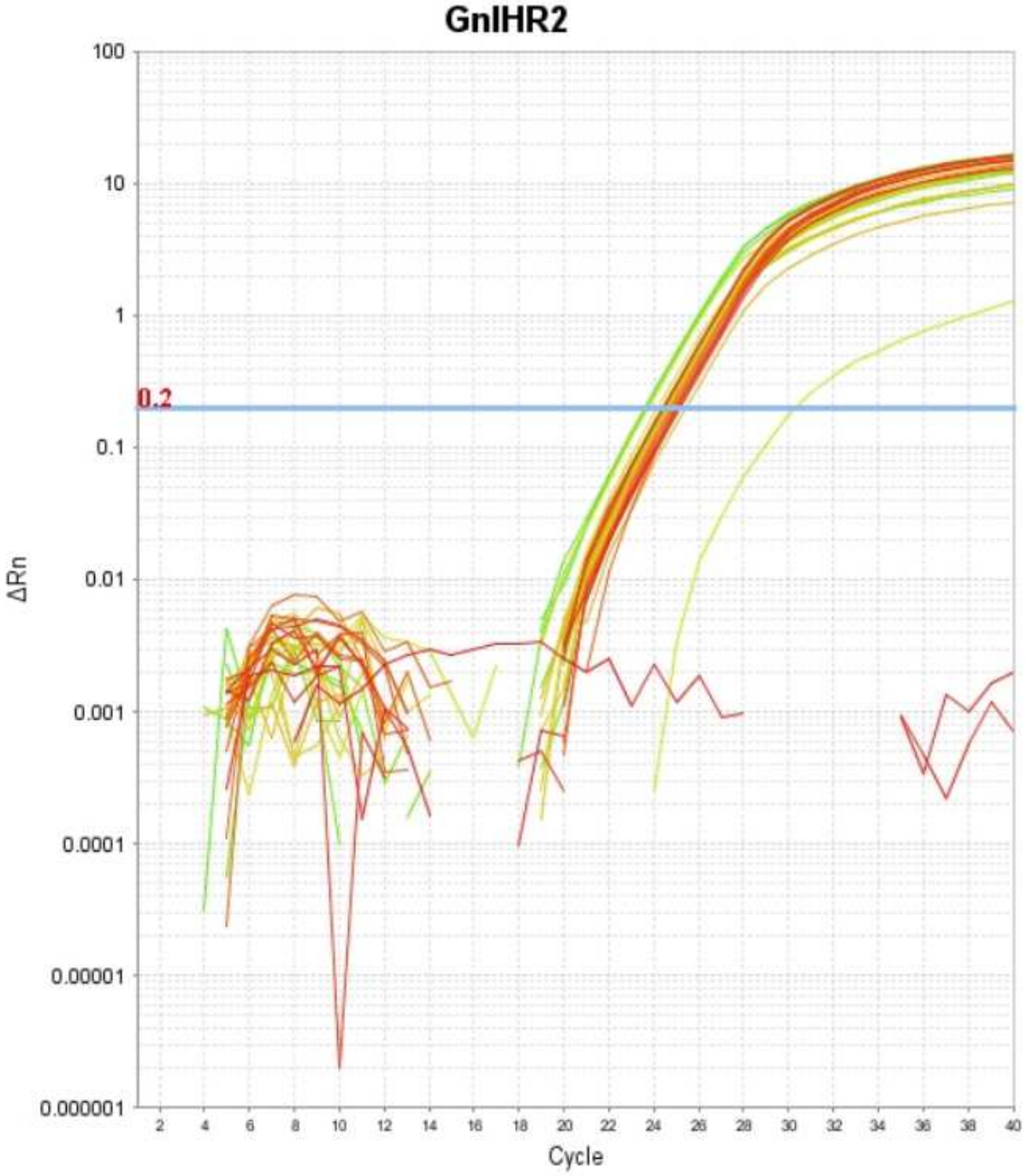


Table 1: Primers designed for all genes examined with associated efficiencies and amplicon lengths. NCBI accession numbers are indicated for annotated genes.

Gene	Accession	Primers	Efficiency	Amp.
β-actin	AF199487.1	Forward GACGAGGCGCAGAGTAAAAG Reverse TCAGGGGCAACTCTCAACTC	2.06	131
GnIH	XM_008112671.1	Forward CATCCCTGCCTTCAAAAGAT Reverse ACAATACTCCCATCCCCACA	1.96	183
GPR147	XM_008106658.1	Forward TTTTTCCCCTTTGCTCACTG Reverse CGCTGTTGTTCCTGTCTGAA	2.10	169
GPR74	XM_008112177.1	Forward TGGACAGCCATTTTCATCAT Reverse CAATCCTCCCGACACCAATA	2.04	158
Kiss1	N/A	Forward GAAGCAACAGGAGCAAGAGC Reverse TGTAAGTGTAGAGGCGTTTTTCC	2.13	119
Kiss2	KT202351	Forward TCGTGAGTCGGAGGTTCTG Reverse GTCATCTGCCAAGGTGTCC	1.98	175
GPR54	XM_008104224.1	Forward CTCAACCCCATTGTCTACGC Reverse TTGTCCTGAATACGCTGACG	2.01	155

Table 2: Sequenced amplicon fragments that align with the anole genome. Bands were cut from gels after target gene replication via PCR and sent to Functional Biosciences for sequencing. The below sequences were aligned with the anole genome.

Gene	**Amplicon Sequences with Genome Alignment**
Kiss1	ATAATCACCATTTTCCAGAGAGCTTTGCTGGTGGAACAGGAAAAACG CCTCTACACTTACAA
Kiss2	GAGACGGAGAGCCAGATCTCATGCCGGCTCCGGTTCACCAGGAGCAA ATTCAATTTCAACCCTTTCGGACTGCGTTTCGGGAAGCGTCAAGGGGA CACCTTGGCAGATGACA
GPR54	TTTCCCTTTCCTGTTTCGTCAGCGTATTCAGGAC
GnIH	CTCAATATTCTTATTATACTCATTTATTCCCATACTATATTCTGATCTC AGCTTGACTCTGTATAGTTGATGTCTCATCAATTGAAAAAAA
GnIHR1	CTTCATGGAGAAGAGGGACAAGGACACCTATTCAGACAGGAACAAC A
GnIHR2	TGTGGTGCTTGGCGAGGGCAACAAGACCAGCCCCATCTATTGGTGTC GGGAGGATTG
β-actin	GGCATCGTCACCAACTGGGACGACATGGAAAAGATCTGGCACCACAC CTTCTACAACG

Table 3: Across species sequence comparison for *kiss1* genes. A BLASTX was done to find conserved nucleotide to protein segments across species. The *kiss1* nucleotide sequences from these species were taken and compared with our anole *kiss1* nucleotide sequences.

Species	**Accession**	**mRNA Base Pairs**	**Percent Similarity to anole *kiss1***
Western Painted Turtle (*Chrysemys picta bellii*)	XP_005308380	69	75%
Garter Snake (*Thamnophis sirtalis*)	XP_013931712	46	80%
House Mouse (*Mus musculus*)	AAW34130	75	73%
Gray Short-Tailed Opossum (*Monodelphis domestica*)	NP_001137604	74	73%
Upper Galilee Mountains Blind Mole Rat (*Spalax galili*)	XP_008828595	34	82%

Table 4: Across species sequence comparison for *kiss1* translated proteins. An anole *kiss1* translated protein sequence was compared across species via the NCBI BLASTP algorithm.

Species	Kiss1
Western Painted Turtle *(Chrysemys picta bellii)*	Query 1 LQFWQGIHPSRKQIITIFQRALLVEQEKRLYTYNWNSL 38 +Q QGIHP+R + I++ Q +LLVE+EK L YNWNS Sbjct 81 VQLGQGIHPARSRAISVPQGSLLVEREKDLSAYNWNSF 118
Chinese Softshell Turtle *(Pelodiscus sinensis)*	Query 5 QGIHPSRKQIITIFQRALLVEQEKRLYTYNWNSL 38 QGIHP++ + I + Q +LL+E+EK L YNWNS Sbjct 83 QGIHPAKSRAIPVPQGSLLMEREKDLSAYNWNSF 116
Gray Short-Tailed Opossum *(Monodelphis domestica)*	Query 3 FWQGIHPSRKQIITIFQRALLVEQEKRLYTYNWNSL 38 W G+ P+R ++IT Q ALLVE+EK + TYNWNS Sbjct 96 LWPGLCPTRSRLITAPQGALLVEREKDMSTYNWNSF 131
House Mouse *(Mus musculus)*	Query 10 SRKQIITIFQRALLVEQEKRLYTYNWNSL 38 SR ++I + A+LV++EK L TYNWNS Sbjct 144 SRSRLIPAPRGAVLVQREKDLSTYNWNSF 172
Ryukyu Mouse *(Mus caroli)*	Query 10 SRKQIITIFQRALLVEQEKRLYTYNWNSL 38 SR ++I + A+LV++EK L TYNWNS Sbjct 87 SRSRLIPAPRGAVLVQREKDLSTYNWNSF 115
Subterranean Mole Rat *(Nannospalax galili)*	Query 6 GIHPSRKQIITIFQRALLVEQEKRLYTYNWNS 37 G+ P+R ++I + +LV++EK L +YNWNS Sbjct 57 GLCPARSRLIPEPRGTVLVQREKDLSSYNWNS 88
Golden Hamster *(Mesocricetus auratus)*	Query 10 SRKQIITIFQRALLVEQEKRLYTYNWNS 37 +R ++I + A+LV++EK L YNWNS Sbjct 80 ARSRLIPAPRGAVLVQREKDLSAYNWNS 107
Brandt Mole *(Lasiopodomys brandtii)*	Query 10 SRKQIITIFQRALLVEQEKRLYTYNWNS 37 +R ++I + A+LV++EK L YNWNS Sbjct 85 ARSRLIPTPRGAVLVQREKDLSAYNWNS 112

References

Alvarado, M. V., Carrillo, M., & Felip, A. (2013). Expression of kisspeptins and their Receptors, gnrh-1/gnrhr-II-1a and gonadotropin genes in the brain of adult male and female European sea bass during different gonadal stages. *General and comparative endocrinology*, *187*, 104-116.

Beck, L. A., O'Bryant, E. L., & Wade, J. S. (2008). Sex and seasonal differences in morphology of limbic forebrain nuclei in the green anole lizard. *Brain research*, *1227*, 68-75.

Burmeister, Sabrina S., Vinita Kailasanath, and Russell D. Fernald (2007). "Social dominance regulates androgen and estrogen receptor gene expression." *Hormones and behavior, 51*(1), 164-170.007

Calisi, R. M., Rizzo, N. O., & Bentley, G. E. (2008). Seasonal differences in hypothalamic EGR-1 and GnIH expression following capture-handling stress in house sparrows (Passer domesticus). *General and comparative endocrinology*, *157* (3), 283-287.

Chartrel, N., Dujardin, C., Leprince, J., Desrues, L., Tonon, M. C., Cellier, E., ... & Vaudry, H. (2002). Isolation, characterization, and distribution of a novel neuropeptide, Rana RFamide (R-RFa), in the brain of the European green frog Rana esculenta. *Journal of Comparative Neurology*, *448*(2), 111-127.

Chowdhury, V.S., Ubuka, T., Osugi, T., Shimura, T., Tsutsui, K., Chen, C. C., Fernald, R.D. (2008). Identification, localization and expression of LPXRFamide peptides, and melatonin-dependent induction of their precursor mRNA in the newt brain. *Journal of Endocrinology*, *209*(2), 211-20.

Chen, C. C. and Fernald, R. D. (2008). GnRH and GnR receptors: distribution, function and evolution. *Journal of fish biology, 73, 1099-1120.*

Cohen, R. E., & Wade, J. (2011). Aromatase mRNA in the brain of adult green anole lizards: effects of sex and season. *Journal of neuroendocrinology*, *23*(3), 254-260.

Crews, D. (1980). Interrelationships among ecological, behavioral, and neuroendocrine processes in the reproductive cycle of Anolis carolinensis and other reptiles. *Advances in the Study of Behavior*, *11*, 1-74.

Dunham, L. A., Lutterschmidt, D. I., & Wilczynski, W. (2009). Kisspeptin-like immunoreactive neuron distribution in the green anole (anolis carolinensis).*Brain, Behavior and Evolution, 73*(2), 129-37.

Felip, A., Zanuy, S., Pineda, R., Pinilla, L., Carrillo, M., Tena-Sempere, M., & Gómez, A. (2009). Evidence for two distinct KiSS genes in non-placental vertebrates that encode kisspeptins with different gonadotropin-releasing activities in fish and mammals. *Molecular and cellular endocrinology*, *312*(1), 61-71.

Findeisen, M., Rathmann, D., & Beck-Sickinger, A. G. (2011). RFamide peptides: structure, function, mechanisms and pharmaceutical potential. *Pharmaceuticals*, *4*(9), 1248-1280.

Forni, P. E., & Wray, S. (2015). GnRH, anosmia and hypogonadotropic hypogonadism. *Frontiers in neuroendocrinology*, *36*, 165-177.

Fortune, J. E., & Eppig, J. J. (1979). Effects of Gonadotropins on Steroid Secretion by Infantile and Juvenile Mouse Ovaries in Vitro*. *Endocrinology*,*105*(3), 760-768.

Hamlett, G. W. (1952). Notes on breeding and reproduction in the lizard Anolis carolinensis. *Copeia*, 183-185.

Herbison, A. E., d'Anglemont de Tassigny, X., Doran, J., & Colledge, W. H. (2010). Distribution and postnatal development of Gpr54 gene expression in mouse brain and gonadotropin-releasing hormone neurons. *Endocrinology*, *151*(1), 312-321.

Iwasa, T., Matsuzaki, T., Murakami, M., Kinouchi, R., Osugi, T., Gereltsetseg, G., ... & Tsutsui, K. (2012). Developmental changes in the mammalian gonadotropin-inhibitory hormone (GnIH) ortholog RFamide-related peptide (RFRP) and its cognate receptor GPR147 in the rat hypothalamus. *International Journal of Developmental Neuroscience*, *30*(1), 31-37.

Jones, R. E., Guillette, L. J., Summers, C. H., Tokarz, R. R., & Crews, D. (1983). The relationship among ovarian condition, steroid hormones, and estrous behavior in Anolis carolinensis. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology*, *227*(1), 145-154.

Kim, D. K., Cho, E. B., Moon, M. J., Park, S., Hwang, J. I., Do-Rego, J. L., ... & Seong, J. Y. (2012). Molecular coevolution of neuropeptides gonadotropin-releasing hormone and kisspeptin with their cognate G protein-coupled receptors. *Frontiers in neuroscience*, *6*, 3.

Knoll, J. G., Clay, C., Bouma, G., Henion, T., Schwarting, G., Millar, R., & Tobet, S. (2013). Developmental profile and sexually dimorphic expression of kiss1 and kiss1r in the fetal mouse brain. *Frontiers in endocrinology*, *4*, 140.

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*(2), 411-419.

Kriegsfeld, L. J., Mei, D. F., Bentley, G. E., Ubuka, T., Mason, A. O., Inoue, K., ... & Silver, R. (2006). Identification and characterization of a gonadotropin-inhibitory system in the brains of mammals. *Proceedings of the National academy of sciences of the United States of America*, *103*(7), 2410-2415.

Lakra, W. S. & S. Ayyappan. (2003). Recent advances in biotechnology applications to aquaculture. *Asian–Australian Journal of Animal Sciences, 16*, 455–462.

Lee, J. H., Miele, M. E., Hicks, D. J., Phillips, K. K., Trent, J. M., Weissman, B. E., & Welch, D. R. (1996). KiSS-1, a novel human malignant melanoma metastasis-suppressor gene. *Journal of the National Cancer Institute*, *88*(23), 1731-1737.

Lee, D. K., Nguyen, T., O'Neill, G. P., Cheng, R., Liu, Y., Howard, A. D., ... & O'Dowd, B. F. (1999). Discovery of a receptor related to the galanin receptors. *Febs Letters*, *446*(1), 103-107.

Lee, Y. R., Tsunekawa, K., Moon, M. J., Um, H. N., Hwang, J. I., Osugi, T., ... & Tsutsui, K. (2009). Molecular evolution of multiple forms of kisspeptins and GPR54 receptors in vertebrates. *Endocrinology*, *150*(6), 2837-2846.

Lehman, M. N., Coolen, L. M., & Goodman, R. L. (2010). Minireview: kisspeptin/neurokinin B/dynorphin (KNDy) cells of the arcuate nucleus: a central node in the control of gonadotropin-releasing hormone secretion. *Endocrinology*, *151*(8), 3479-3489.

Lehman, M. N., Merkley, C. M., Coolen, L. M., & Goodman, R. L. (2010). Anatomy of the kisspeptin neural network in mammals. *Brain research*, *1364*, 90-102.

Lescheid, D. W., Rosen, G. J., Bridge, A. E., Jones, R. E., Warby, C. M., & Sherwood, N. M. (1997). Immunoreactive gonadotropin-releasing hormone (GnRH) is detected only in the form of chicken GnRH-II within the brain of the green anole, Anolis carolinensis. *General and comparative endocrinology*, *108*(2), 247-257.

Li, X., Su, J., Lei, Z., Zhao, Y., Jin, M., Fang, R., ... & Jiao, Y. (2012). Gonadotropin-inhibitory hormone (GnIH) and its receptor in the female pig: cDNA cloning, expression in tissues and expression pattern in the reproductive axis during the estrous cycle. *Peptides*, *36*(2), 176-185.

Lovern, M.B., McNabb, F.M.A., Jenssen, T.A. (2001). Developmental effects of testosterone on behavior in male and female green anoles (*Anolis carolinensis)*. *Hormones and behavior, 39(*2), 131-143.

Lovern, M. B., Holmes, M. M., & Wade, J. (2004). The green anole (Anolis carolinensis): a reptilian model for laboratory studies of reproductive morphology and behavior. *Ilar Journal*, *45*(1), 54-64.

Messager, S., Chatzidaki, E. E., Ma, D., Hendrick, A. G., Zahn, D., Dixon, J., ... & Aparicio, S. A. (2005). Kisspeptin directly stimulates gonadotropin-releasing hormone release via G protein-coupled receptor 54. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(5), 1761-1766.

Moon, J. S., Lee, Y. R., Hwang, J. I., Lee, J. Y., Kim, J. I., Vaudry, H., ... & Seong, J. Y. (2009). Molecular cloning of the bullfrog kisspeptin receptor GPR54 with high sensitivity to Xenopus kisspeptin. *Peptides*, *30*(1), 171-179.

Moore, I. T., & Jessop, T. S. (2003). Stress, reproduction, and adrenocortical modulation in amphibians and reptiles. *Hormones and Behavior*, *43*(1), 39-47.

Moussavi, M., Wlasichuk, M., Chang, J. P., & Habibi, H. R. (2012). Seasonal effect of GnIH on gonadotrope functions in the pituitary of goldfish. *Molecular and cellular endocrinology*, *350*(1), 53-60.

Nabi, G., Muhammad, H., Ali, S., & Ullah, S. (2015). Kisspeptin, its Localization and Physiological Functions in Primates: A Review. *World Journal of Zoology*, *10*(2), 94-101.

O'Bryant, E. L., & Wade, J. (2002). Seasonal and sexual dimorphisms in the green anole forebrain. *Hormones and behavior*, *41*(4), 384-395.

Pasquier, J., Lafont, A. G., Jeng, S. R., Morini, M., Dirks, R., van den Thillart, G., ... & Dufour, S. (2012). Multiple kisspeptin receptors in early osteichthyans provide new insights into the evolution of this receptor family. *PLoS One*, *7*(11), e48931.

Pasquier, J., Lafont, A. G., Rousseau, K., Quérat, B., Chemineau, P., & Dufour, S. (2014). Looking for the bird Kiss: evolutionary scenario in sauropsids. *BMC evolutionary biology*, *14*(1), 1.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucl Acids Res*, *29*(9), 45

Pinilla, L., Aguilar, E., Dieguez, C., Millar, R. P., Tena-Sempere, M. (2012). Kisspeptin and reproduction: physiological roles and regulatory mechanisms. *American Physiological Society, 92*(3), 1235-1316.

Radder, R. S., Shanbhag, B. A., & Saidapur, S. K. (2001). Pattern of plasma sex steroid hormone levels during reproductive cycles of male and female tropical lizard, Calotes versicolor. *General and comparative endocrinology*, *124*(3), 285-292.

Revel, F. G., Ansel, L., Klosen, P., Saboureau, M., Pévet, P., Mikkelsen, J. D., & Simonneaux, V. (2007). Kisspeptin: a key link to seasonal breeding. *Reviews in Endocrine and Metabolic Disorders*, *8*(1), 57-65.

Rizwan, M. Z., Poling, M. C., Corr, M., Cornes, P. A., Augustine, R. A., Quennell, J. H., ... & Anderson, G. M. (2012). RFamide-related peptide-3 receptor gene expression in GnRH and kisspeptin neurons and GnRH-dependent mechanism of action. *Endocrinology*, *153*(8), 3770-3779.

Rosen, G., Sherwood, N., & King, J. A. (1997). Immunoreactive gonadotropin-releasing hormone (GnRHir) is associated with vestibular structures in the green anole (Anolis carolinensis). *Brain, behavior and evolution*, *50*(3), 129-138.

Sawada, K., Ukena, K., Satake, H., Iwakoshi, E., Minakata, H., & Tsutsui, K. (2002). Novel fish hypothalamic neuropeptide. *European Journal of Biochemistry*, *269*(24), 6000-6008.

Selvaraj, S., Kitano, H., Fujinaga, Y., Ohga, H., Yoneda, M., Yamaguchi, A., ... & Matsuyama, M. (2010). Molecular characterization, tissue distribution, and mRNA expression profiles of two Kiss genes in the adult male and female chub mackerel (Scomber japonicus) during different gonadal stages. *General and comparative endocrinology*, *169*(1), 28-38.

Semaan, S. J., Dhamija, S., Kim, J., Ku, E. C., & Kauffman, A. S. (2012). Assessment of epigenetic contributions to sexually-dimorphic kiss1 expression in the anteroventral periventricular nucleus of mice. *Endocrinology*, *153*(4), 1875-1886.

Seminara, S. B., Messager, S., Chatzidaki, E. E., Thresher, R. R., Acierno Jr, J. S., Shagoury, J. K., ... & Colledge, W. H. (2003). The GPR54 gene as a regulator of puberty. *New England Journal of Medicine*, *349*(17), 1614-1627.

Son, Y. L., Ubuka, T., Millar, R. P., Kanasaki, H. & Tsutsui, K. (2012). Gonadotropin-inhibitory hormone inhibits GnRH-induced gonadotropin subunit gene transcriptions by inhibiting AC/cAMP/PKA-dependent ERK pathway in LβT2 cells. *Endocrinology, 153*(5), 2332-2343.

Tsutsui, K., Saigoh, E., Ukena, K., Teranishi, H., Fujisawa, Y., Kikuchi, M., ... & Sharp, P. J. (2000). A novel avian hypothalamic peptide inhibiting gonadotropin release. *Biochemical and biophysical research communications*, *275*(2), 661-667.

Tsutsui, K., & Osugi, T. (2009). Evolutionary origin and divergence of GnIH and its homologous peptides. *General and comparative endocrinology*, *161*(1), 30-33.

Tsutsui, K., Bentley, G. E., Kriegsfeld, L. J., Osugi, T., Seong, J. Y., & Vaudry, H. (2010). Discovery and Evolutionary History of Gonadotrophin□Inhibitory Hormone and Kisspeptin: New Key Neuropeptides Controlling Reproduction. *Journal of neuroendocrinology*, *22*(7), 716-727.

Ukena, K., Iwakoshi-Ukena, E., Osugi, T., & Tsutsui, K. (2016). Identification and localization of gonadotropin-inhibitory hormone (GnIH) orthologs in the hypothalamus of the red-eared slider turtle, Trachemys scripta elegans.*General and comparative endocrinology*, *227*, 69-76.

Untergrasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M., Rozen SG (2012). Primer3 - new capabilities and interfaces. *Nucleic Acids Research, 40*(15), 115.

Wheeler, J. M., & Crews, D. (1978). The role of the anterior hypothalamus-preoptic area in the regulation of male reproductive behavior in the lizard, Anolis carolinensis: lesion studies. *Hormones and behavior*, *11*(1), 42-60.

Yun, S., Furlong, M., Sim, M., Cho, M., Park, S., Cho, E. B., ... & Seong, J. Y. (2015). Pre-vertebrate Local Gene Duplication Facilitated Expansion of the Neuropeptide GPCR Superfamily. *Molecular biology and evolution*, msv179.

Zhao, S., Zhu, E., Yang, C., Bentley, G. E., Tsutsui, K., & Kriegsfeld, L. J. (2010). RFamide-related peptide and messenger ribonucleic acid expression in mammalian testis: association with the spermatogenic cycle. *Endocrinology*, *151*(2), 617-627.