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Examining the effects of Fadrozole, an aromatase inhibitor, on testosterone and estrogen production of domestic chicken embryos (Gallus gallus)

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by

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ABSTRACT:

The hypothalamic-pituitary-gonadal (HPG) axis is responsible for the production of the hormones testosterone and estradiol, and testosterone is thought to contribute to regulation of the axis through a negative feedback mechanism. Regulation by negative feedback involves the product of a pathway turning off that pathway when enough product is made. However, because the enzyme P450 aromatase converts testosterone to estradiol, estradiol may also contribute to regulation of the HPG axis and other phenomena that have been attributed to testosterone, like the inhibition of immune function. Previous studies have injected birds with an aromatase inhibitor (presumably reducing estradiol production) and shown a subsequent increase in the immunity of the injected birds as compared to their controls, implying that estradiol was decreasing immune function. In order to support the hypothesis that it is estradiol, not testosterone, which is inhibiting immune function, it is important to show that a decrease in estradiol and not a subsequent increase in testosterone is what is leading to the decreased immunity. In order to test this hypothesis that estradiol is inhibiting immune function, it is important to determine whether estradiol is a contributor to negative feedback of the HPG axis. In this thesis, I tested the hypothesis that estradiol affects negative feedback in the HPG axis. Chicken embryos were injected with either Fadrozole, an aromatase inhibitor, or a vehicle solution on day 13 of incubation. Two days later, the embryos were bled for hormone quantification and genetic sexing. Genetic sexing was obtained through DNA extraction, PCR, and gel electrophoresis. Hormone levels were compared across treatment and sex through ELISA assays. By using embryos, the immediate effects of

Fadrozole on negative feedback of the HPG axis were assessed. In contrast, recent studies of looking at androgen exposure have mainly focused on the post-hatch stages of development.

Estradiol levels in female Fadrozole treated embryos were significantly lower than in female control embryos, but this pattern was not seen in males. This could be due to the greater amount of estradiol and aromatase in females as compared to males. Additionally, females had a significantly higher level of estradiol than males, which is consistent with published literature and is most likely due to the major role that estrogen plays in female development. There was no significant difference in testosterone levels between treatment groups or sexes, implying that it is the decrease in estradiol, and not a subsequent increase in testosterone, that lead to the increase in immunity in Fadrozole treated embryos in previous studies. This work also implies that estradiol is not affecting negative feedback in the HPG axis of chicken embryos but that it may in fact be affected by testosterone.

I. INTRODUCTION:

Introduction to Testosterone and Estradiol and their role in development:

Hormones are signaling molecules that circulate throughout the body until they reach their target tissues which they act on by binding to receptors expressed inside or on the surface of target tissue cells. Receptors are proteins that act as docking stations to which hormones bind in order to exert their effects. Testosterone ("T") and estradiol ("E") are steroid hormones present in both sexes, but they are seen in higher quantities in the male and female reproductive systems respectively, where they are known for their role in maturation and regulation. Steroid hormones exert their effects early on in development during "critical periods" (Carere & Balthazart 2007), which means that these hormones have the ability to affect an individual's development before they are born. Although the exact roles of testosterone and estradiol in early development are still debated, they are known to have an impact on sexual differentiation. In general, testosterone is known for its role in masculinizing individuals especially through secondary sex characteristics, while estradiol is known for its role in feminizing individuals. Sexual differentiation is mainly genetic but, in birds, the main gene involved in sexual differentiation is the DMRT1 gene, whose activity leads to the masculinization of gonads (Chue & Smith 2011). In addition to the DMRT1 gene, testosterone and estradiol also participate in sexual differentiation. An extreme example was found by Abinawanto et al. (1997) who injected embryos with an inhibitor that blocked estradiol production and saw sex reversal in hatched chicks. The depletion of estradiol in genetic females led them to have the morphological features of males post hatch. Additionally,

steroid hormones also affect sexual behaviors or differences (Carere & Balthazart 2007), reproductive physiology, gamete production, and reproductive regulation in adult birds. More specifically, in birds, testosterone and androgens (a category encompassing all male sex hormones including testosterone) present in the egg affect early embryonic development as shown through earlier hatch time, increased neck muscle, faster post hatch growth and decreased early immune function (Carere & Balthazart 2007). However, due to a process called aromatization, it has been hypothesized that estradiol might actually be exerting some of the developmental effects that were previously attributed to testosterone (Owen-Ashley 2004).

Aromatization

During steroidogenesis, some testosterone is converted to estradiol through a process called aromatization. Cytochrome P9450 aromatase, an enzyme, is responsible for this normal conversion of testosterone to estradiol (Simpson et al. 2002). In the process of aromatization, the enzyme converts the non-aromatic ring in testosterone into an aromatic ring, making the steroid estradiol (Bardal et al. 2011). Converting androgens to estrogens is the only way estradiol can be produced and then goes on to bind to unique receptors on its specific target tissues. Estradiol and testosterone bind to different receptors on different target tissues, resulting in the stimulation and inhibition of different pathways thus triggering different consequences throughout the body.

Hypothalamic-Pituitary-Gonad Axis

One of the ways that testosterone production is regulated within the body is through the hypothalamic-pituitary-gonad (HPG) axis (Figure 1). The HPG axis starts

with the hypothalamus, a region of the brain, secreting gonadotropin releasing hormone (GnRH) which binds to GnRH receptors on the pituitary gland. In response, the pituitary then releases follicle stimulating hormone (FSH) and luteinizing hormone (LH) which bind to receptors on the gonads. In the testes, LH binds to Leydig cells to stimulate testosterone production while FSH binds to receptors on Sertoli cells to stimulate spermatogenesis (Whirledge & Cidlowski 2010). In the ovary, FSH binds to receptors leading to the production of estrogen (which requires the activity of aromatase) while LH stimulates oocyte maturation, ovulation, and follicular luteinization (Whirledge & Cidlowski 2010). Overall the HPG axis leads to the production of testosterone by the testes or estradiol by the ovaries, and the release of these hormones into general circulation (Figure 1).

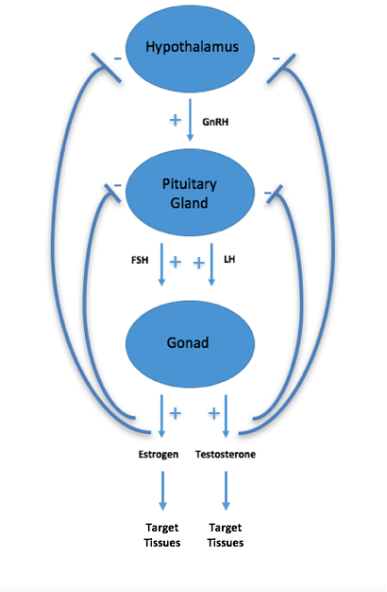


Figure 1. Pathway of hormone secretion of HPG axis showing production of testosterone and estradiol and their possible inhibitory effects. Arrows represent a stimulatory effect on secretion while blunt-ended lines represent an inhibitory effect on secretion

Negative Feedback in the HPG Axis:

Physiological systems like the HPG axis are often regulated by negative feedback mechanisms, through which the product of a pathway is able to shut that pathway off when enough of that product is made. This mechanism prevents hyperactivity of physiological pathways and maintains homeostasis in the body. In addition to being upregulated by the hormonal cascade described above, testosterone and estradiol are strongly regulated by a negative feedback system within the HPG axis. For example, testosterone binds to receptors on the hypothalamus and pituitary gland to stop them from stimulating more testosterone production by reducing the amount of GnRH, LH, and FSH secreted. However, because aromatase converts testosterone to estradiol, a hypothesis has emerged that even in males, estradiol binds to the hypothalamus and pituitary gland and inhibits that axis, rather than testosterone (Tsai et al. 1994; Rochira et al. 2006). The

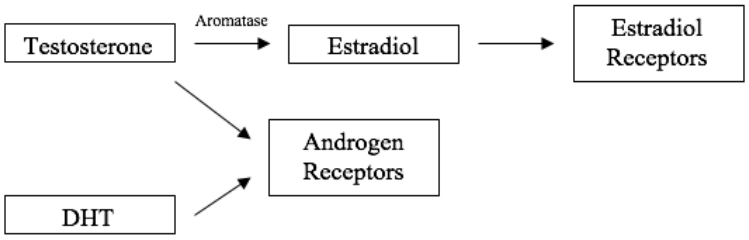
underlying mechanism of how androgens or estrogens might exert negative feedback on the axis remains unclear (O'Hara et al. 2015), but there are known estradiol receptors (mainly estrogen receptor α) on the hypothalamus, pituitary and gonads. These receptors allow estradiol to bind and reduce GnRH secretion from the hypothalamus and ultimately inhibit the axis (Couse et al. 2003; Dorling et al. 2003). Examples of estradiol inhibition of the HPG axis from mammalian models are well-understood, but it is still relatively unknown how this system works in birds. Therefore, further research is still necessary to explore the relative contributions of testosterone or estradiol to HPG axis regulation in birds.

Phenomena attributed to testosterone that could be due to estradiol:

Research has shown some phenomena previously attributed to testosterone are likely due to estradiol. An example of one of these phenomena is decreased immune function (Owen-Ashley et al. 2004; Simkins et al. 2018). A large body of literature describes the ability of testosterone to suppress immune function in birds (Folstad & Karter 1993; Roberts et al. 2004; Cunningham and Gilkeson 2011). Owen-Ashley et al. (2004) injected birds with DHT (a steroid created from testosterone in steroidogenesis), testosterone or a control to see if there was a direct or alternative pathway between testosterone and immune response. Note that both testosterone and DHT bind to the same androgen receptors on target cells and exert their effects, but DHT binds with a much greater affinity than testosterone and, importantly, cannot be aromatized into estrogen (unlike testosterone). They found that injecting birds with DHT did not decrease immune function however, injecting birds directly with testosterone did. Note that testosterone is a

precursor for both DHT and estradiol (Figure 2a). These results suggest that there is an alternative pathway that testosterone is a part of, that does not involve DHT, that explains testosterone's contribution to the decreased immune response seen. That alternative pathway is hypothesized to be aromatization into estrogen (Figure 2b).

A.



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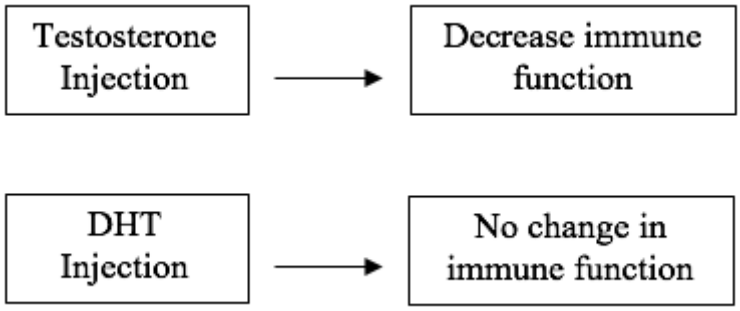


Figure 2. A) Schematic showing testosterone as the precursor of estradiol and DHT. **B)** Schematic of Owen-Ashley et al. (2004) experimental results of testosterone and DHT injections.

Studies supporting estradiol as the regulator of negative feedback:

Many studies in other non-avian vertebrates have manipulated aromatization via aromatase inhibitors to study the role of T and E in HPG axis regulation. Rochira et al. (2006) found that in humans, when aromatase deficient men were injected with estradiol, downstream effects on both the pituitary and hypothalamic levels of the axis were

observed. GnRH-stimulated secretion of LH decreased and the frequency of LH pulses decreased, both indications of inhibition of the axis by the estradiol injections. The hypothesis that estradiol is responsible for the inhibition was also supported by an experiment that gave healthy men an aromatase inhibitor and saw increases in LH and FSH, suggesting that the estradiol that was no longer present had been a main factor in inhibiting this axis. Additionally, Bulun (2016) injected pre-menopausal woman with an aromatase inhibitor and found that with a decrease of estradiol production, there was an increase in the HPG axis activity shown through increased GnRH secretion along with accompanying increasing LH and FSH levels. Tsai et al. (1993) also supported estrogen as the inhibitor with their work in sea turtles. They suggest that the lack of physiological involvement of testosterone in this negative feedback system parallels observations in mammals.

In avian species and domestic chickens in particular, similar results have been found. Rombauts et al. (1993) found that female chickens treated with an aromatase inhibitor exhibited an increase in FSH levels. In other experiments they also found that male chicks injected with estradiol exhibited a decrease in their FSH levels, suggesting that estradiol was inhibiting the FSH secretion (Rombauts et al. 1993). This supports the hypothesis that estradiol is the steroid hormone that leads to the inhibition of the HPG axis in both sexes in these instances. However, the body of literature investigating the role of androgens is still growing, and many suggest that testosterone is still responsible for phenomena like immunosuppression, although some more recent studies have shown that the hormone responsible could actually be estradiol (Owen-Ashley 2004; Simkins et

al. 2018). Therefore, it is important to consider the aromatization process which was not considered in Evans et al. (2000) and Duffy et al. (2000) leading them to suggest testosterone as a key regulator. Therefore, this thesis aims to test the role of aromatization during embryonic development in birds, and to see if there is negative feedback on the HPG axis due to estradiol.

Differences in males in females in hormone levels and Fadrozole effectiveness:

Differing levels of hormones in male vs. female chicken embryos have been reported for testosterone (Woods et al. 1975) and estradiol (Woods & Brazzill 1980). On day 15.5 of embryonic development levels of testosterone were found to be higher in males as compared to females (Woods et al. 1975) and levels of estradiol were found to be higher in females as compared to males (Woods & Brazzill 1980). Males and females have been seen to show different responses when exposed to Fadrozole, an aromatase inhibitor, during early development which is vital information to this experiment. Fadrozole is a competitive inhibitor with high selectivity for aromatase (Yue et al. 1997). Fadrozole inhibits aromatase activity and consequently decreases the aromatization of testosterone to estradiol. Adkins-Regan et al. (1996) found that newly hatched female Zebra finches injected with Fadrozole, an aromatase inhibitor, exhibited male like behavior while males treated with Fadrozole acted similarly to their controls. They explained these results by suggesting that females are more susceptible to estrogen as an organizational hormone early on in their lifetime while males may not be. Simkins et al. (2018) also found that Fadrozole treated females had increased immunity as compared to their controls as measure by IgY antibody levels. This increase in IgY levels was not seen

as strongly in Fadrozole males as compared to their controls (Simkins et al. 2018). This evidence led me to hypothesize that females may be more affected by Fadrozole, as compared to males. Therefore, by quantifying the amount of testosterone and estradiol present in the plasma, I can see the effects Fadrozole may have on these hormones in males compared to females.

Experiment Overview and Hypotheses:

In this thesis, the hypothesis that estradiol affects negative feedback in the HPG axis was tested. This test was done through quantification of the levels of testosterone and estradiol in day 15 chicken embryos that were injected on day 13 with either a control or Fadrozole solution. This analysis made it possible to observe the extent in which testosterone and estradiol act as inhibitors of the HPG axis during the embryonic stage in birds. If the hypothesis that estradiol exerts negative feedback on HPG activity is correct, then embryos injected with an aromatase inhibitor, blocking the conversion of testosterone to estradiol, should show an increase in testosterone and a decrease in estradiol production compared to control embryos. This result will be referred to as outcome 1. If outcome 1 occurs, then we can confirm that the Fadrozole is blocking estrogen synthesis and the estradiol that is lacking would normally be exerting negative feedback on the HPG axis; because estradiol is not present, testosterone is being hypersecreted. Outcome 1 could also be interpreted to mean that the lack of aromatization has left a lot of testosterone behind that was originally destined to be converted to estradiol. An alternative hypothesis is that testosterone is exerting negative feedback. If this is correct then outcome 2 would be seen which is a decrease in estradiol in Fadrozole

treated embryos compared to control embryos, but testosterone will remain the same in Fadrozole compared to control groups (Table 1). If outcome 2 occurs, it would confirm that the Fadrozole is blocking estrogen synthesis but estradiol does not have inhibitory effects on the HPG axis. It could not be implied that it is then testosterone that is inhibiting the axis because it may be something else entirely. The final potential outcome would be that Fadrozole did not block estrogen synthesis or did not exert its effects long enough to be detected 2 days after administration, which would lead to outcome 3. Outcome 3 shows no change in testosterone or estradiol production in Fadrozole treated embryos as compared to the control (Table 1).

In addition to illuminating the role of testosterone and estradiol in negative feedback of the HPG axis, this study also has an additional application. Jeff Simkins (‘19) conducted a similar study in order to measure how aromatase inhibitors and consequently testosterone and estradiol concentrations affect immune function in developing chickens (Simkins et al. 2018). He tested whether testosterones conversion to estradiol is involved in immune function development in birds. However, Simkins did not quantify the actual levels of testosterone and estradiol in the embryonic chicks to ensure that Fadrozole inhibited estradiol production, and to determine whether testosterone levels were altered. He found that chicks treated with Fadrozole did have an increase in immune function but was not able to show that those effects were due to a decrease in estradiol and not an increase in testosterone. Therefore, this study augments Simkins’ data and provides the information on the levels of the hormones at embryonic day 15.

Table 1. Potential outcomes and interpretations

Outcome	Difference in testosterone and estradiol in Fadrozole treated embryos compared to control	Interpretation	Effectiveness of Fadrozole
1	Estradiol decreases and testosterone increases	Estradiol exerts negative feedback so testosterone is being hypersecreted	Effective
2	Estradiol decreases and testosterone remains the same	Estradiol does not exert negative feedback on the axis and negative feedback by testosterone cannot be inferred.	Effective
3	Estradiol and testosterone remain the same	Fadrozole is not working and did not stop the aromatization process	Ineffective or did not persist through our time period of injection to bleeds.

II. MATERIALS AND METHODS:

Egg Care:

97 fertile, unincubated, fertile White Leghorn domestic chicken eggs (*Gallus gallus)* were obtained from Moyer's Chicks in Quakertown, PA and incubated at 37.5 °C and 47-51% humidity in an Ova-Easy Advance Incubator (Brinsea, Titusville, FL, USA). The eggs were placed on a 90-minute turn cycle (Simkins et al. 2018) and checked twice daily to monitor temperature and humidity. These eggs were divided into two batches. Batch one was incubated in September 2018 and Batch 2 was incubated in February 2019. Within each batch there were 2 groups in order to stagger the eggs and make injections and blood sampling manageable and timely. The first group in batch 1, made up of 24 eggs, was put in the incubator followed by the second group of 24 eggs 2 days

later. This ensured that we were only injecting or bleeding 24 eggs at a time. Eggs were also rotated to different shelves of the incubator to ensure that no shelf or egg group was given more or less humidity than another. The same strategies were employed in batch 2.

Fadrozole/Control Injections:

Eggs were weighed, sorted into ascending weight order, then assigned to either the control or Fadrozole group based on this pattern starting with the lightest to the heaviest: CFFCCFFCC (where C is control and F is Fadrozole). This sorting was chosen in order to make sure that the control and Fadrozole groups were of similar weight distributions and weight could not be explaining any treatment differences. The control group had an average weight of 56.96 ± 4.32 g and the Fadrozole group had an average weight of 56.71 ± 3.66 g. The weights of the two groups were not significantly different ($t=_{1,96}.18$, p=.86, α =0.05).

On day 13 of embryonic development, the eggs in the Fadrozole group were injected into the air sac with 0.1 mg of Fadrozole (Sigma-Aldrich, St Louis, MO, USA; F3806) in 0.1 mL of 0.9% NaCl solution using a 0.5 in, 27 g needle as described in Abinawanto et al. (1997). The air sack is located on the blunt end of the egg and provides an easily accessible area to inject into and ensure that either the Fadrozole or control solution will be taken up by the embryo without harming the embryo. The eggs in the control group were injected into the air sac with 0.1 mL of 0.9% NaCl solution as a control. The injection holes were sealed with super glue and eggs were placed back in the incubator. Embryos were bled and euthanized 2 days post-injection, on day 15 of incubation (Simkins et al. 2018).

Blood Sampling:

On day 15 the embryos were sacrificed for blood sampling and tissue collection. Upon removal from the incubator, the egg was broken open exposing the embryo and its blood vessels, and a syringe was used to prick a major visible blood vessel, allowing blood to flow out freely. Blood was then collected with capillary tubes and centrifuged down on a tabletop centrifuge for ~5 minutes at 6,000 rpm to separate into red blood cells and plasma. The plasma was then collected and placed in a separate microcentrifuge tube. Blood was collected from the embryos until it was impossible to collect anymore or the blood appeared to be diluted with albumin. After the blood was collected and separated it was placed in the -20°C freezer.

The embryos were then dissected and visual inspection of the gonads was used to determine gonadal sex of each embryo. The presence of 2 small gonads (testes) indicates male and 1 large gonad and 1 smaller gonad (ovaries) indicating a female. The reason one ovary is larger than the other is because only one ovary undergoes differentiation in chickens.

Genetic Sexing of Embryos

For genetic sexing, the DNA found in red blood cells of the chicken embryos was used to determine the sex. Genetic sexing was determined with PCR and gel electrophoresis using Platinum Green Mastermix (Applied Biosystems, Waltham, MA, USA; L00192), primers AvianSex 2550F (GTTACTGATTCGTCTACGAGA) and AvianSex2718R (ATTGAAATGATCCAGTGCTTG) (Fridolfsson and Ellegren 1999), and 2.5 high-resolution agarose gels. In chickens, females are the heterogametic sex

(ZW) while males are the homogametic sex (ZZ). Heterogametic means that the sex chromosomes are different (Z and W) while homogametic means that the two sex chromosomes are the same (2 Z chromosomes). In humans we see the opposite where males are heterogametic (XY) and females are homogametic (XX). Therefore, due to the ZW, ZZ nature of birds, Fridolfsson and Ellegren (1999) found 2 genes, 1 that was specific to the Z chromosome and one specific to the W chromosome, and developed primers that could amplify Z and W specific regions during PCR. The genes that they found were CHD1W and CHD1Z. The primers listed above are specific to regions of introns that flank the CHD1W and CHD1Z regions. Therefore, if the chick is a female, both of those genes will be amplified (Z and W gene) and shown on the gel as two different bands, and if it is a male only 1 gene will be amplified (the Z gene) and shown on the gel as a single band (Figure 3).

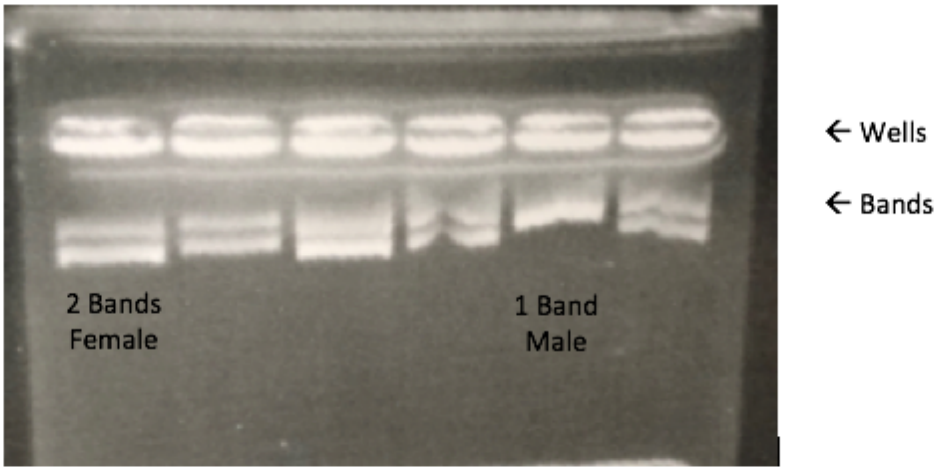


Figure 3. Gel Image for genetic sexing using gel electrophoresis

Hormone Extraction from Plasma

In order to extract the hormones from the plasma and to prevent other lipids from interfering in the assay, a dichloromethane extraction was done. 80 samples were separated into testosterone and estradiol groups. Sex, treatment, experiment number, and batch were distributed evenly between hormones. 40 samples were set aside for testosterone extraction and 40 were set aside for estradiol extraction. For this experiment the range of plasma available for extraction was from 20-200 μL.

During extraction some amount of hormone is always lost, and it is important to quantify that proportion lost in order to factor that into the final concentration calculations. This proportion remaining in each sample after extraction is referred to as the individual recovery. To determine recoveries, 20 μL of radioactive T or E was then added to the sample, as the radioactive hormone is expected to be lost in the same proportions as the hormone present in the sample that I am trying to quantify. The average CPM/20μL was counted on a scintillation counter, in triplicate, to give us total recoveries (the amount of radioactivity that would be recovered if no hormone was lost). 300 μL of distilled water was added to each sample. The samples were then vortexed and set to equilibrate overnight at 4°C to ensure that the radioactivity was evenly distributed throughout the sample. The following day 5 mL of distilled dichloromethane was added, vortexed and incubated at room temperature for 2 hours. The supranatant was then removed and dried down in a water bath using nitrogen gas. The samples were then reconstituted in either 250 μL or 350 μL of PBSg (phosphate buffered saline with gelatin) for testosterone and estradiol respectively, vortexed and left to sit for 24 hours at 4°C.

They were then vortexed and 100 μL was removed and counted on a scintillation counter to determine individual hormone recovery for each sample. The scintillation counter measures the amount of radioactivity in each sample and report that value which we then can classify as individual recovery. Each individual recovery is converted to the percent of the total recovery (from above) recovered from the sample. The individual recoveries were then used to correct the raw concentration values from the EIA to determine how much testosterone and estradiol was present in each individual sample.

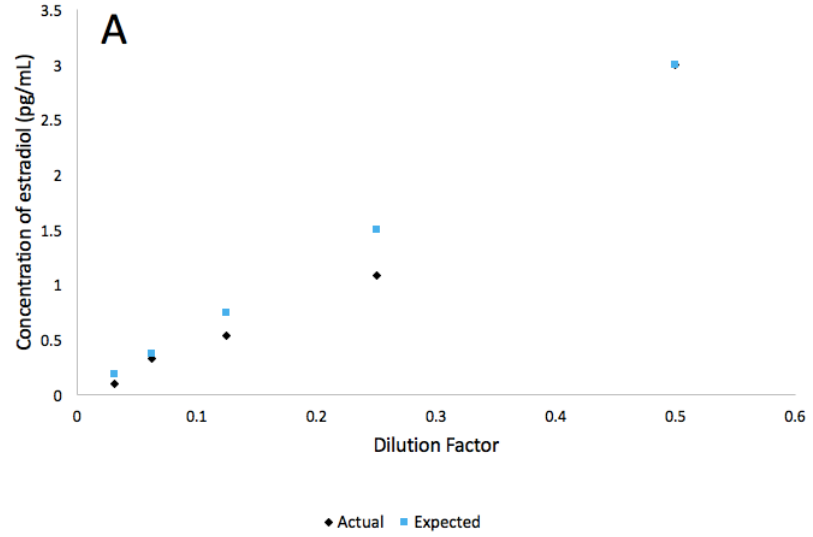
Quantification of Testosterone and Estradiol:

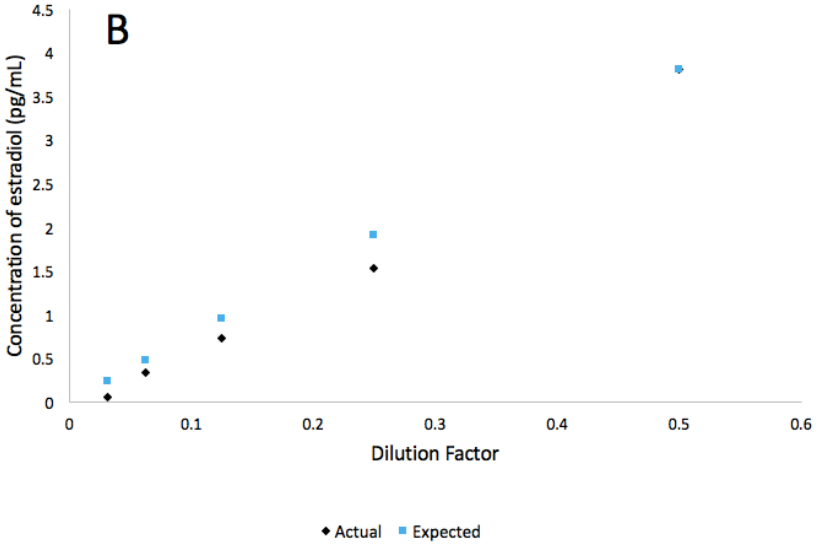
The amount of testosterone and estradiol in the samples was then quantified using Salivary Testosterone (Salimetrics, State College, PA, USA 1-2312) and Salivary Estradiol (Salimetrics, State College, PA, USA 1-4702) kits. After running a serial dilution, a 50% dilution of the samples was determined to be optimal for both the testosterone and estradiol assays. Testosterone was measured in duplicate but estradiol was assayed without duplicates due to the shortage of sample. The average % CV for the testosterone assay was 6.05%. %CV for estradiol were not able to be determined because the samples were not run in duplicate. However, the relatively low %CV obtained from the testosterone EIA gave confidence in the estradiol values despite the lack of replicates.

Validation of ELISA

Validations were conducted on both testosterone and estradiol ELISA kits. Validations are done through serial dilutions of the plasma in order to confirm that with each dilution the concentration of hormone in each sample is changing proportionally to the dilution. A serial dilution was done for testosterone and estradiol for each sex (Figure

4) with each subsequent dilution being half the concentration of the previous. Dilutions were validated by ensuring that the concentration found was half the concentration of the previous well. These validations were also conducted to ensure that the dilution chosen would fall on the standard curve. The dilution of 50% was selected for both T and E samples after ensuring those values met the needed requirements.





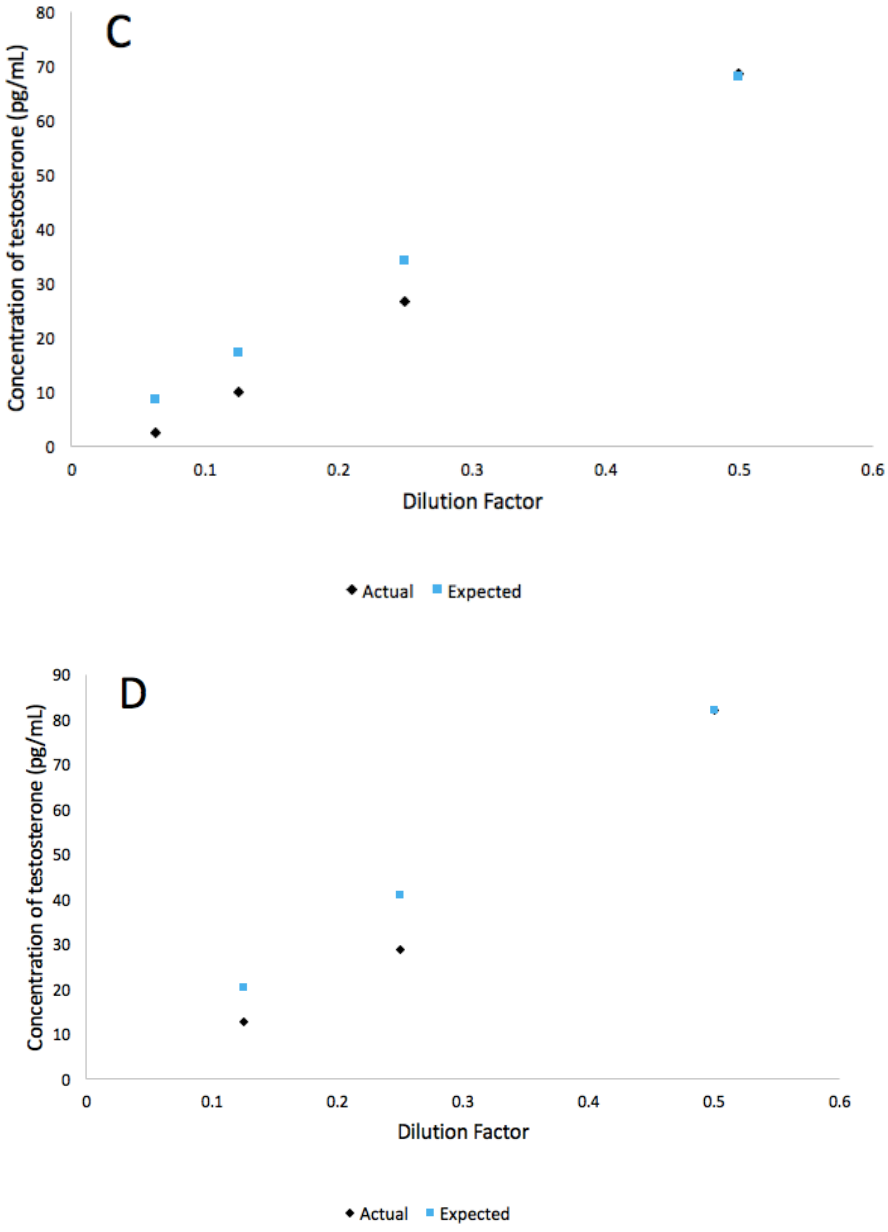
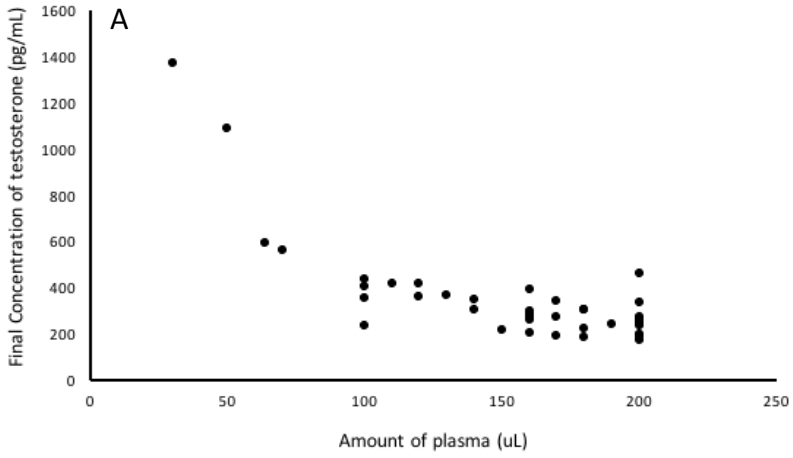


Figure 4. (A) Validation data for estradiol in males where blue square markers represent a perfect dilution and black diamond markers represent actual dilution values. **(B)** Validation data for estradiol in females where blue square markers represent a perfect dilution and black diamond markers represent actual dilution values. **(C)** Validation data for testosterone in males where blue square markers represent a perfect dilution and black diamond markers represent actual dilution values. **(D)** Validation data for testosterone in females where blue square markers represent a perfect dilution and black diamond markers represent actual dilution values.

Data Analysis and Statistics

There was negative relationship between plasma volume and final concentration, which suggests that samples with lower plasma levels had inflated final concentrations (Figure 5). Therefore, samples with <100 μL were removed from the data set and all further analyses. The remaining samples were used for analysis. (Table 2).

Hormone concentrations were tested for normality using a Shapiro Wilkes W test by genetic sex and by hormone. There was no significant deviation from normality and therefore we proceeded with parametric analyses. A full linear model was run with JMP Pro 14 to test for sex, treatment, and sex by treatment interaction effects. Post hoc t-tests were then run to identify effects indicated by significant sex by treatment interactions. Effects of batch and group were also analyzed and although there was a suggestion that Fadrozole had different effects on what? seasonally, there was no significance in the overall model. Need to say clearly somewhere that batch and group were not significant



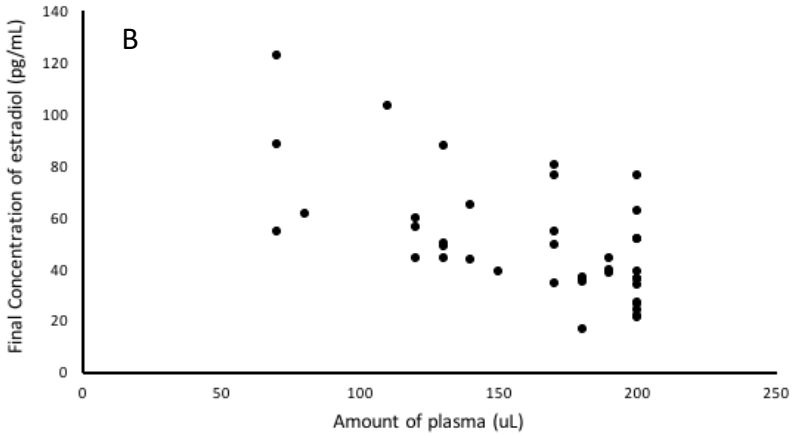


Figure 5. (a) A negative relationship between amount of plasma and final testosterone concentration suggested that lower plasma concentrations had an inflated final concentration. Samples <100μL were left out of all analyses. **(b)** A negative relationship between amount of plasma and final estradiol concentration suggested that lower plasma concentrations had an inflated final concentration. Samples <100μL were left out of all analyses.

Table 2. Final sample sizes for testosterone and estradiol used in analysis

	Estradiol		Testosterone	
	Male	Female	Male	Female
Control	7	11	8	12
Fadrozole	10	8	9	7

III. RESULTS:

Development of Embryos:

There was no significant difference in the survival of Control vs. Fadrozole embryos

($Z_{1,96}$=.547, p=.58, α=0.05) (Table 3).

Table 3. Development success of embryos at day 15 in both control and Fadrozole groups.

Group	Developed	Undeveloped	% Developed
Control	41 (26 females, 15 males)	7	85.4%
Fadrozole	39 (19 females, 20 males)	9	81.2%

Male and Female Distribution between Treatment

This experiment included 15 control males, 26 control females, 20 Fadrozole males and 19 Fadrozole females (Table 3). There was no significant difference in the distribution of males and females between control and Fadrozole groups. ($X^2_{1,1}$= 1.75, p= 0.185, α=0.05).

Linear Model

For estradiol, there was a significant effect of the treatment by sex interaction ($t_{3,35}$=2.87, p=0.0072, α=0.05) (Figure 6). There was also significant effect due to treatment alone ($t_{3,35}$=2.13, p=.0411, α=0.05). There were no significant effects with testosterone of sex, treatment, or sex by treatment interactions (Figure 7).

Post-Hoc tests:

Post-hoc t-test revealed a significant difference in estradiol concentrations in control vs Fadrozole females ($t_{1,16}$=-4.98, p<0.0001, α=0.05) (Figure 6). Post hoc t-test reveal a significantly higher level of estradiol in control females as compared to control males ($t_{1,16}$=-3.57, p=0.0025, α=0.05) and that significant difference disappeared in the Fadrozole females as compared to Fadrozole males ($t_{1,12}$=1.27, p=0.226, α=0.05) further illustrating the effects of Fadrozole on estradiol levels in females.

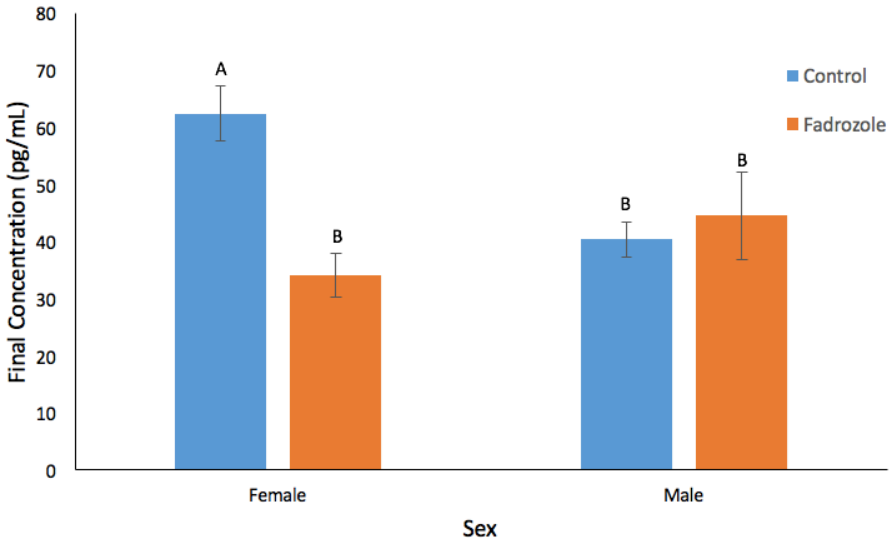


Figure 6. Estradiol concentration in female vs. male embryos. The estradiol concentration of the control females was significantly higher than that of Fadrozole females, control males or Fadrozole males. A and B represent groups that are significantly different from each other.

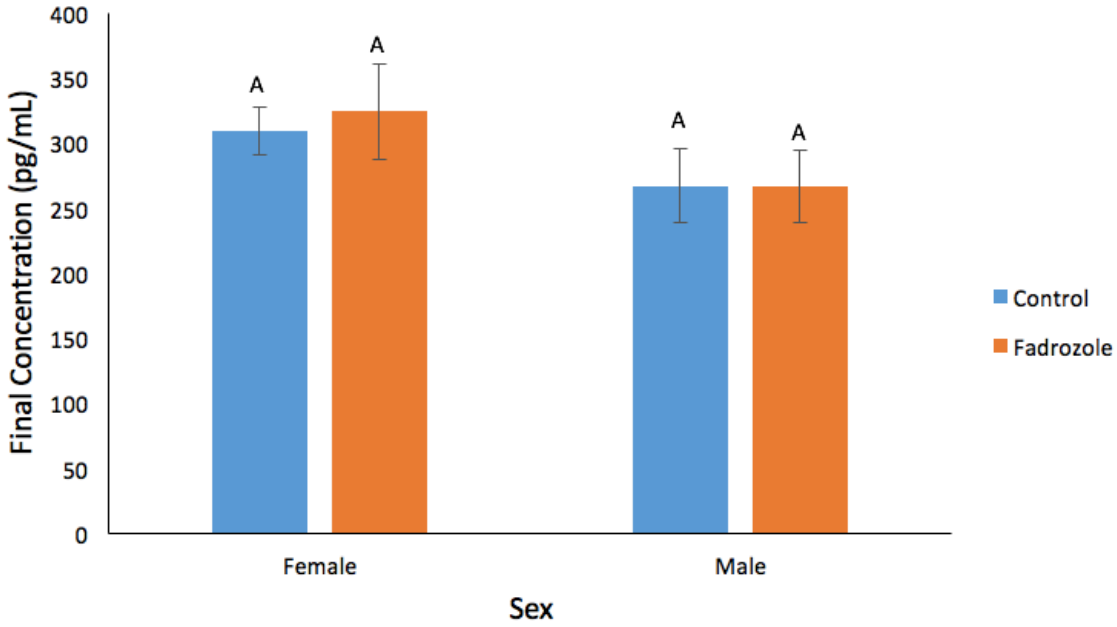


Figure 7. Testosterone concentration in female vs. male embryos. There was no significant difference in the testosterone concentration by sex or treatment.

IV. DISCUSSION:

Summary:

The goal of this thesis was to quantify the amount of testosterone and estradiol in Fadrozole treated vs. control embryonic chicks in order to see if testosterone or estradiol affects regulation of the HPG axis through negative feedback. Estradiol concentrations were significantly affected by the treatment by sex interaction, in which estradiol concentration was significantly lower in female embryos treated with Fadrozole as compared to controls, but males did not show this difference. There were no significant effects with testosterone in sex, treatment, or sex by treatment interactions. This evidence suggests that estradiol does not affect regulation of the HPG axis through negative feedback. There is also no definite role of testosterone in regulation seen through these results.

Effect of Fadrozole on Development:

No significant difference was found in the development of chicks treated with Fadrozole as compared to those in the control group (Table 3). This confirms that Fadrozole did not significantly affect the development of the embryos. Although this same dosage of Fadrozole was used by Simkins et al. (2018), there was still the fear that those embryos treated with Fadrozole may have significantly lower rates of development. However, this was not the case in the present study affirming that the dose of Fadrozole used is sufficient enough to exert effects on the embryos without lethal effects.

Expected Concentrations as compared to actual concentrations:

The comparative concentrations for testosterone at embryonic day 15.5 were 210.1 ± 3.6 pg/mL for males and 116.2 ± 2.2 pg/mL for females. (Woods et al. 1975). Our results showed an average of 267.11 ± 79.35 pg/mL for control males and 309.24 ± 64.60 pg/mL for control females. The comparative concentrations for estradiol at embryonic day 15.5 were 716.6 ± 8.8 pg/mL for males and 1229.6 ± 26.9 pg/mL for females. (Woods et al. 1981). Our results showed an average of 40.41 ± 10.18 pg/mL for control males and 62.45± 15.99 pg/mL for control females. The plasma testosterone levels found in the present study differ than the literature cited. However as described by Weniger (1991) there has been a range of testosterone values seen during embryonic development that differs across labs. Additionally, day 15 is a crucial day for production of these hormones by the gonads. Guichard et al. (1977) explained that up until day 15, the pairs of gonads in females produce more testosterone than the males. After day 15 however, the production of testosterone by the pairs of gonads is higher in males than females. (It is important to realize however that the relative production of testosterone compared to other steroids produced is higher in males than females for all of development. For females, the highest relative steroid produced is estrogens). The plasma estradiol levels found in the present study are higher in females than males, which is consistent with the literature. However, the concentration values obtained in this study are not as high as those cited in the literature which could be due to the use of an EIA as compared to the RIA used in Woods et al. (1981), the breed of chicken and the seasonality. It is also highly possible that the variation seen in testosterone levels across labs as describe in Weniger (1991) is applicable to estradiol levels as well.

Testosterone Levels in control vs. Fadrozole embryos:

There was no significant difference in testosterone levels for Fadrozole males or females as compared to their controls. Because testosterone did not change significantly in either sex, it is difficult to make a conclusion about the role of testosterone in negative feedback of the HPG axis. Rombauts et al. (1993) found a large increase in FSH levels in chicks treated with an aromatase inhibitor suggesting that estradiol is a key regulator of negative feedback. They also found a large decrease in FSH levels in those chicks injected with estradiol (Rombauts et al 1993), but this same effect of inhibition by estradiol at the level of testosterone production was not seen. An explanation for the lack of rise in testosterone concentrations, implying that estradiol may not regulate the negative feedback of HPG axis, could be that most studies up to date have dealt with chicks (Rombauts et al. 1993) and not embryos. Additionally, studies that have suggested that aromatase inhibitors increased testosterone levels have been done in either non-avian species or post hatch chicks. The present study is a novel study in the sense that embryos were injected with Fadrozole and the immediate, not post-hatch, effects of the aromatase inhibitor on steroid concentrations was observed. Other studies looked at the hormone levels after the chicks had hatched (Simkins et al 2018). Some possible explanations for these results could include that the negative feedback mechanism is regulated differently at this stage or that the negative feedback mechanism is not fully in place yet for these embryos. Further studies could help answer this discrepancy by repeating this experiment but also injecting the eggs with DHT, an androgen that binds androgen receptors but cannot be aromatized, to see how testosterone and estradiol levels change. If testosterone

levels decrease with DHT injection then that supports the hypothesis that testosterone is the regulating hormone. However, if testosterone does not change with DHT injection, that supports that testosterone does not affect regulation of the HPG axis.

Testosterone and Immune Function:

As explained before, one of the goals of this thesis was to serve as a follow up to Simkins et al. (2018). Simkins et al. (2018) tested the hypothesis that estradiol leads to decrease immune function in chicks. He injected embryos on day 13 with either Fadrozole or a control and then put the chicks through a series of immune function tests. He found that overall, those chicks that were injected with Fadrozole saw an increase in immune function suggesting that estradiol, that was now absent, was originally suppressing immune function. However, lacking in the Simkins et al. (2018) study was the quantification of hormone levels in the day 15 embryos. Without quantification, Simkins et al. (2018) cannot be sure that it was the decrease in estradiol that was leading to the increase in immune function or whether it is something else such as an increase in testosterone. Without confirming that there is no change in testosterone levels, Simkins cannot be certain that the decrease in estradiol is leading to the improvement in immune function. Since the present study saw no significant change in testosterone concentration between control and Fadrozole embryos for both sexes, the results found in Simkins et al. (2018) can be attributed to the decrease in estradiol and not a decrease or increase in testosterone. This holds true especially for the females in which Simkins found a significant increase in immune function of females treated with Fadrozole accompanied with the significant decrease in plasma estradiol concentration found in this study. This

confirms that the significant decrease in estradiol is the only significant change that is occurring among testosterone and estradiol levels and therefore can be the cause for the results seen. Therefore, this study has supported Simkins' et al. (2018) results, and shows that the decreased immune function that was previously attributed to testosterone can now be attributed to estradiol.

Estradiol Levels in Control vs. Fadrozole embryos in both males and females

There was a significant difference in the levels of estradiol for control vs. Fadrozole treated females. This difference shows that Fadrozole was able to decrease estradiol synthesis in females. This effect was not seen in males treated with Fadrozole which suggests a difference in the way Fadrozole may affect male vs. female embryos. One reason for this difference could be that the overall concentration of estradiol in males as compared to females is much lower and so Fadrozole does not exert the dramatic effects as seen in females. Another possibility could be attributed to the higher levels of testosterone found in females as compared to males. Since there is a greater amount of testosterone in females as compared to males, aromatase will be more active and seen in higher quantities in females. Therefore, when aromatase inhibitors are present, they are inhibiting a greater amount of aromatase activity in females as compared to males so we see more potent inhibition in females. In a future study, it would be interesting to look at the decrease in estradiol and compare that to the aromatase expression seen in male vs. female embryos. This would allow us to correct for the amount of aromatase present in each sex and see if Fadrozole is inhibiting aromatase to the same degree in both sexes or if it is more potent in one sex.

Simkins et al. (2018) also saw a stronger effect of Fadrozole in females as compared to males. In their study, Fadrozole exposure increased day 18 IgY levels in females for given bursal mass; however, males did not show as strong of a relationship and Fadrozole only dampened the already existent negative trend. IgY antibodies are the major immunoglobulin in birds and an increased level of IgY would imply a stronger acquired immunity (Simkins et al. 2018). An increase in IgY levels in those females treated with Fadrozole indicates an increase in immunity as well, implying that the decrease in estradiol with Fadrozole injections subsequently increased immunity. This trend was not seen in males implying that the Fadrozole did not exert as great of an effect on the males as it did for the females. This is similar to the results found in the present study in which Fadrozole significantly decreased estradiol biosynthesis in females but not in males. This evidence in Simkins et al. (2018) supports the present study's results that Fadrozole affects males and females differently, and therefore helps explain why we also saw discrepancies in estradiol's biosynthesis in male vs. female embryos.

The decrease in estradiol in the female embryos, however, accompanied by no significant change in testosterone, is Outcome 2 (Table 1). Outcome 2 shows that Fadrozole is working but that estradiol does not exert negative feedback on the system due to the consistent levels of testosterone. These results also suggest that testosterone may be inhibiting the axis from over-secreting testosterone, because levels did not significantly increase, but in order to confirm this an additional experiment with DHT should be done as mentioned above. If embryos injected with DHT do not show a change

in testosterone levels, then that would provide information that there may something else altogether that is inhibiting the axis that is not an androgen or estrogen.

Estradiol Levels in Control Females vs. Control Males

The levels of estradiol were significantly higher in control females as compared to control males, which is consistent with the data presented in the literature (Woods et al. 1981). This likely is due to the crucial role that estradiol has in female development that that is not as apparent in male development of chicken embryos (Carere & Balthazart 2007). The embryonic stage is an important window in organizational development and the concentrations of the sex specific hormones will be key in the sexual development of these embryos. There is also a higher level of aromatase protein expression in female ovaries as compared to male testes during avian development (Shimada 1998). This explains the increased levels of estradiol found in females as compared to males because an increase in aromatase activity would lead to an increase of estradiol biosynthesis.

Major Conclusions:

There are three major conclusions that can be gathered from the results found in this thesis.

1. Fadrozole significantly decreased estradiol levels in females but testosterone levels remained the same. This, along with the results in Simkins et al. (2018) showing an increase in immunity in Fadrozole treated embryos, supports the hypothesis that estradiol, not testosterone, decreases immunity in chicks.
2. In the present study, Fadrozole was effective but it cannot be concluded that estradiol exerts negative feedback. Testosterone could be the steroid that is

affecting negative feedback in the HPG axis in these embryos but further tests, preferable with DHT, would be needed to confirm.

3. Fadrozole can successfully exert its effects on chick embryos, but has a stronger effect in females as compared to males, which may be due to the larger concentration of estradiol in females as compared to males.

Through these conclusions, this thesis was able to show that estradiol does not affect negative feedback of the HPG axis during development. Additionally, after adding the results of the present study to the results found in Simkins et al., it was seen that estradiol affects immunity post hatch. This study served as a foundation for the effects of steroid hormone exposure during development and laid the ground work for future research in the role of testosterone and estradiol during development.

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