ABSTRACT

Evidence suggests that birds bias sex ratios prior to hatch. It is likely that females, as the heterogametic sex, can facultatively control offspring sex ratios through a variety of potential mechanisms. Hormones, as important regulators of reproductive behavior and physiology, have been suggested as potential mediators of primary sex ratio adjustment, but the roles of steroid hormones, such as the stress hormone corticosterone and the reproductive hormone testosterone, are not well understood. Chronic corticosterone had stimulated breeding female birds to produce more female offspring, while chronic or non-targeted increases in testosterone stimulated females to produce more male offspring; however the roles of corticosterone and testosterone or the potential mechanisms had not been determined. We administered corticosterone and testosterone injections to laying hens during meiotic segregation and quantified sexes from the subsequently laid eggs to determine the effects of these hormones on primary sex ratios. Results of these experiments provided a further understanding of the role of steroid hormones in primary sex ratio manipulation in birds. Results presented here suggest that an acute pharmacological elevation of corticosterone beginning at 5 h prior to ovulation stimulated females to produce more male offspring, likely through non-random chromosome segregation; however, if the elevation was not within the pharmacological range or did not occur until 4 h prior to ovulation,
there was no significant change in sex ratios, suggesting a critical role of the timing and magnitude of the hormone elevation. Also, an acute increase of testosterone during meiotic segregation stimulated females to produce more male offspring and the sex ratio adjustment likely occurred through non-random chromosome segregation. To our knowledge, until these experiments were conducted, the effects of acute corticosterone or testosterone elevations during meiotic segregation were unknown in the chicken system. The ability to intentionally bias sex ratios or understand the range of factors influencing primary sex ratios, such as circulating maternal steroid hormones, could have profound impacts on the poultry industry as well as captive breeding and conservation programs.

INDEX WORDS: primary sex ratio manipulation, maternal effects, corticosterone, testosterone, chicken, bird
THE EFFECTS OF STEROID HORMONE TREATMENT DURING MEIOTIC SEGREGATION ON PRIMARY SEX RATIOS IN THE DOMESTIC CHICKEN

by

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BSA, University of Georgia, 2008

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2011
THE EFFECTS OF STEROID HORMONE TREATMENT DURING MEIOTIC SEGREGATION ON PRIMARY SEX RATIO MANIPULATION IN DOMESTIC CHICKENS

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Dean of the Graduate School
The University of Georgia
December 2011
DEDICATION

I would like to dedicate this dissertation to the memory of Mary Jo Schureck. Nanny, I miss you so much and wish you could see me now. You would be so proud of me!

I would also like to dedicate this dissertation to the memory of Russell O. Moore. I miss you Daddy Russell.

Finally, I would like to dedicate this work to my friend Michael Hendrickson. You left this world way too soon.
ACKNOWLEDGEMENTS

First, a special thank you to Kristen. You opened my eyes to the joys of research. Thank you for providing me such amazing opportunities as a member of your lab and helping me become a better student, presenter, and researcher. Thank you for seeing potential in me and continuing to push me to succeed. I am very fortunate to have you as a mentor.

Also, a big thank you to all of the past and present members of the Navara Lab. An additional thank you to Josh Cartmill, Ashley Gam, Erin Anderson, Christina Parr, Claire Stice, Anna Stojanovik, Jose Portillo, and Sheilena Brookshire. Your help, especially during all those 5am mornings, was greatly needed and even more appreciated.

Thanks also to my committee. Dr. Davis, thank you for always taking the time to answer my endless questions and pushing me to continue to improve as a student and researcher. Dr. Wilson, I cannot thank you enough for helping me learn a little bit about what it’s like to be a poultry scientist and trying to share your endless wealth of knowledge about proper animal care and husbandry with me. Without you, I can’t imagine where I’d be. Dr. Cooper, thank you serving on my committee, taking the time to answer all of my questions, and trying to teach me statistics and experimental design. Dr. Mary Mendonça, thank you for agreeing to serve on my committee, supporting me, and introducing me to so many people in our field of research.

I would also like to thank the Department of Poultry Science for supporting me. Dr. Lacy, I doubt I’ll ever find another department head who is as supportive of his faculty, staff, and students as you are. Thank you for seeing the potential in me, encouraging me to become a
graduate student, and continuing to support me throughout my time here. Your confidence in me as a researcher and presenter has provided me so many opportunities. I would also like to thank all of the members of the farm crew who have helped me in too many ways to mention. There is no way I could have done this without you. I’d also like to thank Beverly Wills who provided me so much assistance throughout my time here. Also, a big thank you to Melanie who kept everything organized.

Lastly, I need to thank my amazing family. This would have been much more difficult without your support. Mama and Daddy, thank you for loving me and supporting me as I strive to achieve my goals. Thank you for working so hard to provide opportunities for your children to succeed in all avenues of life. You have set the best examples and I can only hope that I can do the same for someone else. Kimberly and Daniel, thank you for always making me laugh. Carla and John thank you for making an effort to understand a little bit of what I’ve been doing these last few years, supporting me, and bringing little Mary Jo in to this world so we have another excuse to get the family together. To the Pinsons, thank you for loving me and praying for me. Grandma, Pop and Sara Ann, and Grammy and Daddy Larry, thank you for being such amazing grandparents. To Andrew, a simple thank you is not enough. I could not have done this without you by my side as my husband. Thank you for being there for me, suffering through the innumerable pre-dawn mornings and delayed dinners, and loving and understanding me as only you can. I know that being my husband can be difficult at times, but you’re doing a great job.
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CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW: A BRIEF REVIEW OF PRIMARY SEX RATIO MANIPULATION IN BIRDS

Introduction

Facultative sex ratio adjustment, once reported as rare in birds [14], appears to occur in many avian species. Biases in avian sex ratios have been reported in relation to a variety of environmental, social, seasonal, and parental factors (reviewed in [1, 56]). Several hypotheses have been proposed to explain these biases, but we still understand very little about the precise mechanisms involved.

Female birds are the heterogametic sex and may have unique mechanisms to control offspring sex. It has been suggested that females can manipulate the sex of their offspring prior to oviposition (reviewed in [1, 56] and described later). Also, steroid hormones, important regulators of behavioral and physiological functions involved in reproduction, are good candidates as mediators of sex ratio adjustment, even though their precise roles in the mechanisms of sex ratio adjustment are still not understood. Understanding the roles of hormones in sex ratio adjustment, as well as the precise mechanisms involved, has practical applications because avian sex ratios could potentially be intentionally manipulated. These techniques would be advantageous to commercial and exotic bird industries as well as captive breeding programs for threatened and endangered species.
**Hypotheses of Sex Ratio Manipulation**

Many researchers have focused on identifying ecological factors influencing sex ratio manipulation. Birds appear to manipulate sex ratios in response to environmental conditions, such as food availability and quality [4, 10, 26, 53, 63, 75], and social conditions, such as mate attractiveness [18, 21, 36, 41, 48, 60, 65, 67, 68] and presence of helpers [17, 35, 45, 50]. Biases have also been associated with maternal body condition [2, 53, 57, 59, 73-75], clutch position [3, 5, 8, 27, 28, 44, 46, 49, 66, 72], season position [15, 50, 71], territory quality [42, 43], and incubation temperature [19, 34].

Several sex allocation hypotheses have been proposed to explain observed biases. According to Trivers and Willard [69] sex ratio adjustment occurs when the fitness benefits of producing male and female offspring differ. This hypothesis applies to a mating system where the variance in reproductive success among members of one sex is greater than the variance among the other sex, which will probably mate regardless of quality. Females that are in good condition and can invest more heavily in their offspring could benefit from producing more of the high variance sex (typically males) who will have many opportunities to produce good offspring. In contrast, females that are not in good condition should produce more of the low variance sex to increase her probability of producing offspring that produce a next generation. Similarly, others hypothesize that females mated to attractive males should bias towards more sons if the sexually selected traits are heritable because attractive sons have more reproductive potential than attractive daughters, thus increasing their fitness [11, 12].

Sex ratio biases are not only driven by the effects of differential offspring quality on fitness but provisioning costs as well. Females who are in poor condition should not invest in the sex with the greater demands on resources so she can adequately provide for her young and
prevent failure of her clutch, thus maximizing her reproductive output [33, 52, 75]. Similarly, cooperatively breeding species can maximize reproductive output by producing more of the “helper” sex so they can help the parents raise the most recent clutch [23, 47].

Sex ratio manipulation in birds is particularly interesting to study because female birds, unlike female mammals, are the heterogametic sex and can potentially control the sex of offspring prior to ovulation. This characteristic of the avian reproductive system, in addition to others discussed in the next section, provide birds several unique opportunities to facultatively manipulate offspring sex in response to social and environmental conditions.

**Avian Female Reproductive System**

The avian female reproductive system consists of an ovary containing thousands of follicles – small white, large white, small yellow, and large yellow – and, in most species, an oviduct located on only the left side of the body. The ovarian follicles develop slowly until approximately 2 weeks prior to ovulation when a small proportion of the small yellow follicles, and the oocyte contained within each of the follicles, is selected to enter the ovarian hierarchy. Once selected into the ovarian hierarchy, rapid yolk deposition occurs, as lipids, proteins, and carotenoids are added in concentric layers. This stage of follicular development completes at least 24 h prior to ovulation [39]. The follicles in the ovary are in a strict hierarchy and the order of ovulation can be determined by the size of the follicle, with the largest follicle (F1) being the first to ovulate, followed by the next largest follicle (F2), and so on. At any stage of development, follicles may experience atresia (degeneration and resorption of the follicle), eliminating any further development of the associated oocyte and genetic material.

During the completion of Meiosis I, which occurs 2 – 4 h prior to ovulation [54], one set of chromosomes is retained in the oocyte while the other set is segregated to the polar body with
no further potential for development. Because the female is the heterogametic sex, the oocyte will contain either the WW or ZZ set of chromosomes following Meiosis I. The oocyte will then enter a second meiotic division. During Meiosis II, one chromosome is retained in the oocyte and the other is segregated to the polar body, yet again with no potential for further development, resulting in only a W or Z sex chromosome, depending on which set was retained during the first meiotic division, in the oocyte.

Approximately 2 h after the completion of meiosis I, the follicle ruptures and the oocyte is ovulated. If it is successfully ovulated, the haploid oocyte is captured by the infundibulum; occasionally, the oocyte may be internally ovulated into the abdomen, instead of the infundibulum, and potentially absorbed in the peritoneal cavity. If sperm are present in the infundibulum, they can fertilize the oocyte. The nucleus of the haploid sperm which contains genetic material, including the Z chromosome, fuses with the genetic material of the egg and fertilization has occurred, resulting in diploid cells. The egg then passes through the magnum, where the majority of albumen is deposited around the egg, and the isthmus, where albumen and egg shell membranes are deposited around the egg. It then passes through the egg shell gland where the egg shell is deposited on the egg before being laid at oviposition, approximately 24 h after ovulation in domestic chickens. [39]

**Primary Sex Ratio Manipulation**

There is evidence that birds, like many other animals, influence offspring sex ratios by secondary sex ratio manipulation through sex-specific mortality of embryos or nestlings. Contrary to Clutton-Brock’s conclusion that biased sex ratios at hatch are unusual in birds [14], evidence from the last two decades, since the development of molecular sexing techniques, also suggests that female birds, as the heterogametic sex, bias offspring sex prior to or at the time of
fertilization (reviewed in [1, 56]). This type of manipulation in birds is known as primary sex ratio manipulation.

Molecular sexing techniques provide reliable methods to determine the sex of embryos, thus offering a means to measure primary sex ratios [29, 37]. Instead of relying on morphological characteristics to determine phenotypic sex of the offspring, researchers can now accurately determine the genetic sex of the offspring at an early stage of incubation. Using genomic DNA extracted from embryos, polymerase chain reaction (PCR) amplifies portions of the genes linked to the sex chromosomes. The PCR products can then be visualized on agarose gel. Molecular sexing techniques allow for accurate estimations of primary sex ratios by removing biases associated with sex-specific embryonic and nestling mortality because samples can be collected early in incubation, prior to the time that most secondary manipulation occurs.

Primary sex ratio manipulation may provide adaptive benefits because biasing offspring sex prior to egg production, instead of in a secondary manner, occurs without wasting the nutrients and resources needed to create an unwanted egg, embryo, or nestling. Even though benefits of primary sex ratio manipulation are great, there is still little understanding of the proximate mechanisms controlling these biases. A more complete understanding of the mechanisms could provide valuable information regarding the cost and benefit trade-offs of sex ratio manipulation faced by breeding female birds. Several proposed mechanisms of primary manipulation are discussed in the next section.

Potential Mechanisms of Primary Sex Ratio Adjustment

There are several potential mechanisms through which offspring sex could be adjusted in a primary manner. Several mechanism influence offspring sex during follicular development which occurs days – potentially weeks – prior to ovulation and subsequent egg laying while
others occur a few short hours before, at the time of, or immediately following ovulation (Figure 1.1).

**Preferential selection into hierarchy and follicular atresia**

If the sex of oocytes are predetermined, follicles containing oocytes of the desired sex could be preferentially selected to experience rapid yolk deposition and enter the ovarian hierarchy in a specific order so that eggs containing the most valuable sex are laid at the beginning of the sequence [39]. It is also possible for follicles containing oocytes destined to be the undesired sex to experience atresia (degeneration and resorption) before or after selection into the hierarchy. The regular occurrence of atresia among pre-hierarchical follicles has been reported [30] and may provide breeding females the ability to control ovulation order of those follicles containing a particular sex. While atresia of hierarchical follicles is rare in a natural setting [30], there is evidence that it can occur following exogenous hormone treatment [76]. It is currently unknown if atresia of hierarchical follicles actually occurs in a sex-specific manner.

**Asynchronous follicular development**

Asynchronous follicular development is another proposed method of primary sex ratio manipulation. Pike and Petrie [56] suggested that rapid yolk development rates could differ in a sex-specific manner with oocytes destined to be male or female offspring acquiring yolk proteins at different rates (see also [3]). Using house finches, Badyaev et. al. [7] demonstrated that growth rates can differ between follicles; ovulation order may not be strictly controlled by the order in which follicles are selected into the hierarchy, thus providing breeding females control of sex ratios in relation to sequence position. If females use this method to bias sex ratios, the faster-growing sex would appear at the beginning of clutches (sequences) while the slower-growing sex would be at the end of the clutch. In this way, more of one sex would be laid at the
beginning of clutches while the other sex would be laid at the end. To date, there is no evidence that breeding females can distinguish between follicles containing oocytes destined to be male or female, so it is difficult to discuss a mechanism through which the female could purposefully induce differential growth of the follicles. It may be that variation in growth rates exposes the follicles to different concentrations of circulating hormones and the hormones are accumulated in the yolk of the growing follicle at different concentrations. While controversial, there is some evidence that eggs containing male and female embryos differ in androgen content [20, 51, 55, 64], so it may be that hormonal milieu during follicular development, not selective manipulation of growth rates, drives this potential mechanism of sex ratio manipulation.

*Non-random chromosome segregation*

Changes in sex ratios may also be mediated through non-random chromosome segregation just prior to ovulation. Female birds, as the heterogametic sex, have oocytes containing either Z or W chromosomes, while sperm contains only the Z chromosome. During the first meiotic division, which completes 2 – 4 h prior to ovulation [54], one sex chromosome is retained in the oocyte and the other is segregated to the polar body with no further potential for development. It is possible that chromosome segregation could occur in a non-random manner so that that the chromosome of the preferred sex (Z if male, W if female) is retained in the oocyte. This is a possible mechanism of sex ratio adjustment for birds because the two avian sex chromosomes differ in size, shape, and content (reviewed in [61]), which could facilitate non-random chromosome segregation. Non-random chromosome segregation seems to be a rather parsimonious option because it results in less waste of invested energy and material compared to adjustments that occur at later stages and result in the loss of an ovulated oocyte. Also, since this mechanism affects only the largest pre-ovulatory follicle (F1), it allows for adjustment of
offspring sex with each egg laid, providing plasticity for the female to respond appropriately to dynamic environmental or social conditions.

**Selective follicular abortion**

It is also possible for primary sex ratios to be influenced at the time of ovulation through selective follicular abortion [22]. This occurs when the post-meiotic oocyte does not pass through the oviduct to be oviposited due to either non-random resorption of the oocyte and follicle just prior to ovulation (similar to atresia except it occurs after meiosis) or absorption of the oocyte ovulated into the abdomen instead of the oviduct. Selective follicular abortion has been suggested as an adaptive mechanism used to discard an oocyte containing the undesirable sex chromosome so that a secondary oocyte, possibly containing the desirable sex chromosome, can then be ovulated. It has even been suggested that a female could abort oocytes until one with the desired sex chromosomes is produced.

This mechanism has been used to explain sex ratio biases in species with small clutch sizes, but seems an unlikely method of mid-clutch sex ratio adjustment in species with larger clutch sizes because abortion and re-absorption of follicles would produce a gap, of approximately 24 h, in laying sequences [22]. The costs associated with these delays, such as wasted energy and resources in addition to increase hatching asynchrony and predation, are too great for those birds laying clutches of more than a few eggs; however, selective follicular abortion could occur at the beginning of the clutch without some of the associated cost [5, 8].

Pike [59] argued that follicular abortion and secondary ovulation could occur without a gap in the laying sequence. Domestic pigeons, that had reduced body condition caused by repeated egg laying and limited food intake, produced female biases from both the first and second egg in the clutch without a significant delay in laying. Pike suggested that if a follicle is
aborted and the next-in-line follicle contains the chromosome of the desired sex, the secondary oocyte could be ovulated immediately, perhaps with a reduction of yolk volume. While this post-hoc model fit the results of his experiment perfectly, it was not observed whether the hormonal milieu – increased secretion of progesterone from the pre-ovulatory follicle coincident with an elevation of circulating luteinizing hormone – was sufficient to trigger a second ovulation within such a short time period.

Follicular abortion, like non-random chromosome segregation, provides female birds the ability to adjust offspring sex with each egg laid in response to her current environmental and social conditions. However, there are considerable costs, in addition to gaps in the laying sequence, associated with this mechanism. If the oocyte is ovulated into the abdomen, it may cause peritonitis, which is often fatal [39]. Also, the follicle, containing up to several grams of yolk lipid and protein, may not be completely re-absorbed by the female, thus resulting in wasted energy and resources.

**Selective fertilization**

Finally, birds could also influence primary sex ratios immediately following ovulation through sex-specific selective fertilization. Preferential release of sperm from sperm storage tubules and changes in the fluid composition of the oviduct as well as prevention of sperm penetration and parental control [56], have been suggested as potential mechanisms of selective fertilization. Alonso-Alvarez suggested in his review [1] that the female could alter characteristics of the oviduct fluids that influence sperm fecundity, such as calcium content, pH, or viscosity of oviduct fluids [6], when the ovum is not of the desired sex. Even though the costs are reduced, compared to loss of an embryo during incubation or nestling after hatch,
selective fertilization still represents a significant cost for the female because she wastes the nutrients and resources need to produce the infertile egg.

**Steroid Hormones and Primary Sex Ratios**

It is likely that hormones, important regulators of biochemical, physiological, and reproductive functions, are mediators in the mechanisms of sex ratio adjustment. Indeed, elevated concentrations of reproductive and stress hormones have stimulated biases in avian primary sex ratios [9, 16, 31, 57, 58, 62, 70]. Specifically, experimental evidence suggests that the stress hormone corticosterone [9, 57, 58] and reproductive hormones progesterone [16] and testosterone [31, 62, 70] may be involved in the mechanism of sex ratio adjustment as females with elevated levels of these hormones have produced biased primary sex ratios.

**Corticosterone**

Corticosterone is the primary stress hormone in birds and is of particular interest as a potential mediator of primary offspring sex for several reasons. Corticosterone is released from the adrenal glands, one of which is located within a close proximity to the ovary, and is elevated during and participates in ovulation [24]. Corticosterone also regulates many cues that have been associated with biased sex ratios, such as maternal body condition [2, 53, 57, 59, 73-75] and food availability and quality [4, 13, 40, 63].

Studies in several avian species suggest that chronic elevations of corticosterone can stimulate birds to produce a female bias [9, 57, 58]. White-crowned sparrows and peafowl with chronic, physiological elevations of corticosterone produced more female offspring [9, 57] while white-crowned sparrows and Japanese quail with experimentally elevated corticosterone via implants also produced more female offspring [9, 58]. In these studies, corticosterone was elevated over the entire ovulatory process, including the stages of follicular development and
meiotic segregation, as well as immediately following ovulation when follicular abortion and selective fertilization could occur. This makes it difficult to pinpoint when corticosterone was playing a role in sex ratio manipulation during those studies. It is also unclear whether long-term corticosterone elevation is necessary to influence offspring sex, or whether an acute increase of corticosterone concentrations during meiotic segregation is sufficient.

**Progesterone**

As the primary hormone elevated during meiotic segregation [25], progesterone has also been suggested as a hormone that may be involved in the mechanism of sex ratio adjustment in birds. Indeed, acute treatment with progesterone stimulated a significant bias in laying hens. Hens injected with 2 mg progesterone several hours prior to ovulation produced significantly fewer male offspring than hens injected with control oil or 0.25 mg progesterone [16]. However, injections of exogenous progesterone have been shown to prevent ovulation in chickens and zebra finches [24], so it seems an impractical hormone to use to study the mechanism of sex ratio adjustment in birds.

**Testosterone**

Testosterone, a reproductive hormone produced by follicular cells that lie in very close proximity to the oocyte, stimulated male-biased sex ratios in three avian species. An injection of testosterone administered after oviposition of the first egg in the clutch induced a testosterone elevation in zebra finches that lasted several days after the time of injection and stimulated the production of more males in eggs laid late in the clutch [62]. Also, chronic elevations of testosterone via implants in spotless starlings and homing pigeons stimulated male biases that persisted after the removal of implants [31, 70]. However, when similar chronic elevations were induced in homing pigeons and Japanese quail, no significant effect on offspring sex ratios was
observed [32, 58]. In experiments where testosterone did induce a bias in offspring sex ratios, the treatments were either chronic implants [31, 70] or injections that increased testosterone concentrations for several days prior to the observed sex ratio adjustment [62]. It was impossible to determine how testosterone influenced offspring sex in these studies and or if an acute elevation of testosterone would be sufficient to manipulate offspring sex.

**Domestic Chicken as an Experimental Model**

Sex ratio manipulation has largely been evaluated from an ecological standpoint and, until recently, little research has been done to determine the potential mechanisms controlling primary sex ratio manipulation. In several avian species, treatments with the reproductive hormone testosterone and stress hormone corticosterone stimulated sex ratio biases very early in development [9, 31, 58, 62, 70], but these hormones have yet to be tested with the chicken model.

The domestic chicken is a particularly useful bird to use for sex ratio studies for several reasons. First, the birds used in the studies featured in this dissertation (Hy-Line W-36, Hy-Line International, West Des Moines, IA) are from a commercially available strain of chickens, so sufficient quantities of genetically similar birds with the same hatch date can be acquired. Also, several molecular sexing techniques for chickens have been validated [29, 37], so it is possible to determine the primary sex ratio by sexing embryos early in development. Biases associated with mate attractiveness can be avoided by using a chicken system because semen (collected from roosters by abdominal massage) can be used to artificially inseminate the hens. Also, all eggs can be incubated in the same large incubator at a standard temperature and humidity, preventing any bias associated with parental incubation, such as altered temperature [19, 34].
Several characteristics of single-comb white Leghorn laying hens (Hy-Line W-36, Hy-Line International, West Des Moines, IA) make them ideal for manipulative sex ratio studies. First, the hens are prolific layers and lay an egg almost every day under appropriate nutritional and environmental conditions. Additionally, egg-laying patterns can be used to predict ovulation timing. Laying hens have been shown to ovulate approximately 30 minutes after oviposition of the previous egg [38], so egg laying patterns can be used to predict ovulation timing of the target oocyte. Finally, by using laying hens, treatments can be targeted for the critical period of genetic sex determination of the embryo because the timing of completion of the first meiotic division is well-documented, completing 2 – 4 h prior to ovulation [54].

Dissertation Content

Our aim was to gain a further understanding of primary sex ratio manipulation in birds and how hormones can influence these mechanisms. Initially, to know if the strains of chickens used in our experiments were capable of sex ratio manipulation, we determined if any variation in primary sex ratios existed between eggs laid at different sequence positions or ages (Chapter 2). We then manipulated circulating hormone concentrations in the females when the primary follicle was experiencing chromosome segregation to determine if hormone treatment during this time could influence primary offspring sex ratios. First, we tested the effects of a pharmacological dose of corticosterone on sex ratios (Chapter 3). Based on the results of this study, we then manipulated the time and magnitude of corticosterone elevation to further pinpoint the timing of the hormone’s influence and determine if a physiological dose of corticosterone would be sufficient to bias offspring sex (Chapter 4). Then, we tested the effects of acute, targeted testosterone treatment on offspring sex to determine if acute treatment of other steroid hormones during meiotic segregation could influence primary sex ratios (Chapter 5).
Finally, in Chapter 6, the findings of these chapters are summarized and future directions for the continuation of this research are suggested. Also, implications for the industry as well as conservation efforts are discussed to provide a practical application for working towards a better understanding how primary sex ratio manipulation occurs in birds.
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Figures

Figure 1.1: Shown above the time scale are the stages of follicular development and egg formation of the primary (F1) follicle, including rapid yolk deposition (initiated by selection of follicle into hierarchy), completion of meiotic segregation (when one sex chromosome is retained in the oocyte and the other is segregated to the polar body), ovulation of the oocyte from the ovary into the infundibulum, fertilization of the oocyte by sperm in the infundibulum, albumin deposition, shell formation, and oviposition. Approximate timing of potential mechanisms of primary sex ratio manipulation are shown below the time scale with white arrows or open brackets.
CHAPTER 2

PRIMARY SEX RATIOS VARY WITH SEQUENCE POSITION, BUT NOT AGE, IN DOMESTIC CHICKENS, GALLUS GALLUS DOMESTICUS

Abstract

Evidence suggests that birds are capable of biasing sex ratios prior to hatch in relation to a variety of social and environmental conditions. Several studies have found relationships between age or sequence position and biased sex ratios in wild and captive bird populations. There is little information with regards to the influence of these factors on sex ratios of domestic chickens, which are often used as the study species in experimental sex ratio studies. The purpose of this study was to determine if there was variation between the proportion of males produced at different ages or sequence positions in commercially available birds. We collected fertile eggs from randomly selected hens when they were 32, 35, 53, 56, 59, and 63 weeks of age. We also collected fertile eggs from randomly selected hens and assigned the appropriate sequence position to each egg. We incubated the eggs for 8 days and quantified the sexes using a molecular sexing technique. In our study, laying hens produced statistically similar proportions of males for all sampled weeks of age, so age does not appear to be a factor influencing primary sex ratios in chickens; however, there was significant variation between sequence positions. A significantly greater proportion of male offspring was produced from the third egg in the sequence compared to the earlier ($\chi^2 = 5.085, p=0.02$) and later ($\chi^2 = 3.734, p=0.05$) sequence positions. Results of this study suggest that sequence position can influence sex ratios in hens, so
it is a factor that should be considered when designing and executing manipulative sex ratio studies in domestic chickens. Understanding that the proportion of males can be related to factors such as sequence positions could also prove beneficial to the poultry industry where incubator and hatchery space is currently being wasted for chicks of the unwanted sex.

Introduction

Birds have demonstrated that they are capable of biasing sex ratios prior to hatch in relation to a variety of social and environmental conditions (reviewed in [2, 17]). Several studies have found relationships between age or sequence position and biased sex ratios in wild and captive bird populations [1, 3-5, 8, 14, 16, 19]. There is, however, little information with regards to the influence of these factors on primary sex ratios of domestic chickens. In fact, most have reported even sex ratios for hens of productive laying age [7, 10, 11, 13]. Even though there was no bias in overall sex ratios, Hays reported extreme variation in the proportion of males produced among hens, but provided no information regarding relationship between either age or sequence position and the observed biases [10]. Leonard and Weatherhead reported a relationship between dominance rank and tendencies towards the production or more or less males [13]. Significant deviations from parity as well as variation between sequence positions were reported for several strains of domestic hens, but these biases were observed from only the first 15 eggs laid by the hens [11].

The purpose of this study was to determine if there was variation between the proportion of males naturally produced at different ages or sequence positions in commercially available birds. Determining if either of these factors was correlated to variations in sex ratios in domestic hens would provide valuable information to not only researchers who use chickens as a study
species but also the commercial layer industry where approximately 50% of all chicks are killed immediately after hatch.

**Materials and Methods**

**Animals**

Single Comb White Leghorn laying hens and roosters (Hy-Line W-36, Hy-Line International, West Des Moines, IA) were housed in individual cages in a single climate-controlled room, provided *ad libitum* access to food and water, and maintained on a standard breeding light cycle of 14h L: 10h D.

**Egg Collection and Incubation**

Leghorn hens were artificially inseminated twice weekly with pooled semen from 35 roosters. Egg were collected manually every 2h during peak laying hours and egg collection times were recorded for each hen. To examine the effect of age on primary sex ratios, fertile eggs were collected from randomly selected hens at 32, 35, 53, 56, 59, and 63 weeks of age (n=53, 57, 202, 35, 37, and 41 respectively). To evaluate the effect of sequence position on primary sex ratios, we determined sequence positions for eggs collected from 32, 35, 53, 56, and 59 week old hens by counting the number of consecutive days of egg laying from the last pause day to the day of egg collection. Positions one through four were assigned accordingly (n=87, 50, 58, and 93 respectively). Positions five through nine (categorized as 5-9, n=64) and positions ten and greater (categorized as 10+, n=32) were grouped due to the low number of eggs collected at these positions.

All eggs were collected and stored for no more than 72h prior to incubation. Eggs were incubated for 8 days at 37.5°C and 58% relative humidity in a Natureform incubator. Embryos
were manually removed from eggs and stored in 70% ethanol solution until molecular sexing was performed.

**Molecular Sexing**

Genomic DNA was extracted from embryonic tissue using a standard salt extraction method [12]. Portions of CHD-W and CHD-Z genes from the sex chromosomes were amplified using polymerase chain reaction (PCR) with primers 2550F and 2718R (adapted from [9]). For PCR amplification, we used a reaction volume of 25ul, containing 3mM MgCl₂, 40mM Tris-HCl, 100mM KCl, 200uM dNTP Mix (Bio-Rad Laboratories, Hercules, CA USA, cat# 170-8874), 5pmol each primer, 0.18U iTaq© DNA polymerase (Bio-Rad Laboratories, Hercules, CA USA, cat# 170-8870), 500ng DNA, and water. PCR was performed in a Bio-Rad thermal cycler as described by Pinson, Wilson, and Navara [18]. We visualized PCR products on a 3% agarose gel stained with ethidium bromide.

**Statistical Analyses**

Logistic regression analyses were used to compare the proportion of male offspring produced at different ages and sequence positions by the hens. The proportion of males produced at each age or sequence position was then compared to an even sex ratio (50% males) of the same sample size, using individual logistic regression to determine whether any age or sequence position differed significantly from an even sex ratio. All statistical analyses were carried out using Statview software (SAS Institute, Cary, NC USA).
Results

Age

Hens produced proportions of males that ranged from 42.11% for 35 week old hens to 59.46% males for 59 week old hens. Even though 35 week old hens produced 17% fewer males than 59 week old hens, this difference was not significant ($\chi^2 = 2.67, p = 0.10$). At no age did hens produce a proportion of males that differed significantly from the hypothetical sex ratio of 50% males (32 weeks: 54.72%, $\chi^2 = 0.24, p = 0.63$; 35 weeks: 42.11%, $\chi^2 = 0.72, p = 0.40$; 53 weeks: 49.01%, $\chi^2 = 0.40, p = 0.84$; 56 weeks: 54.29%, $\chi^2 = 0.86, p = 0.36$; 59 weeks: 59.46%, $\chi^2 = 0.68, p = 0.41$; 63 weeks: 48.78%, $\chi^2 = 0.01, p = 0.91$) (Figure 2.1).

Sequence Position

The proportions of male embryos collected from all sequence positions were statistically similar to the hypothetical sex ratio of 50% males (1: $\chi^2 = 0.05, p = 0.82$; 2: $\chi^2 = 0.64, p = 0.42$; 3: 63. $\chi^2 = 0.223, p = 0.14$; 4: $\chi^2 = 0.05, p = 0.83$; 5-9: $\chi^2 = 0.28, p = 0.59$; 10+: $\chi^2 = 0.57, p = 0.45$). There were, however, significant differences among the sequence positions. Eggs from position 3 tended to contain more male embryos (63.79%), while all other positions, except for positions 5-9 (54.69%), contained more females than males (1: 48.28%; 2: 42%; 4: 48.39%; 10+: 40.63%). Eggs collected from sequence position 3 contained significantly more male embryos than those collected from position 2 ($\chi^2 = 5.04, p = 0.02$) as well as positions categorized as 10 or greater ($\chi^2 = 4.38, p = 0.03$)(Figure 2.2). If the proportion of males collected from position 3 is compared to the proportions collected from earlier and later positions (categorized as 1 & 2 and 4+), then significantly more male embryos were collected from position 3 than were collected from earlier and later positions (1 & 2: $\chi^2 = 5.09, p = 0.02$; 4+: $\chi^2 = 3.73, p = 0.05$)(Figure 2.3).
Discussion

Similar proportions of male offspring were collected from all ages, but not sequence positions, of domestic laying hens. Results of this study suggested that hens of laying age (here, ranging from 32 to 63 weeks of age) produce sex ratios that do not deviate significantly from 50% males and agreed with a study that examined the sex ratios of several Leghorn varieties at 45 weeks of age [11]. To our knowledge, this is the first study to demonstrate variation between sex ratios at different sequence positions for hens of prime laying age. Klein and Grossman [11] reported significant deviations from an even sex ratio among the first 15 eggs produced by several Leghorn hen varieties but did not evaluate the relationship between sequence position and sex ratios in hens after their first few weeks of production. Unlike Klein and Grossman, we found no significant deviations from 50% male offspring, but there were significant differences between proportions of males produced at the different sequence positions.

It is difficult to apply an adaptive context to the observed variations for several reasons. First, it is unlikely that the variations were a result of social rank of the female because social interactions were minimized by individually caging hens. It is also unlikely that mate attractiveness or quality contributed to the observed variations because hens had no contact with the roosters and were artificially inseminated with mixed semen from all available males. Furthermore, hens were provided ad libitum access to food and water, so hens presumably maintained the same body condition throughout the laying sequence, which makes it unlikely that any changes in body condition influenced the observed variations.

Unlike studies where biases were reported from eggs at the beginning or ending of a clutch [1, 3-5, 8, 14, 16, 19], in this study, the greatest proportion of males was produced from the third position in the laying order. According to Bortolotti [6], parents should adjust the
sequence of sexes within a clutch to maximize offspring survival while minimizing parental costs because survival probability decreases with hatching order; however, this hypothesis may not apply to this system because *Galliformes*, such as chickens, produce precocial young which require little parental care after hatch. It may be that factors not evaluated in this study were responsible for the differences between sequence positions. Factors that may have contributed to the observed variations, such as egg mass and yolk hormone concentrations [8, 15, 19], were not measured. Future experiments should include measurements of these factors to determine if variations in sex ratios in relation to sequence position are correlated with egg mass or yolk hormones.

Results of this study are applicable to sex ratio manipulation research as well as the poultry industry. The domestic chicken has been used for studies of primary sex ratio manipulation in birds because they are prolific egg layers. Laying order has been dismissed as a confounding factor in sex ratio and yolk hormone studies of domestic hens because they lay an egg almost every day without defined clutches [15]; however, results of this study suggest that sequence position can influence sex ratios in hens and is a factor that should be considered when designing and executing manipulative sex ratio studies in domestic chickens.

Keeping records of the proportion of male offspring produced from each sequence position could also prove beneficial to the poultry industry. While a similar pattern of more males produced from sequence position 3 than any other position may not hold true for all flocks of hens, it is likely that variation does exist between sequence positions. Currently, approximately 50% of all chicks are killed immediately after hatch because they are males, so if eggs collected from particular sequence positions tend to contain significantly more males, then it could potentially be cost effective to avoid incubating and hatching eggs collected from those
positions because only a minority of the offspring produced would be of the desired sex. Until a method exists to intentionally manipulate hens to produce less of the undesired sex, using factors, such as sequence position, that may influence offspring sex may be a way for the industry to take advantage of primary sex ratio manipulation in birds.
References


Figures

**Figure 2.1:** Comparison of proportions of male offspring produced by hens at 32, 35, 53, 56, 59, and 63 weeks of age. Actual proportions are shown at the bottom of the bars directly above the age. There were no significant differences among the ages and at no age did the hens produce a proportion of males that differed significantly from 50% males.
**Figure 2.2:** Comparison of the proportions of male offspring collected from hens at each sequence position. Actual proportions are shown at the bottom of the bars directly above the sequence position. Different letters above the bars denote statistical differences. The proportion of males at no sequence position differed significantly from 50% males.
Figure 2.3: Comparison of the proportions of male offspring collected from the first two sequence positions (1 & 2) the third position, and the forth and later positions (4+). Actual proportions are shown at the bottom of the bars directly above the sequence position. Different letters above the bars denote statistical differences. The proportion of males collected from no group of positions differed significantly from 50% males.
CHAPTER 3

ACUTE CORTICOSTERONE ADMINISTRATION DURING MEIOTIC SEGREGATION STIMULATES FEMALES TO PRODUCE MORE MALE OFFSPRING

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1 Sara E. Pinson, Christina M. Parr, Jeanna L. Wilson, Kristen J. Navara. 2011. Physiological and Biochemical Zoology. 84: 292-298
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Abstract

Birds have demonstrated a remarkable ability to manipulate offspring sex. Previous studies suggest that treatment with hormones can stimulate females to manipulate offspring sex prior to ovulation. For example, chronic treatments with corticosterone, the primary stress hormone produced by birds, stimulated significant skews towards female offspring. It has been suggested that corticosterone acts by influencing which sex chromosome is donated by the heterogametic female bird into the ovulated ovarian follicle. However, it is difficult to pinpoint when in developmental time corticosterone affects offspring sex because corticosterone treatment in previous studies was given over a long period of time. We treated laying hens with acute high-dose corticosterone injections 5h prior to the predicted time of ovulation and quantified the sexes of the subsequently ovulated eggs to determine whether mechanisms exist by which corticosterone can skew offspring sex ratios just prior to ovulation. We hypothesized that an injection of corticosterone coincident with segregation of the sex chromosomes would stimulate hens to produce more female than male offspring. Contrary to our predictions, hens injected with corticosterone produced a significant bias towards male offspring, nearly 83%. These results suggest that acute corticosterone treatment during meiosis I can influence primary sex ratios in birds, potentially through non-random chromosome segregation. Furthermore, acute corticosterone exposure, compared with chronic exposure, may act through different mechanisms to skew offspring sex.

Keywords: Primary sex ratio, offspring sex, corticosterone, stress, maternal effects
Introduction

Biases in avian primary sex ratios have been documented in relation to a variety of social and environmental conditions (reviewed in [2, 32]); however, little is known about the mechanism controlling adjustment of primary sex ratios in birds. An understanding of how social and environmental cues are translated mechanistically to bias offspring sex ratios would be helpful when discussing the adaptive significance of these biases.

Females are likely in control of the primary sex ratio in birds because they are heterogametic, contributing either a W or Z chromosome to offspring. In chickens and quail, the sex of the offspring is determined 2-4 hours prior to ovulation during the first meiotic division when one sex chromosome is retained in the oocyte and the other segregates to the polar body with no further potential for development (Olsen and Fraps 1950; Johnson 1996). It has been suggested that females can manipulate the sex of their offspring prior to oviposition, possibly by non-random segregation of sex chromosomes during the first meiotic division [2, 23, 32, 38]. This proposed method of sex ratio adjustment, as opposed to follicular reabsorption or post-fertilization adjustment, seems to be the most parsimonious option because nutrients and resources used to create the follicle and oocyte are not wasted. In addition, there are cytological features of the avian oocyte that make segregation distortion during meiosis a plausible mechanism for sex ratio adjustment (reviewed in [37]).

Hormones are good candidates as mediators of offspring sex ratios because they are important regulators of behavioural and physiological functions involved in reproduction. Indeed, treatment with both reproductive and stress hormones stimulates biases in avian sex ratios very early in development. Acute or chronic elevations of testosterone stimulate male biased sex ratios [16, 38] while acute elevations of progesterone stimulate the production of
more females [8]. Corticosterone, the primary stress hormone in birds, is also of particular interest because this hormone regulates responses to the many cues that have previously been shown to stimulate sex ratio biases in birds such as food abundance and quality [4, 7, 20, 39] and maternal body condition [3, 29, 33, 35, 42-44]. Furthermore, corticosterone is present during and plays a role in ovulation [11].

Studies in other avian species suggest that chronically elevated corticosterone concentrations in breeding females bias the primary sex ratios towards females [6, 33, 34]. Specifically, Japanese quail and white-crowned sparrows with corticosterone implants produced significantly more female offspring than those without corticosterone implants [6, 34] while peafowl and white-crowned sparrows with naturally, chronically elevated corticosterone concentrations also produced more female offspring [6, 33]. In these studies, corticosterone was elevated over the entire ovulatory process, including during yolk deposition, follicular growth, and meiotic segregation, as well as immediately following ovulation when follicular resorption could occur. This makes it difficult to pinpoint when corticosterone was playing a role in sex ratio manipulation during those studies. It is also unclear whether long-term corticosterone elevation is necessary to influence offspring sex, or whether an acute increase of corticosterone concentrations during meiotic segregation is sufficient.

In the current experiment, we administered an acute, exogenous corticosterone treatment at the critical time when sex chromosomes were segregating to determine if corticosterone can act during that time to bias sex ratios. We purposely administered an acute corticosterone treatment at this time point, rather than inducing a chronic elevation of corticosterone, in an attempt to further pinpoint the precise time period during which sex ratio adjustment can occur. Based on the results of the above-mentioned studies examining the effects of corticosterone on
sex ratios in birds, we hypothesized that a short-term elevation of corticosterone at this critical time would bias the primary sex ratio towards females.

**Materials and Methods**

Single comb laying hens (Hy-Line International) at peak laying age were housed in individual laying cages in a single room, given *ad libitum* access to food and water, and were maintained on a standard breeding light schedule (14h L: 10h D). We chose to work with laying hens because of the ease of predicting ovulation timing on a daily basis. Previous studies in laying hens have shown that ovulation occurs within 30 minutes of oviposition of the previous egg [18]; therefore, we can use egg laying patterns to predict the timing of ovulation of the target egg. In addition, the timing of meiotic segregation, during which sex chromosomes segregate, has been fully documented in the domestic hen. The attachment of spindle fibers and physical segregation occurs approximately 2-4h prior to ovulation [31], and this is the time window during which treatments were targeted.

Hens were artificially inseminated twice weekly with pooled semen from 8 roosters to ensure egg fertilization. Eggs were collected manually every 2h during peak-laying hours and egg collection times were recorded for each individual hen. Before the experiment began, eggs were collected from 61 hens to quantify the sexes produced naturally by untreated hens. The same hens were then randomly assigned to one of the following injection treatments: a corticosterone treatment containing 1.5mg corticosterone dissolved in 0.5ml of peanut oil (CORT, n=33) or a control group receiving on 0.5ml of peanut oil (CONT, n=28). We administered injections subcutaneously in the back of the neck 5h prior to the predicted time of ovulation with the intent of elevating corticosterone concentrations 2-4h prior to ovulation, when the sex chromosomes were segregating. After treatment, follicles ovulated and took 24h to pass
through the remainder of the reproductive tract, after which the resulting targeted eggs were collected at oviposition (Figure 1). Eggs were incubated for 10 days at 37.5°C and 58% relative humidity in a Natureform incubator.

**Molecular Sexing**

Embryos were manually removed from eggs and stored in a 70% ethanol solution. Genomic DNA was extracted from embryonic tissue using a standard salt extraction method [26]. Portions of the CHD alleles on Z and W chromosomes were amplified using polymerase chain reaction (PCR) with primers 2550F and 2718R (adapted from [13]). For PCR amplification, we used a reaction volume of 20μl, containing 1.75 mM MgCl₂, 2μl 10X iTaq buffer, 200μM dNTP, 4pmol each primer, 0.65U iTaq polymerase, 500ng DNA, and water. Reaction parameters were the same as those described in Fridolfsson and Ellegren [13]. We visualized PCR products on a 3% agarose gel stained with ethidium bromide.

Not all hens laid a target egg at the predicted time and not all target eggs were fertile, so target embryo samples sizes were decreased to 32 untreated eggs, 23 CORT-treated eggs, and 16 CONT-treated eggs. The number of eggs that were either undeveloped/infertile (untreated n=7, CORT n=2, CONT n=0) or were not laid at target times (CONT n=12, CORT n=8) did not differ among treatment groups. Sexes of embryos in eggs that were not laid at the target time were not included in statistical analyses of offspring sex because any delay in oviposition could indicate that sex ratio adjustment had occurred as a result of follicular resorption rather than segregation distortion.

**Blood Samples and Radioimmunoassays**

Blood samples were collected from a separate set of hens treated in a similar manner to those described above to verify that CORT treatment elevated plasma corticosterone during the...
completion of meiotic segregation. We did not quantify offspring sexes from eggs laid by these hens because the process of blood collection represents a stressor and may have stimulated endogenous corticosterone production. Blood samples were collected from half of the CORT, CONT, and untreated birds 1h after injection (4h prior to ovulation) and from the other half 4h after injection (1h prior to ovulation)(Figure 1). In all cases, blood samples were collected from the brachial vein within 3min of initial handling to avoid variation due to handling stress [36]. Because samples were collected during the dark period using infrared head lamps and hens were carried to an adjacent room for blood collection, hens in the room remained undisturbed (i.e. did not stir from sleep) until they were removed from their cages for blood sampling. However, we also recorded how much time had elapsed between initial entry into the room and blood sampling for later analysis to ensure that general room disruptions did not alter corticosterone concentrations in our samples. Blood samples were centrifuged and the plasma was frozen at -20°C until hormone analysis.

Extraction and radioimmunoassay of plasma corticosterone were completed in 3 assays as described by Wingfield and Farner [46] and Etches [10]. Briefly, a small amount of tritiated corticosterone (1,000cpm) was added to each 20ul plasma sample for later calculation recovery efficiency after which corticosterone was extracted from samples using 3ml of diethyl ether. Samples were then snap-frozen and supernatant was collected in a fresh tube and dried under a N₂ stream. Plasma samples were resuspended in 300 ul of phosphate buffered saline (PBS) gel and duplicate aliquots of 100 ul each were added to assay tubes. An additional 50 ul sample was used to determine extraction efficiencies. To each assay tube and to additional tubes containing a graduated curve of corticosterone, 50ul of tritiated corticosterone (approximately 10,000 cpm) and 50ul of a rabbit-derived anti-corticosterone antibody (MP Biomedicals, Solon, OH USA,
cat# 07-120016) were added. All of the tubes were incubated for 18h after which 500ul of a solution contained dextran-coated charcoal was added to each tube. Tubes were incubated for 10min and centrifuged at 4,500 rpm for 10min to separate bound and free fractions. Supernatant was decanted into scintillation vials and radioactivity was counted after 4ml of scintillation fluid was added. The final concentration for each sample was corrected for its individual extraction recovery percentage. All samples were run in three 46 lucocorticoids. Average intra-assay variation was 3.72% and interassay variation was 8.4%. The average extraction recovery was 84.8%.

We analyzed 23 blood samples from UN-treated hens (12 and 11 at 1h and 4h, respectively), 13 from CONT-treated hens (5 and 8 at 1h and 4h, respectively), and 12 from CORT-treated hens (6 from each time period).

Statistical Analyses

We initially intended to compare sexes produced by hens in each treatment group to the sexes produced by the same hens the day before treatment to determine whether the injection itself exerted an effect on offspring sex. However, there were several hens from which we obtained either untreated or treated embryos, but not both because some eggs were undeveloped/infertile or were not ovulated at the target time. We were able to include all of these samples by categorizing untreated hens as a separate treatment group and comparing them with CONT and CORT-treated hens using a general linear mixed model (GLIMMIX). Because samples were taken from only some hens on both the pre-target and target day, bird ID was included as a random variable to correct for the lack of independence [5]. The GLIMMIX procedure provides a method of analyzing binary data while incorporating both random and repeated effects and thus handles over-dispersion [1]. In addition, we also examined sex ratios
as we had initially intended, as repeated measures from the same hen using a repeated measures logistic regression with sex as the response variable (male=1). Results from both analyses were similar and are included below. Finally, we also compared the proportion of males produced by each group of hens (untreated, CONT, and CORT) with a hypothetical 50:50 sex ratio of the same sample size using individual logistic regressions to determine if any treatment resulted in a proportion of males that differed significantly from the predicted 50:50 sex ratio. We felt this was an important comparison because natural sex ratios of offspring produced by domestic hens rarely deviate from 50:50 [12, 17, 22].

All hormone data were non-normally distributed and were log-transformed for statistical analysis. Plasma corticosterone concentrations were analyzed among treatment groups at each time point using an ANOVA and differences among individual treatment groups were analyzed using Fisher’s PLSD. A simple regression analysis was used to determine if time since initial disturbance influenced corticosterone concentrations. Statistical analyses were carried out using Statview and SAS 9.1 software (SAS Institute, Cary, NC USA).

**Results**

Treatment had a significant effect on offspring sex ($F_{2,28}=3.87, p=0.03$); hens treated with corticosterone produced significantly more male offspring (82.6%) than when hens were untreated ($p=0.01$). While hens receiving a control injection produced a slightly male-biased sex ratio (62.5%), this was not statistically different from sex ratios produced when hens were untreated (Figure 2; $p=0.28$). There was no significant difference between the percentage of male offspring produced by CORT and CONT hens ($\chi^2 = 1.927, p = 0.16$). There was, however, a significant difference between the proportion of male offspring produced by CORT hens and a hypothetical 50:50 sex ratio ($\chi^2 = 5.011, p=0.02$) while CONT and untreated hens did produce
similar proportions of males compared to a hypothetical 50:50 sex ratio (CONT: $\chi^2 = 0.505$, p=0.4773; Untreated: $\chi^2 = 0.251$, p=0.6166). When we analyzed data using repeated measures logistic regression analyses, we obtained similar results; hens treated with corticosterone produced significantly more male offspring after treatment than before treatment (p=0.02), but hens treated with control injections did not (Figure 3; p=0.5).

Blood samples collected from a separate set of hens 1h and 4h after injection showed that treatment had a significant effect on plasma corticosterone levels (Figure 4; 1h:$F_{2,20}=45.807$, p<0.0001; 4h:$F_{2,22}=11.949$, p=0.0003). CORT injections significantly raised corticosterone levels compared to CONT and untreated hens 1h after injection (p<0.0001 in both cases) and continued to be significantly elevated 4h after injection (CONT: p=0.01, untreated: p<0.0001). Plasma corticosterone levels in CORT treated hens averaged nearly 160 ng/ml at 1h and decreased to an average of 42 ng/ml at 4h while untreated hens averaged less than 3 ng/ml at both collection times. CONT treatment also significantly raised corticosterone levels compared to untreated hens 1 and 4h after injection (p=0.01, p=0.04), suggesting that the injection and handling method induced a handling stress in the hens. Plasma corticosterone levels in CONT treated hens averaged approximately 30 ng/ml at 1h and 9 ng/ml at 4h. Time since initial disturbance did not influence measured corticosterone concentrations ($r^2=0.02$, p=0.57).

**Discussion**

Corticosterone had a significant effect on offspring sex when administered at the critical time of meiotic segregation. However, contrary to our predictions, corticosterone stimulated the production of significantly more males. Our results differ from those of recent studies in other avian species that show a female bias when plasma concentrations of corticosterone were chronically elevated with corticosterone implants or chronic stress [33, 34], but concur with a
recent study in zebra finches showing a male-bias after acute treatment with a pharmacological dose of corticosterone [15]. Perhaps acute and chronic corticosterone elevations differentially regulate a similar mechanism. For example, chronic exposure to corticosterone can down-regulate the expression of corticosterone receptors [28]. In this way, chronic exposure to corticosterone could inhibit the acute actions of corticosterone that would normally stimulate the production of males. Alternatively, perhaps acute and chronic elevations act through other hormones in different ways. Chronic corticosterone elevations are known to influence other hormones that have been shown to affect offspring sex in birds, such as progesterone or testosterone [8, 16, 38] and could act by influencing or down-regulating these hormones over time. In the acute sense, corticosterone may act directly on the developing follicle, as glucocorticoids receptors and enzymes that metabolize glucocorticoids have been identified in the avian ovary [24].

The dose of corticosterone that we injected resulted in an elevation of corticosterone concentrations that extended beyond the physiological range. This dose was used to see if a mechanism exists by which corticosterone exposure at any dose can induce a sex ratio skew at the critical period of meiotic segregation and because negative results at a lower dose would have been inconclusive. In addition, the dose we provided elevated corticosterone concentrations over the entire time period during which meiotic segregation could occur, while physiological doses are metabolized within 2 hours (as occurred with the endogenous corticosterone levels produced during control injections). It is possible that the pharmacological nature of the injection produced different effects compared to what a physiological dose would. However, we were able to determine through this work that there is an existing mechanism by which a hormone can influence the sex of offspring at a very early developmental stage. If the
dose administered had been too low, we would not have known whether a negative result was because of the corticosterone dose or because a mechanism for skewing sex ratios did not exist. Whether lower concentrations of corticosterone would have similar effects on offspring sex needs to be tested.

We initially expected that, if corticosterone exerted an effect on offspring sex, the number of males produced by hens in CORT and CONT treatment groups would differ. Instead, while CORT treated hens produced more males than when hens were untreated, sexes of offspring produced by CORT and CONT hens were not significantly different from one another. It is possible that the sex ratio skews were caused by the injected vehicle (peanut oil); however, peanut oil was purposefully chosen for the current experiment because of its innocuous nature and because it does not contain the phytoestrogenic qualities of other oils (e.g. sesame oil) commonly used in similar experiments [41]. The stressful nature of the injection treatment stimulated hens to produce slightly elevated levels of endogenous corticosterone, and hens in the CONT treatment group produced numerically, though not statistically, more males compared to untreated hens. We suspect that this slight male bias, which prevented a difference between CONT and CORT hens, may be related to the production of endogenous corticosterone by CONT hens. This remains to be tested. However, sexes produced by the CONT group were not statistically different from untreated hens or the hypothetical 50:50 ratio, while sexes from the CORT group were, which supports a role of corticosterone in the determination of offspring sexes in this experiment. The results shown here may result from a dose-dependent response to corticosterone elevation, since the CONT group experienced significant corticosterone elevation. The effects of varying doses of corticosterone using several different vehicles must be tested.
Using an acute injection treatment, we have shown that avian sex ratio biases can be influenced if corticosterone is administered during the critical period of meiotic segregation. This means that the mechanistic basis exists for sex ratio adjustments prior to ovulation. Whether the adjustment is occurring via non-random segregation of sex chromosomes remains to be tested; however, it is unlikely that adjustments occurred after ovulation given that few eggs were undeveloped/infertile and that the number of undeveloped eggs did not differ between treatment groups. The minimum length of an ovulatory cycle for hens maintained on a 14L:10D schedule is 24h [11] and ovulation of the largest follicle occurs every 24-26h in response to a surge of luteinizing hormone [14, 19, 25, 40, 45]. We would expect that resorption of a follicle would result in a delay of oviposition of 24h; however, King [21] showed that the largest two follicles in the hierarchy do not differ dramatically in size, which could allow for a quick secondary ovulation following abortion of the first follicle. Indeed, Pike [35] suggested that this effect could explain how maternal condition stimulates a female bias in pigeon eggs. While this mechanism may occur in the pigeon system, it seems an unlikely method of sex ratio adjustment in our experiment. Ovulation of the primary oocyte in chickens and other galliform birds is triggered by increased secretion of progesterone from the granulosa cells surrounding the oocyte coincident with an elevation of circulating luteinizing hormone [30, 47]. Since the surge of luteinizing hormone occurs only every 24-26h and only the granulosa cells surrounding the primary oocyte increase secretion of progesterone [30], it is unlikely that follicular resorption and secondary ovulation occurs without delayed oviposition of the secondary oocyte in the chicken system. We monitored the timing of oviposition for our hens very strictly to ensure that we could detect even a short time delay from the expected time of oviposition. Eggs were collected every 2h and a qualitative temperature description (cold, room temperature, warm, or
hot) was assigned to each egg so that an approximate timing of oviposition could be determined for each collected egg. All of the embryos included in our analyses were collected from eggs laid on the predicted day and at the predicted time. Emlen [9] suggested that evidence of follicular resorption may be hidden if sex ratio adjustment occurred only for the first egg in the laying sequence. In our study, however, all hens had laid an egg on the previous day, suggesting that follicular resorption had not occurred prior to the production of the target egg to influence the sex of the offspring in the target egg. Thus, our methods of data collection and analyses make it unlikely that the male bias we saw resulted from preferential follicular resorption.

Our study provides additional evidence for the role of corticosterone in the adjustment of avian primary sex ratios, although the male bias we saw was the opposite of the results seen in studies in which corticosterone concentrations for chronically elevated. Because body condition is often closely associated with chronic elevations in corticosterone concentrations, some have suggested that there should be clear adaptive benefits of a link between corticosterone and offspring sex ratios [6, 27, 34]. It is currently difficult, however, to expand the findings of our study in a similar adaptive context for several reasons. First, our treatment was purposely designed to create an acute spike in corticosterone rather than a long-term elevation. Additionally, we induced levels of corticosterone that exceeded what would be observed in a bird with natural chronic elevations. Finally, the chicken model has experienced a high level of inbreeding and many of its evolutionary strategies may have been artificially selected against.

The benefits of adjusting sex ratios in relation to a single acute stressful event are less clear. However, we show here that a mechanism exists by which hormones can modulate offspring sex in birds, and these effects likely occur prior to ovulation. By gaining further understanding of how this mechanism works through additionally studies, we can gain insight into the costs and
benefits of sex allocation, which will ultimately allow us to address why hormone-mediated sex allocation occurs at an adaptive level.

Acknowledgements

We thank J. Cartmill, B.Wills, and J.Hannah for technical assistance. Financial support was provided by U.S. Poultry and Egg Association, grant #F027. This experimental protocol was approved by the University of Georgia Institutional Animal Care and Use Committee, AUP#2008-10079.
References


Figures

Figure 3.1: Injections were timed to elevate corticosterone during the completion of Meiosis I. Blood samples were collected from separate sets of hens 1 and 4h after injection (4 and 1h prior to ovulation) and the target egg was collected at oviposition, approximately 29 hours after the injection.
**Figure 3.2:** Comparison of proportion of male offspring produced among corticosterone-treated (CORT: n=23), control oil (CONT: n=16), and untreated (UN: n=32) hens. Different letters above the bars denote statistical differences.
**Figure 3.3:** Comparison of proportion of male offspring collected from pre-target and target eggs by corticosterone-treated (CORT: n=17) and control oil (CONT: n=13) hens. Different letters above the bars denote statistical differences.
**Figure 3.4:** Plasma corticosterone concentrations (mean + standard error) of hens in the corticosterone (CORT), control oil (CONT), and untreated (UN) groups at 1h (n=6, 5, 12) and 4h (n=6, 8, 11). Hormone concentrations were compared only among treatment groups, not among time points. Statistics were determined using log-transformed values because hormone data were not normally distributed, but untransformed values are presented here. Different letters above the bars denote statistical differences.
CHAPTER 4

EXAMINING THE EFFECTS OF MAGNITUDE AND TIMING OF CORTICOSTERONE ELEVATIONS ON PRIMARY SEX RATIOS IN LAYING HENS

2 Sara E. Pinson, Jeanna L. Wilson, Kristen J. Navara. To be submitted to Physiological and Biochemical Zoology.
Abstract

Evidence suggests that birds influence offspring sex prior to egg laying and may use hormones to mediate these skews. Corticosterone, the primary stress hormone in birds, is of particular interest as a potential mediator of offspring sex because it regulates responses to environmental and social stimuli that trigger sex ratio biases. It is also elevated during and participates in ovulation in birds. In previous studies, chronic elevations of corticosterone stimulated female-biased sex ratios while acute, pharmacological elevations stimulated male-biased sex ratios. Here, we aimed to determine the magnitude of corticosterone necessary to influence offspring sex by inducing acute or chronic elevations during chromosome segregation. We also aimed to further pinpoint the timing of the hormonal influence by increasing corticosterone prior to or at the time of the continuation of meiotic segregation. We hypothesized that females with acute, pharmacological elevations of corticosterone would produce more male offspring than controls and that females with acute, physiological elevations would produce an intermediate proportion of males. We tested our hypotheses in domestic laying hens by elevating corticosterone in the physiological or pharmacological range through injections of corticosterone administered 4 or 5 h prior to chromosome segregation. Contrary to our hypothesis, neither pharmacological nor physiological corticosterone hens produced sex ratios that differed significantly from controls or hypothetical 50:50 ratios. Results of this study suggest that elevation of corticosterone concentrations within the physiological range for just a few hours prior to ovulation is not sufficient to bias sex ratios in birds and that the timing and magnitude of hormone elevation is critical to influence offspring sex.

Keywords: Primary sex ratio, offspring sex, corticosterone, stress, maternal effects
Introduction

Birds have shown a unique ability to bias sex ratios prior to hatch. These primary sex ratio manipulations have been observed in many types of birds (reviewed in [1, 19]) and in relation to a variety of factors, including maternal body condition, social status, presence of helpers, mate quality, and food availability and quality (reviewed in [1, 19]). Even though many of the factors associated with sex ratio biases have been identified, we know little about the mechanisms controlling sex ratio manipulation in birds.

It is likely that the female can manipulate the sex of her offspring prior to ovulation because she is the heterogametic sex, contributing either a W or Z chromosome to offspring. During the first meiotic segregation, which completes 2-4h prior to ovulation [12, 18], one sex chromosome is retained in the oocyte and the other is segregated to the polar body with no further potential for development. It has been proposed that a female bird can control primary sex ratios through a variety of mechanisms due to her heterogametic nature. If the sex of the follicles are predetermined, follicles programmed to be the desired sex could be preferentially selected into the ovulatory hierarchy or grow at a different rate than other follicles. Primary sex ratio biases could also occur just prior to ovulation through non-random chromosome segregation, so that the desired chromosome remains in the oocyte, or at the time of ovulation through selective follicular abortion and secondary ovulation, so that any oocyte containing the undesired chromosome is discarded.

Hormones are good candidates as mediators of offspring sex ratio adjustment because they are important regulators of behavioral, biochemical, and physiological functions involved in reproduction. Indeed, biases in primary sex ratios have been observed in birds with elevated reproductive and stress hormones. Acute elevations of progesterone several hours prior to
ovulation stimulated the production of more females [6] while acute or chronic elevations of testosterone have stimulated male-biased sex ratios [11, 24, 26, 28]. Male and female-biased sex ratios have been observed following acute [10, 23] and chronic [4, 20, 21] elevations of the stress hormone corticosterone.

Corticosterone is the primary stress hormone in birds and is of particular interest as a potential mediator of primary offspring sex ratios for several reasons. Corticosterone is released from the adrenal glands (the left adrenal gland is located very close to the ovary) and is elevated during and necessary for ovulation [8]. Corticosterone also regulates many cues that have been associated with biased sex ratios, such as maternal body condition [2, 17, 20, 22, 29-31] and food availability and quality [3, 5, 14, 27].

Studies in several avian species suggest that chronically elevated corticosterone concentrations in females bias sex ratios towards females [4, 20, 21]. White-crowned sparrows and peafowl with naturally chronically elevated corticosterone produced more female offspring [4, 20]. White-crowned sparrows and Japanese quail with experimentally elevated corticosterone via implants also produced more female offspring [4, 21].

More recently, the effect of acute corticosterone on offspring sex has been studied. Zebra finches and domestic laying hens injected with high doses of corticosterone just prior to chromosome segregation produced significantly more male offspring than untreated females, possibly through non-random chromosome segregation [10, 23]. Results of these studies suggest that long-term corticosterone elevation is not necessary to induce a sex ratio bias and that acute and chronic corticosterone may act through different mechanisms to influence offspring sex. Furthermore, laying hens injected with the control vehicle had elevated corticosterone 1h after injection and produced an intermediate proportion of male offspring compared to the untreated
and corticosterone-treated females [23], suggesting the potential for biased sex ratios following an acute, physiological increase of corticosterone during meiotic segregation. To our knowledge, there is no evidence supporting or refuting the role of acutely elevated corticosterone in the physiological range during meiotic segregation in offspring sex ratio manipulation in birds.

The purpose of this current study was twofold. First, by injecting hens to induce an elevation in plasma corticosterone that reflected either a physiological or pharmacological increase, we intended to determine if an acute, physiological increase of corticosterone just prior to chromosome segregation was sufficient to influence offspring sex. Additionally, we intended to further pinpoint when corticosterone could influence offspring sex by injecting hens either 4 or 5h prior to the expected time of ovulation. We previously showed that a high, pharmacological dose of corticosterone injected 5 h prior to ovulation stimulated the production of more male offspring [23]. We hypothesized that females injected with the low, physiological dose at the same time point would produce an intermediate proportion compared to control females. Also, we hypothesized that if it was critical for corticosterone to be elevated prior to the continuation of meiotic segregation (4 h prior to ovulation), we would see an effect of corticosterone in those hens injected 5 h prior to ovulation but not those injected 4 h prior to ovulation.

Materials and Methods

Single-comb laying hens (Hy-Line International) of laying age were housed in a single room in individual layer cages, provided ad libitum access to food and water, and maintained on a stimulatory light cycle (14h L: 10h D). Laying hens were selected for this study because of their regular ovulation-oviposition cycles and ease of predicting ovulation timing on a daily basis. Ovulation occurs within 30 min of oviposition of the previous egg [13], so egg laying
patterns can be used to predict the ovulation timing of the target egg in the domestic hen. Additionally, the timing of meiotic segregation has been well-documented and segregation of the chromosomes completes 2–4 h prior to ovulation [18]. It is this critical time period, 2–4 h prior to ovulation, that was targeted by our treatments.

Our aim was to provide two doses of corticosterone, one within the pharmacological range and one within the physiological range, at two different time points before and after the continuation of meiotic segregation. Since we showed previously that an injection 5 h prior to ovulation elicits an effect on sex ratios, we aimed to repeat the same dose and add a physiological dose at the same time point as well as 1 h later (4 h prior to ovulation, Figure 4.1).

**Experimental Design**

Two experiments were conducted. Experiment I (5 h injection protocol) was conducted when the hens were 33–36 weeks old. Hens were artificially inseminated twice weekly with pooled semen from 36 roosters to ensure egg fertilization. Eggs were collected manually every 2 h during peak laying hours and egg collection times were recorded for each individual hen so that egg laying patterns could be determined. Once ovulation timing could be predicted for an individual hen, she was randomly assigned to one of the following treatment groups: untreated (UN), control oil (C: 1 ml peanut oil), physiological corticosterone (LCORT: 0.5 mg corticosterone dissolved in 1ml peanut oil), or pharmacological corticosterone (HCORT: 1.5mg corticosterone dissolved in 1ml peanut oil). Injections were administered subcutaneously in the back of the neck 5h prior to the predicted time of ovulation (Figure 4.2).

Blood samples were collected from a separate set of hens treated in the manner to those described above (Figure 4.2). We did not quantify offspring sexes from eggs laid by these hens because the process of blood collection represents a stressor and may have stimulated
endogenous corticosterone production. Blood samples were collected from subsets of hens in the UN, C, LCORT, and HCORT groups 20 min, 1 h, 2 h, and 4 h after injection, which coincided with 4.6 h, 4 h, 3 h, and 1 h prior to the predicted time of ovulation for Experiment I. Blood samples were collected from the brachial vein within 3 min of initial handling to avoid variation in plasma hormone concentrations due to handling stress [25] and were then centrifuged and the plasma was frozen at -20°C until hormone analysis.

Experiment II (4 h injection protocol) was conducted on the same population of hens several weeks after the completion of Experiment I, to ensure that the treatment from Experiment I was no longer influencing the hens. The experimental procedure was similar to the one described above, except that injections were administered 4 h prior to ovulation. Blood samples were also collected from a subset of hens and coincided with 3.6 h, 3 h, and 2 h prior to ovulation as well as the predicted time of ovulation (Figure 4.2).

For both experiments, oocytes took 24 h to transverse the oviduct and were collected at oviposition (Figure 4.2). All collected eggs were incubated for 8 days at 37.5°C and 58% relative humidity in a Natureform incubator prior to embryo sexing.

**Molecular Sexing**

Embryos were manually removed from eggs and stored in 70% ethanol solution until genomic DNA was extracted from embryonic tissue using a standard salt extraction method [16]. Portions of CHD-W and CHD-Z genes from the sex chromosomes were amplified using polymerase chain reaction (PCR) with primers 2550F and 2718R (adapted from [9]). For PCR amplification, we used a reaction volume of 25 µl, containing 3 mM MgCl₂, 40 mM Tris-HCl, 100 mM KCl, 200 µM dNTP Mix (Bio-Rad Laboratories, Hercules, CA USA, cat# 170-8874), 5 pmol each primer, 0.18 U iTaq® DNA polymerase (Bio-Rad Laboratories, Hercules, CA USA,
cat# 170-8870), 500ng DNA, and water. PCR was performed in a Bio-Rad thermal cycler as described by Pinson, Wilson, and Navara [24]. We visualized PCR products on a 3% agarose gel stained with ethidium bromide.

**Radioimmunoassay**

Extraction and radioimmunoassay of plasma corticosterone were completed as described by Wingfield and Farner [32] and Etches [7]. Briefly, a small amount of tritiated hormone (1,000cpm) was added to each 20ul plasma sample for later calculation recovery efficiency after which corticosterone was extracted from samples using 3ml of diethyl ether. Samples were then snap-frozen and supernatant was collected in a fresh tube and dried under a N₂ stream. Plasma samples were resuspended in 300ul of phosphate buffered saline (PBS) gel and duplicate aliquots of 100ul were added to assay tubes. An additional 50ul sample was used to determine extraction efficiencies. To each assay tube and to additional tubes containing a graduated curve of corticosterone, 50ul of tritiated hormone (approximately 10,000cpm) and 50ul of rabbit-derived anti-corticosterone antibody (MP Biomedicals, Solon, OH USA, cat#07-120016) were added. All assay tubes were then incubated for 18h after which 500ul of a solution containing dextran-coated charcoal was added to each tube. Tubes were incubated for 10min and centrifuged at 4,500 rpm for 10min to separate bound and free fractions. Supernatant was decanted into scintillation vials and radioactivity was counted after 4ml of scintillation fluid was added. The final concentration for each sample was corrected for its individual extraction recovery percentage. All samples were run in 15 radioimmunoassays (Exp. I: 7 assays, Exp. II: 8 assays). Average intra-assay variation was 2.67% (Exp. I: 2.42%, Exp. II: 2.9%) and interassay variation was 29.54%. The average extraction recovery was 86.25% (Exp. I: 85.25%, Exp. II: 87.25%).
From hens injected 5h prior to ovulation (Experiment I), blood samples were analyzed from 29 UN hens (n = 5, 10, 6, and 8 at 20min, 1h, 2h, and 4h respectively), 30 C hens (n = 7, 8, 7, and 8 at 20min, 1h, 2h, and 4h respectively), 32 LCORT hens (n = 8 for all collection times), and 31 HCORT hens (n = 7, 9, 7, 8 at 20min, 1h, 2h, and 4h respectively). From hens injected 4h prior to ovulation (Experiment II), blood samples were analyzed from 46 UN hens (n = 11, 12, 11, and 12 at 20min, 1h, 2h, and 4h respectively), 45 C hens (n = 10, 13, 10, and 12 at 20min, 1h, 2h, and 4h respectively), 45 LCORT hens (n = 12, 11, 10, and 12 at 20min, 1h, 2h, and 4h respectively), and 45 HCORT hens (n = 12, 12, 10, and 11 at 20min, 1h, 2h, and 4h respectively).

**Statistical Analyses**

Logistic regression analyses were used to compare the proportions of male offspring produced among treatments for both experiments. We used individual logistic regression to determine whether the proportion of males produced by any treatment group differed significantly from a 50:50 sex ratio of the same sample size.

All hormone data were normally distributed and were log-transformed for statistical analyses. The effect of treatment on plasma corticosterone concentrations was analyzed at each time point with analysis of variance (ANOVA), using Fisher’s protected least significant difference (PLSD) to determine differences among individual treatment groups. Statistical analyses were performed using Statview software (SAS Institute, Cary, NC).

**Results**

**Plasma Corticosterone Concentrations**

We aimed to elevate corticosterone concentrations into the physiological and pharmacological range at two different time points – prior to and at the start of continuation of
meiotic segregation. For the pharmacological dose, we used the same treatment of corticosterone (1.5 mg of corticosterone dissolved in 1 ml peanut oil) as we used previously in the same strain of birds to induce a sex ratio skew. Interestingly, while this treatment initially resulted in a pharmacological elevation within 20 min (120 ng/ml), concentrations quickly dropped such that they were significantly lower at 1 h following injection compared to those in the previous experiment (69 ng/ml versus 160 ng/ml). As a result, our attempts at a pharmacological injection at the -5 h timepoint in this study resulted in a high physiological elevation at the critical time when sex chromosomes were likely influenced (Figure 4.1), and we now tested the effect of two different physiological elevations (high and low) on sex ratios produced by these hens.

The same injection doses given at 4 h prior to ovulation produced similar corticosterone concentrations to those in the previous experiment at both 20 min and 1 h following injection (150 ng/ml versus 160 ng/ml). The reasons for this difference are unknown; however, for the second experiment, when injections were administered 4 h prior to ovulation, we were able to make the intended comparisons between a pharmacological and physiological elevation of corticosterone.

For both experiments, treatment significantly affected plasma corticosterone 20 min (Exp. 1: F_{3,23}=26.62, p<0.0001; Exp. 2: F_{3,27}=6.31, p<0.0015), 1h (Exp. 1: F_{3,31}=106, p<0.0001; F_{3,44}=313.06, p<0.0001), and 2h (Exp. 1: F_{3,24}=21.15, p<0.0001; Exp. 2: F_{3,41}=184.56, p<0.0001) after injection. Corticosterone concentrations in HCORT and LCORT birds were significantly elevated compared to controls 20 min, 1 h, and 2h after injection (HCORT: p < 0.01 at all time points; LCORT: p < 0.05 at all time points), falling back to uninjected levels 4 h after injection in birds injected 5 h prior to ovulation (p = 0.08) but not those injected 4 h prior to ovulation (p =
Plasma corticosterone concentrations from untreated and control hens were statistically similar at all blood collection times in both experiments (p > 0.35 for all; Figures 4.3 and 4.4).

**Offspring Sex Ratio**

When injected 5 h prior to ovulation, HCORT females produced 35.6% males, which was not significantly different from the ratios produced by UN ($\chi^2=2.06$, p=0.15), C ($\chi^2=0.08$, p=0.77), or LCORT ($\chi^2=2.86$, p=0.09) females. Even though the HCORT group tended to produce more females than males, they produced a proportion of male offspring that was not significantly different from a hypothetical 50:50 ratio ($\chi^2=1.84$, p=0.18). LCORT females produced 53.2% males, which was not significantly different from the ratios produced by UN ($\chi^2=0.77$, p=0.38) or C ($\chi^2=2.73$, p=0.1) females or the hypothetical 50:50 ratio ($\chi^2=0.25$, p=0.62). UN and C females produced 48.9% and 38.7% males respectively, neither of which was statistically different from the hypothetical 50:50 (UN: $\chi^2=0.02$, p=0.89; C: $\chi^2=2.14$, p=0.15) or each other ($\chi^2=1.47$, p=0.23)(Figure 4.4).

When injected 4 h prior to ovulation, HCORT females produced 37.5% males, which was not significantly different from the ratios produced by UN ($\chi^2=3.3$, p=0.06), C ($\chi^2=1.48$, p=0.22), or LCORT ($\chi^2=0.007$, p=0.93) females. LCORT females produced 38.3% males, which was not significantly different from the ratios produced by UN ($\chi^2=2.52$, p=0.11 ) or C ($\chi^2=1.2$, p=0.27 ) females. Even though HCORT and LCORT females tended to produce more females than males, neither group produced a ratio statistically different than the hypothetical 50:50 sex ratio (HCORT: $\chi^2=1.77$, p=0.18; LCORT: $\chi^2=1.29$, p=0.26). UN and C females produced balanced sex ratios (UN: 50.5% ; C: 50%), which were not statistically different from one another ($\chi^2=0.01$, p=0.91) or the hypothetical 50:50 sex ratios (UN: $\chi^2=0.01$, p=0.93; C: $\chi^2=0.01$, p=0.91)(Figure 4.5).
Discussion

We show here that a dose of corticosterone given 5 h prior to ovulation that induces a physiological rise at 1 h after injection was not sufficient to stimulate a skew in offspring sex ratios. Also, a pharmacological dose of corticosterone given after meiotic segregation had resumed (4 h prior to ovulation) was likely too late to produce the male bias we saw in previous experiments when the pharmacological dose was given at the -5 h time point. Our results were contrary to our hypothesis that physiological corticosterone treatment would stimulate females to tend towards producing more males, although not as many as pharmacological corticosterone. At both time points, a high physiological dose, resulting in concentrations from 60-80 ng/ml, tended to produce more female offspring (< 40% males), even though these proportions were not significantly different from UN or C females or a hypothetical 50:50 ratio. This trend agrees with previous studies in which physiological doses of a chronic nature stimulated production of more females [4, 20, 21]; however, lack of statistical significance in the current study prevents us from drawing any true relationships among these studies. Females injected 5 h prior to ovulation with a dose of corticosterone that resulted in levels within the mid-physiological range (20 ng/ml) produced an even sex ratio (~53% males).

Even though no significant bias was seen in this study, results suggest that there are some important factors with regards to the influence of corticosterone on offspring sex ratios in birds. First, the magnitude of the corticosterone increase is important. Previous studies have shown that elevations of corticosterone beyond the physiological range (approximately 150 ng/ml in chickens and 70 ng/ml in zebra finches) that extended to an hour after injection resulted in the production of more male than female offspring [10, 23]. In the current study, plasma corticosterone concentrations in hens that were injected with HCORT 5h prior to ovulation (in a
similar manner to Pinson et. al. [23]) averaged less than 70 ng/ml 4h prior to ovulation while those hens injected at the same time with LCORT averaged less than 20 ng/ml 4h prior to ovulation. Results of this experiment suggest that the male bias seen in the previous experiments [10, 23] may have been a result of the pharmacological nature of the corticosterone treatment and that there is a threshold that corticosterone concentrations must exceed to influence offspring sex in an acute manner during chromosome segregation. It is possible that high concentrations of corticosterone could (1) influence or up-regulate other hormones that have induced a male-bias when administered in an acute manner, such as testosterone [24, 26], or (2) bind directly to glucocorticoids receptors on the ovary [15, 33], but this remains to be tested.

Also, results of this study suggest that the time, in relation to chromosome segregation and ovulation, that corticosterone is elevated is also a critical factor involved in sex ratio manipulation. In previous studies where a male bias was seen, females were injected 5h prior to ovulation which induced a pharmacological increase in corticosterone at least 4h prior to ovulation [10, 23]. In chickens, meiotic segregation completes 2-4 h prior to ovulation [18], so if a hormone is to influence offspring sex during this critical time, it needs to be increased in circulation and near the pre-ovulatory follicle by this time. In Experiment II of this study, injections were administered 4h prior to ovulation, which resulted in corticosterone concentrations averaging 160 and 80 ng/ml from HCORT and LCORT females 3.6 prior to ovulation, so that corticosterone was not increased in treated females until after the critical stage of chromosome segregation had resumed. HCORT females had corticosterone concentrations similar to those in previous experiments where a male-bias was observed, but it may not have been increased early enough to influence chromosome segregation.
Even though corticosterone concentrations in this study did not meet the magnitude and timing restrictions to bias offspring sex through non-random chromosome segregation, the hormone was elevated several hours prior to the time that offspring sex could be manipulated through follicular abortion and secondary ovulation; however, no bias was observed in either experiment, so it seems unlikely that corticosterone treatment just a few hours prior to ovulation induces a sex ratio bias through this mechanism of sex ratio adjustment.

It is not known why an injection of 1.5 mg corticosterone dissolved in 1 ml peanut oil administered 5 h prior to ovulation produced a lower concentration in hens in this experiment than hens of the same strain did in the previous experiment using the same treatment and protocol [23]. Even though the same strain of hens was used and the birds were of similar age, the hens in this current experiment seemed to more quickly metabolize the hormone. The difference could be contributed to differences in receptor expression or availability, but remains to be tested. It is also not known why hens injected 4 h prior to ovulation had higher corticosterone concentrations 1 h after injection than those injected 5 h prior to ovulation. The only difference between the 5 h and 4 h protocols was that females injected 5 h prior to ovulation were injected before the light period while those injected 4 h prior to ovulation were injected soon after the light period started. It is possible that the hens were capable of more quickly metabolizing the hormone just prior to the start of the light period, during the natural peak in corticosterone production. Differences in receptor expression and availability could affect whether or not corticosterone, or any steroid hormone, influences changes in offspring sex and should be considered for future experiments.

Future experiments should manipulate timing as well as magnitude of corticosterone elevations to determine if and when corticosterone influences offspring sex in birds. Results of
this study suggest that a short-term, physiological increase in corticosterone just a few hours prior to ovulation is not sufficient to bias offspring sex and that a concentration of corticosterone exceeding natural concentrations is needed. Efforts should be made to determine the lowest effective dose of corticosterone. The effects that pharmacological concentrations of corticosterone have on the synthesis and concentrations of other hormones as well as its ability to bind to receptors on the ovary should be studied. In addition, a more precise time frame during which corticosterone can influence offspring sex through non-random chromosome segregation should be determined. It appears that corticosterone must be increased more than 4h prior to ovulation and remain elevated in the pharmacological range until at least 4h prior to ovulation, but future experiments should further pinpoint the timing. By further elucidating the circumstances under which corticosterone can influence offspring sex, we can gain a more completely understanding of the mechanisms through which hormones influence primary sex ratio manipulation in birds.

Acknowledgements

We thank J. Cartmill and B. Wills for technical assistance. Financial support was provided by the U.S. Poultry and Egg Association, grant F027. This experimental protocol was approved by the University of Georgia Institutional Animal Care and Use Committee, AUP #2008-10079.
References


Figures

Figure 4.1: The aim of this study was to examine the effects of two doses of corticosterone. We intended for the injections to induce a hormone elevation within either the pharmacological range (HCORT) or physiological range (LCORT). We also aimed to further pinpoint when corticosterone could influence offspring sex by administering injections either 5 h (prior to the continuation of meiosis I) or 4 h (after the continuation of meiosis I) prior to ovulation. Here, we illustrate the intended hormone profiles for plasma corticosterone in the injected females.
**Figure 4.2:** Injections were administered 5h (Experiment I) or 4h (Experiment II) prior to ovulation to increase maternal plasma corticosterone during the critical period of chromosome segregation of the oocytes. Target eggs were collected the following day, approximately 29h after injection. In separate sets of hens, blood samples were collected 20min, 1h, 2h, and 4h after injection.
Figure 4.3: Plasma corticosterone concentrations (mean + standard error) of hens in the untreated (UN), control oil ©, physiological corticosterone (LCORT), and pharmacological corticosterone (HCORT) groups 20min (n= 5, 10, 6, 8), 1h (n= 7, 8, 7, 8), 2h (n= 8 for all groups), and 4h (n= 7, 9, 7, 8) after injection 5h prior to ovulation (Experiment I). Hormone concentrations were compared only among treatment groups at each time point, not between time points. Statistical differences were determined using log-transformed values in ANOVA because hormone data was not normally distributed, but actual values are shown in the graph. Different letters above the bars denote statistical differences.
**Figure 4.4:** Plasma corticosterone concentrations (mean + standard error) of hens in the untreated (UN), control oil †, physiological corticosterone (LCORT), and pharmacological corticosterone (HCORT) groups 20min (n= 11, 12, 11, 12), 1h (n= 10, 13, 11, 12), 2h (n= 12, 11, 10, 12), and 4h (n= 12, 12, 10, 11) after injection 4h prior to ovulation (Experiment II).

Hormone concentrations were compared only among treatment groups at each time point, not between time points. Statistical differences were determined using log-transformed values in ANOVA because hormone data was not normally distributed, but actual values are shown in the graph. Different letters above the bars denote statistical differences.
Figure 4.5: Comparison of the proportion of male offspring collected in Experiment I from eggs laid by hens in the untreated (UN), control oil ©, physiological corticosterone (LCORT), and pharmacological corticosterone (HCORT) treatment groups that were treated 5h prior to ovulation. Statistical differences between the treatment groups and hypothetical 50:50 ratios were determined using logistic regression analyses.
Figure 4.6: Comparison of the proportion of male offspring collected in Experiment II from eggs laid by hens in the untreated (UN), control oil (C), physiological corticosterone (LCORT), and pharmacological corticosterone (HCORT) treatment groups that were treated 4h prior to ovulation. Statistical differences between the treatment groups and hypothetical 50:50 ratios were determined using logistic regression analyses.
CHAPTER 5

ELEVATED TESTOSTERONE DURING MEIOTIC SEGREGATION STIMULATES LAYING HENS TO PRODUCE MORE SONS THAN DAUGHTERS³


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Abstract

Biases in avian sex ratios have been documented in relation to a variety of social and environmental conditions. Previous studies suggest that treatment with hormones can stimulate females to manipulate offspring sex, and that this effect occurs before ovulation. For example, acute and chronic treatments with testosterone stimulated significant skews towards male offspring. Hormones may act by influencing which sex chromosome is donated by the heterogametic female bird into the oocyte. However, it is difficult to pinpoint when effects of testosterone on offspring sex occurred in previous experiments because testosterone treatments were given either chronically over the entire period of follicular development or many hours before the critical period of chromosome segregation. We injected laying hens with testosterone injections 5h prior to ovulation to target this critical period and quantified the sexes of the subsequently ovulated eggs. We hypothesized that an injection of testosterone coincident with segregation of sex chromosomes would stimulate hens to produce more male than female offspring. As hypothesized, hens injected with testosterone produced a significant bias towards male offspring compared to controls, nearly 70%. These results suggest that acute testosterone elevation during meiotic segregation may mediate skews in avian primary sex ratios.

Keywords: Primary sex ratio, offspring sex, testosterone, maternal effects
Introduction

Birds have demonstrated a remarkable ability to bias offspring sex prior to egg laying in relation to a variety of social and environmental conditions (reviewed in [1, 36]). Sex ratio skews have been observed in response to maternal body condition [2, 32, 37, 49-51], social conditions such as presence of helpers [9, 27, 31] and mate quality [10, 19, 26, 45, 46], and environmental conditions such as food abundance and quality [3, 7, 24, 43]; however, little is known about the mechanisms controlling adjustment of these primary sex ratios. An understanding of how social and environmental cues affect potential mechanisms of sex allocation would provide insight into the adaptive significance of sex ratio biases.

In avian species the female is likely in control of primary sex ratios because she is the heterogametic sex, contributing either a W or Z chromosome to offspring. Genetic sex of the offspring is determined during the first meiotic division which completes just a few hours prior to ovulation [34, 41, 55, 56] when one sex chromosome is retained in the oocyte and the other is segregated to the polar body with no further potential for development. The heterogametic nature of the female makes it possible for her to manipulate the sex of her offspring prior to ovulation and thus control primary sex ratio of her clutch through a variety of mechanisms [1, 28, 36, 41].

Hormones are important regulators of behavioral and physiological functions involved in reproduction, and, as such, are good candidates as mediators of offspring sex ratio adjustment. Treatments with reproductive and stress hormones have stimulated biases in avian primary sex ratios. Specifically, experimental evidence suggests that corticosterone [6, 16, 37-39], progesterone [8], and testosterone [17, 42, 48] may be involved in the mechanism of sex ratio adjustment as females with elevated levels of these hormones have produced biased sex ratios.
Testosterone, in particular, is produced in follicular cells that lie in very close proximity to the oocyte and has been shown to stimulate male-biased sex ratios in three avian species. An injection of testosterone administered after oviposition of the first egg in the clutch induced a testosterone elevation in zebra finches that lasted from the first egg date through the end of the clutch and stimulated the production of more males in eggs laid late in the clutch [42]. Similarly, chronic elevations of testosterone via implants in spotless starlings and homing pigeons stimulated male biases that persisted after the removal of implants [17, 48]. However, when similar chronic elevations were induced in homing pigeons and Japanese quail, no significant effect on offspring sex ratios was observed [18, 38]. Goerlich and colleagues [18] suggested that the effects of testosterone on sex ratios may depend on maternal body condition, particularly body weight. The role of testosterone in sex ratio manipulation requires further examination. In addition, in cases where testosterone did induce a bias in offspring sex ratios, the treatments were either chronic implants [17, 48] or injections that increased testosterone concentrations several days prior to observed sex ratio adjustment [42], making it impossible to determine how testosterone acted to influence offspring sex.

There are six potential mechanisms by which offspring sex could be altered prior to hatch: 1) If sexes of oocytes are predetermined, testosterone could act to preferentially select follicles programmed to be the desired sex into the ovulatory hierarchy [4, 5]. It has also been suggested that testosterone treatments may influence offspring sex by (2) altering yolk hormones or (3) influencing follicular growth rates [1, 4, 5, 17, 36]. Alternatively, testosterone may influence offspring sex in a more acute manner (4) just prior to ovulation by affecting the sex chromosomes as they segregate [1, 17, 28, 36, 41] or (5) immediately after ovulation by stimulating selective follicular resorption or selective ovulation [1, 11, 17, 35, 36]. (6) Finally,
oocytes of a particular sex may be selectively fertilized [1, 36]. To understand where in the developmental process testosterone is manipulating offspring sex, it is necessary to restrict the window during which testosterone treatment is administered.

In chickens, the final stages of meiosis have been well-documented and complete 2-4h prior to ovulation [21, 34], so it is possible to target this critical period of sex determination with hormone treatment. Indeed, biased sex ratios have been observed in birds following targeted hormonal treatment at this time. For example, an injection of the stress hormone corticosterone in zebra finches and domestic chickens just prior to the period of chromosome segregation stimulated treated females to produce more male offspring [16, 39] while treatment with the reproductive hormone progesterone in domestic chickens during the same time period stimulated treated females to produce more female offspring [8]. Results of these experiments suggest that an acute elevation of a hormone during this critical period of genetic sex determination can influence the mechanism of sex ratio adjustment and that a chronic increase of hormones is not necessarily needed to bias offspring sex (see [6, 37, 38] for biases in offspring sex associated with chronic corticosterone).

In the current experiment, we administered a testosterone injection to elevate the concentration of circulating testosterone in treated females at the critical time when sex chromosomes were segregating, 2-4h prior to ovulation. We aimed to determine if testosterone can act during this short time period to influence offspring sex. Based on results of the previously mentioned studies examining the effects of testosterone on offspring sex, we hypothesized that a short-term elevation of testosterone within this time frame would stimulate females to produce a male-biased sex ratio.
Materials and Methods

Animals

Single Comb White Leghorn laying hens (Hyline International) were housed in individual layer cages in a single room, provided *ad libitum* access to food and water, and maintained on a standard breeding light cycle (14h L: 10h D). Laying hens were selected for this experiment because of the ease of predicting ovulation timing on a daily basis using egg-laying patterns. Previous studies in laying hens have shown that ovulation occurs within 30 minutes of oviposition of the previous egg [22], so egg laying (oviposition) patterns can be used to accurately predicting ovulation timing. Additionally, the timing of the final stages of meiotic segregation has been fully documented in domestic hens as 2-4 prior to ovulation [34], so treatments could be targeted for this critical period of genetic sex determination of the embryo.

Experimental Design

Hens were artificially inseminated twice weekly with pooled semen from 35 roosters. Eggs were collected manually every 2h during peak-laying hours. Egg collection times and qualitative temperature measurements of the eggs (warm or cold) were recorded for each individual hen. Eggs remain warm for approximately 1h following oviposition (S. Pinson personal observation) which allows us to further pinpoint when collected eggs were laid within approximately 1h. Egg collection times were used to determine egg laying patterns and predict the timing of ovulation for the target eggs. Once egg laying patterns were determined, hens were randomly assigned to one of three treatment groups: untreated (UN: n = 49), control (0.5ml control peanut oil, C: n = 35), or testosterone (1.5mg testosterone dissolved in 0.5ml peanut oil, T: n = 36). Peanut oil was selected as the control vehicle for the current experiment because it does not contain the phytoestrogenic qualities of other oils (e.g. sesame oil) commonly used in
similar experiments [47]. Injections were administered subcutaneously in the back of the neck 5h prior to the predicted time of ovulation to elevate circulating testosterone concentrations during meiotic segregation of the target follicle (Figure 5.1). Oocytes were ovulated approximately 5h after treatment and took 24h to pass through the remainder of the reproductive tract. Target eggs were collected at oviposition.

From the same females, we also collected eggs that were already in the shell gland during injection (pre-target eggs) and were therefore uninfluenced by the injection. In this way, we were able to compare sexes of eggs produced by hens in the different treatment groups and also make comparisons using the hens as controls for themselves to account for individual variation in sexes produced. All collected eggs were incubated for 8 days at 37.5°C and 58% relative humidity in a Natureform incubator prior to embryo sexing.

**Molecular Sexing**

Embryos were manually removed from eggs and stored in 70% ethanol solution. Genomic DNA was extracted from embryonic tissue using a standard salt extraction method [30]. Portions of CHD-W and CHD-Z genes from the sex chromosomes were amplified using polymerase chain reaction (PCR) with primers 2550F and 2718R (adapted from [14]). For PCR amplification, we used a reaction volume of 25ul, containing 3mM MgCl₂, 40mM Tris-HCl, 100mM KCl, 200uM dNTP Mix (Bio-Rad Laboratories, Hercules, CA USA, cat# 170-8874), 5pmol each primer, 0.18U iTaq© DNA polymerase (Bio-Rad Laboratories, Hercules, CA USA, cat# 170-8870), 500ng DNA, and water. PCR was performed in a Bio-Rad thermal cycler. An initial denaturing step at 94°C for 3min was followed by 30 cycles of 48°C for 45s, 58.3°C for 45s, and 94°C for 30s. A final cycle of 48°C for 1min and 72°C for 5min completed PCR and
samples were held at 4°C. We visualized PCR products on a 3% agarose gel stained with ethidium bromide.

Not all hens laid a target egg at the predicted time and not all target eggs were fertile, so final target embryo samples sizes were reduced to 41 untreated eggs (n=7 infertile eggs, n=1 egg without sufficient embryonic development), 28 control oil eggs (n=3 eggs not laid on target day, n=1 egg not laid at predicted time, n=3 infertile eggs), and 25 testosterone eggs (n=4 eggs not laid on target day, n=5 eggs not laid at predicted time, n=2 infertile eggs). Additionally, only those pre-target embryos that had an accompanying target embryo were molecularly sexed, so the pre-target samples sizes were 31 eggs from untreated hens, 22 eggs from control oil hens, and 21 eggs from testosterone treated hens.

**Hormone Analysis**

**Blood Sampling**

Blood samples were collected from a separate set of hens treated in a similar manner to those described above to verify that testosterone treatment elevated circulating levels of plasma testosterone during the completion of meiotic segregation. We did not quantify offspring sexes from eggs laid by these hens because the process of blood collection represents a stressor and may have stimulated endogenous corticosterone production, a hormone that has demonstrated influences on avian sex ratios [6, 16, 37-39]. Blood samples were collected from subsets of hens in the untreated, control oil, and testosterone groups 1h after injection (4h prior to ovulation), 2h after injection (3h prior to ovulation), and 4h after injection (1h prior to ovulation). In all cases, blood samples were collected from the brachial vein within 3min of initial handling to avoid variation in plasma hormone concentrations due to handling stress [40]. Blood samples were centrifuged and the plasma was frozen at -20°C until hormone analysis.
Extraction and Radioimmunoassay

Extraction and radioimmunoassay of plasma testosterone and corticosterone were completed as described by Wingfield and Farner [53] and Etches [12]. Briefly, a small amount of tritiated hormone (1,000cpm) was added to each 20ul plasma sample for later calculation recovery efficiency after which testohormonosterone was extracted from samples using 3ml of diethyl ether. Samples were then snap-frozen and supernatant was collected in a fresh tube and dried under a N₂ stream. Plasma samples were resuspended in 300ul of phosphate buffered saline (PBS) gel and duplicate aliquots of 100ul each were added to assay tubes. An additional 50ul sample was used to determine extraction efficiencies. To each assay tube and to additional tubes containing a graduated curve of either testosterone or corticosterone, 50ul of tritiated hormone (approximately 10,000cpm) and 50ul of rabbit-derived anti-hormone antibody (MP Biomedicals, Solon, OH USA, testosterone: cat# 07-189016, corticosterone: cat#07-120016) were added. All testosterone assay tubes were vortexed (corticosterone assay tubes were not vortexed) and then incubated for 18h after which 500ul of a solution containing dextran-coated charcoal was added to each tube. Tubes were incubated for 10min and centrifuged at 4,500 rpm for 10min to separate bound and free fractions. Supernatant was decanted into scintillation vials and radioactivity was counted after 4ml of scintillation fluid was added. The final concentration for each sample was corrected for its individual extraction recovery percentage. All samples were run in three radioimmunoassay per hormone assayed. For testosterone, average intra-assay variation was 2.6% and interassay variation was 13.8%. The average extraction recovery was 85.3%. 
Blood samples were analyzed from 32 untreated hens (n = 11, 9, and 12 at 1h, 2h, and 4h respectively), 34 control oil hens (n = 11, 12, and 11 at 1h, 2h, and 4h respectively), and 35 testosterone treated hens (n = 12, 11, 12 at 1h, 2h, and 4h respectively).

**Statistical Analyses**

**Plasma Hormone Concentrations**

All raw hormone data were non-normally distributed and were log-transformed to meet the normal distribution requirement for statistical analyses. Plasma testosterone and corticosterone concentrations were analyzed using an ANOVA and Fisher’s PLSDs were used to compare plasma concentrations among treatment groups at each time point.

**Offspring Sex and Fertility**

We used logistic regression analyses to compare the proportions of male offspring produced among treatments in both pre-target and target eggs. Then, for each treatment, the proportions of male offspring in pre-target versus target eggs were analyzed using repeated measures logistic regression analyses. This allowed us to determine whether treatment influenced the same hens to change the proportion of males produced compared with their own production the day before. Finally, we also compared the proportion of males produced by each group of hens with a 50:50 sex ratio of the same sample size, using individual logistic regressions to determine whether any treatment resulted in a proportion of males that differed significantly from the 50:50 sex ratio.

We used logistic regression analyses to compare the fertility (proportion of fertile eggs) among treatment groups using only those eggs laid at the predicted time on the target day. All statistical analyses were carried out using Statview and SAS 9.1 software (SAS Institute, Cary, NC USA).
Results

Plasma Hormone Concentrations

Treatment significantly affected plasma testosterone concentrations overall ($F_{2,98}=105.692$, $p<0.0001$) and at all collection times (1h: $F_{2,31}=33.31$, $p<0.0001$; 2h: $F_{2,29}=17.215$, $p<0.0001$; 4h $F_{2,32}=83.234$, $p<0.0001$). Concentrations of testosterone for untreated and control hens were similar to one another at 1h ($p=0.83$), 2h ($p=0.79$), and 4h ($p=0.86$) post injection. Plasma testosterone concentrations were significantly higher in testosterone-treated females than control or untreated females 1h, 2h, and 4h after injection ($p<0.0001$ for comparing T to UN and C at all collection times)(Figure 5.2). During the time that meiotic segregation was completing (2-4h prior to ovulation coinciding with 1-3h after injection), corticosterone concentrations did not exceed 1ng/ml and there was no significant effect of treatment (1h: $F_{2,32}=0.09$, $p=0.91$; 2h: $F_{2,31}=1.97$, $p=0.1573$).

Offspring Sex and Fertility

Hens from the untreated and control groups produced statistically similar proportions of male offspring compared both before ($\chi^2=0.59$, $p=0.44$) and after ($\chi^2=0.60$, $p=0.44$) treatment. To maximize sample sizes and reduce the number of comparisons, we combined these two groups and categorized the new combined group as the CONT group for all analyses. It should be noted, however, that the results were also similar when comparing only the testosterone and uncombined control group without incorporating the results from the untreated hens.

Prior to treatment, the proportion of male offspring did not differ significantly between groups (T=42.9%, CONT=45.3%, $\chi^2=0.11$, $p=0.74$). After treatment, testosterone-injected hens produced significantly more male offspring (68%) compared to those in the combined CONT group (44.9%, $\chi^2=3.78$, $p=0.05$)(Figure 5.3). When comparing the proportion of males produced
between pre-target and target groups using repeated measures logistic regression, testosterone-treated hens produced 28.5% more males than they did prior to treatment (z=1.96, p=0.05) while CONT hens produced only 1.9% more males (z=0.19, p=0.85). Thus, testosterone treatment induced a change in the proportion of male offspring produced that did not occur in CONT hens (Figure 5.4). Neither the combined CONT group nor the testosterone group produced a proportion of male offspring that differed significantly from the 50:50 ratio (CONT: $\chi^2=0.36$, p=0.55; T: $\chi^2=1.68$, p=0.19); however, the statistical comparisons between treatment groups and pre-target and target eggs are more realistic comparisons for this study because these particular hens naturally produced a slightly female-biased sex ratio (45.2% males for eggs collected from all hens prior to treatment).

The percentage of fertile eggs exceeded 85% for all treatment groups (untreated = 85.7%, control oil = 90.3%, testosterone = 92.6%). There was no statistical difference in the proportion of fertile eggs between those produced among treatments ($\chi^2=0.91$, p=0.64). If eggs from untreated and control oil hens were combined as they were for statistical analyses of the proportion of male offspring, the fertility for the combined CONT group (87.5%) was not statistically different than that of the testosterone group ($\chi^2=0.51$, p=0.47).

**Discussion**

Testosterone had a significant effect on offspring sex when administered just prior to the time of chromosome segregation. Those hens that received testosterone injections and thus had elevated levels of plasma testosterone produced significantly more male offspring than control hens as well as compared to the same hens prior to treatment. These results are consistent with biases observed following testosterone injections or implants in other studies [17, 42, 48]. Results of our study provide further evidence that testosterone can influence offspring sex ratios in birds
and that these changes can occur when testosterone is elevated just a few hours prior to ovulation.

**Potential mechanisms occurring days, weeks, or months prior to ovulation**

Three of the six potential mechanisms of primary sex ratio manipulation occur well before ovulation. If sex chromosomes retained by oocytes are predetermined, hormones could influence the selection of oocytes into the ovulatory hierarchy or adjust follicular growth rates, ultimately influencing offspring sex ratios [1, 36]. Alternatively, the incorporation of hormones into yolk during the phase of rapid yolk deposition could influence the process of sex chromosome segregation [1, 4, 5, 36]. However, in the current study, plasma testosterone was increased for no more than 5h prior to the predicted time of ovulation, well after follicles were selected into the hierarchy and also at least 24h after rapid yolk deposition had completed [22]. As a result, the first three potential mechanisms can be eliminated when considering how testosterone acted on sex ratios in our study. It is possible that injected testosterone localized in the outer layer of the yolk and subsequently affected embryo sex; however, Hackl et al [20] showed that only 1% of injected testosterone could be localized to the yolk. This makes it unlikely that sex ratios were influenced by changes in yolk testosterone that resulted directly from our injections. Still, it would be helpful to quantify a suite of hormones in the outermost layer of yolk in future studies of hormone-mediated sex ratio adjustment.

**Non-random chromosome segregation**

By targeting injections to increase concentrations of plasma testosterone for no more than 5h prior to the predicted time of ovulation, we demonstrated that it is possible for circulating levels of testosterone in the female to influence offspring sex through more immediate pathways compared to changes in follicular recruitment and growth or yolk hormone levels. During the
hours leading up to ovulation, the germinal disc (which contains the sex chromosomes) is located in the periphery of the follicle which is highly vascularized, making it possible for it to be exposed to circulating testosterone. Androgen receptors have been identified on the granulosa cells, interstitial cells, and fibroblasts in the theca interna and externa of the pre-ovulatory follicle 4h prior to the predicted time of ovulation [54], so it is possible that testosterone could bind directly to the follicle during the critical period of chromosome segregation to influence offspring sex.

Non-random chromosome segregation during the completion of meiosis I has been suggested as a mechanism of primary sex ratio adjustment and it is particularly appealing for several reasons. First, controlling offspring sex at the chromosome level, as opposed to post-fertilization adjustment or follicular resorption, seems to be the most parsimonious option because it results in less waste of invested materials and energy compared to adjustments at a later stage that would result in the loss of an ovulated egg or fertilized offspring. Also, because this mechanism involves an effect that influences only the largest preovulatory follicle immediately prior to ovulation, it allows for adjustment of the sex-allocation strategy with each egg laid. Given that sex ratio adjustment often takes place in response to very dynamic environmental conditions, this plasticity would be beneficial. Finally, the avian sex chromosomes exhibit differences in size, shape, and content that could make segregation distortion possible (reviewed in [41]).

More work needs to be done to determine if and how segregation distortion occurs in the avian oocyte. Further research should address the question of whether testosterone acts directly on the oocyte or through other mediators to influence this process. Additionally, the cytological mechanisms that permit hormonal control of segregation distortion should be studied.
Selective follicular abortion and secondary ovulation

It is also possible that reproductive hormones could have influenced offspring sex at the time of ovulation through selective follicular abortion, which encompasses the proposed mechanisms termed follicular resorption and selective ovulation [1, 11, 17, 35, 36]. Selective follicular resorption and selective ovulation have been used interchangeably to explain the phenomenon that occurs when the primary post-meiotic oocyte does not pass through the oviduct to be oviposited due to either non-random reabsorption of the follicle just prior to ovulation or absorption into the abdomen following ovulation of the oocyte into the abdominal cavity instead of the oviduct [17, 36]. Selective follicular abortion has been suggested as an adaptive mechanism used to discard an oocyte containing the undesirable sex chromosome so that a secondary oocyte, possibly containing the desirable sex chromosome, can then be ovulated.

This mechanism has been used to explain sex ratio biases in species with small clutch sizes [11, 35], but seems an unlikely method of sex ratio adjustment in our experiment. Follicles in the avian ovary grow in a hierarchical fashion and the primary follicle suppresses further development of other hierarchical follicles leading up to ovulation of the primary follicle [22], so if selective abortion and secondary ovulation occur, we would expect a gap in the timing of oviposition. According to Etches [13], the minimum length of an ovulatory cycle for hens maintained on a 14L: 10D schedule is 24h and ovulation of the largest follicle occurs every 24-26h in response to a surge of luteinizing hormone [15, 23, 29, 44, 52]. Because the luteinizing hormone surge takes approximately 24h to generate, we would expect that follicular abortion would result in a delay in oviposition of approximately 24h. King [25] argued that the secondary follicle is of sufficient size to be ovulated, reducing the potential delay to a period of hours;
however, he did not observe whether the hormonal milieu was sufficient to trigger a second
ovulation within such a short time period.

Ovulation of the primary oocyte in chickens and other galliform birds is triggered by an
increased secretion of progesterone from the granulosa cells surrounding the oocyte coincident
with an elevation of circulating luteinizing hormone [33, 55]. Only the granulosa cells
surrounding the primary oocyte increase secretion of progesterone [33] and the luteinizing
hormone surge occurs only ever 24-26 h, so it is unlikely that selective follicular abortion and
secondary ovulation occur without delayed oviposition in the chicken system. Even if secondary
ovulation could occur with a delay of only hours, which is unlikely in the chicken system, the
timing of oviposition for treated hens was monitored strictly in our study so that even a short
delay of oviposition of 1h could be noticed. Only embryos from those eggs laid during the
predicted time (less than 1h before or after the predicted time) were included in the analyses, and
still more males were produced by testosterone-treated hens. Therefore, methods of data
collection and analyses make it unlikely that the male bias following testosterone treatment
resulted from selective follicular abortion.

Sex-specific fertility or embryo mortality

Rates of fertility and/or embryonic mortality can influence the proportion of male
offspring at hatch if they differ in a sex-specific manner [1, 36]. However, in the current study,
the proportion of fertile eggs collected from hens in the testosterone group was similar to
proportions from control and untreated hens. This makes it unlikely that our biases resulted from
preferential fertilization of Z-bearing oocytes or through differential mortality of female embryos
after fertilization.
Conclusion

Results of this study suggest that an increase in testosterone just a few hours before ovulation can bias offspring sex ratios. To our knowledge, this is the first study to examine the effect of elevating testosterone to target the critical period of chromosome segregation and it provides insight into the possible mechanisms controlling sex ratio manipulation in birds. Further research is needed to determine the potential mechanisms of testosterone action on the oocyte during the critical period of chromosome segregation. Even though the concentration of testosterone induced by our injections exceeded concentrations produced naturally by chickens, we were able to determine that there is an existing mechanism through which testosterone can influence offspring sex during this stage of development. Future experiments should be used to determine the lowest effective concentration of testosterone on offspring sex. Given that a majority of studies have documented a male bias in response to elevated testosterone concentrations, it appears that the mechanism by which testosterone acts could be shared by many distantly related avian species. This suggests that testosterone may play an adaptive role in sex ratio manipulation, but it is difficult to understand what the role may be until we determine the precise mechanisms by which testosterone acts. We should also focus on determining the costs and benefits that result from testosterone-induced sex ratio manipulation.

Acknowledgements

We thank J. Cartmill and B. Wills for technical assistance. Financial support was provided by U.S. Poultry and Egg Association, grant #F027. This experimental protocol was approved by the University of Georgia Institutional Animal Care and Use Committee, AUP#2008-10079.
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Figures

Figure 5.1: Injections were administered 5h prior to ovulation to increase maternal plasma testosterone during the critical period of chromosome segregation of the pre-ovulatory follicles. In separate sets of hens, blood samples were collected 1h, 2h, and 4h after injection, which coincided with 4h, 3h, and 1h prior to ovulation. Pre-target eggs were collected on the day of injection and target eggs were collected the following day, approximately 29h after injection.
**Figure 5.2:** Plasma testosterone concentrations (mean + standard error) of hens in the uninjected (UN), control oil (C), and testosterone (T) groups 1h (n=11, 11, 12), 2h (n=9, 12, 11), and 4h (n=12, 11, 12) following injections. Hormone concentrations were compared only among treatment groups at each time point, not between time points. Statistical differences were determined using log-transformed values because hormone data was not normally distributed, but actual values are shown in the graph. Different letters above the bars denote statistical differences.
Figure 5.3: Comparison of the proportion of male offspring collected from eggs laid by hens in the testosterone and combined control oil group before (T n=21, CONT n=53) and after treatment (T n=25, CONT n=69). Eggs from the uninjected (UN) and control oil (C) group were combined and categorized as the CONT group for statistical analyses. Statistical differences between treatment groups were determined using logistic regression and different letters above the bars denote statistical differences.
**Figure 5.4:** Comparison of proportion of male offspring collected from pre-target and target eggs laid by hens in the testosterone group (T n=21) and combined control oil and uninjected group (CONT n=53). Eggs from the uninjected (UN) and control oil (C) group were combined and categorized as the CONT group for statistical analyses. Statistical differences between pre-target and target groups were determined using repeated measures logistic regression and different letters above the bars denote statistical differences.
CHAPTER 6
SUMMARY, FUTURE DIRECTIONS, AND PRACTICAL IMPLICATIONS

Dissertation Summary

Experiments presented in this dissertation provide a further understanding of the role of hormones in primary sex ratio manipulation in birds. Prior to the start of these experiments, we knew that long term natural or artificial increases in corticosterone stimulated breeding females to produce more female offspring [1, 10, 11], but the mechanisms of the adjustments were unknown. We now know that an acute pharmacological elevation of corticosterone beginning at 5 h prior to ovulation stimulated females to produce more male offspring, likely through non-random chromosome segregation; however, if the elevation was not within the pharmacological range or did not occur until 4 h prior to ovulation, there was no significant change in sex ratios, suggesting a critical role of the timing and magnitude of the hormone elevation. Also, chronic or non-targeted increases in testosterone stimulated females to produce more male offspring in three avian species [7, 15, 16], while no bias was observed in similar experiments [8, 11], so the role of testosterone or the potential mechanisms involved was not well understood. We now know that an acute increase of testosterone within the high physiological range during meiotic segregation stimulated females to produce more male offspring and that the sex ratio adjustment likely occurred through non-random chromosome segregation.
Future Directions

While understanding that acute and chronic elevations of steroid hormones can influence breeding females to bias their sex ratios [1, 3, 6, 7, 10-13, 15, 16] is a step in the right direction, we still do not understand how steroid hormones are influencing offspring sex. Future experiments should not focus only on determining what hormones influence offspring sex or when the manipulations occur, but should also work to determine how the hormones are actually influencing the developing follicle. The availability, expression, and location of hormone receptors during the periods that sex ratio manipulation may occur should be determined. Also, it is unlikely that hormones act independently to bias offspring sex, so interactions between hormones should be studied. We suggest in this dissertation that pharmacological corticosterone could have mediated changes in offspring sex through other hormones, such as testosterone, so the effect of high concentrations of corticosterone on testosterone synthesis, circulating concentrations, and receptor availability and expression should be studied. Treating females with a combination of hormones and determining the effect on primary sex ratios and offspring fitness as well as circulating and yolk hormones would be recommended.

Most importantly, researchers should strive to target treatments so that specific mechanisms can be tested. Targeted treatments allow a more accurate assignment of which mechanism may have been used to bias offspring sex, thus bringing us one step closer to understanding how sex ratio manipulation occurs in birds. Understanding the proximate mechanisms of sex ratio adjustment can have profound impacts on not just our knowledge of an interesting adaptive phenomenon but the poultry industry and conservation programs as well.
Practical Implications

In the commercial laying industry, approximately 50% of all chicks are killed immediately after hatch because they are male. In 2002, approximately 226 million male chickens were killed in the US alone [4]. This practice may seem wasteful, but it is currently the only financially feasible option for the industry. For more than 50 years, chickens have been selectively bred for either egg production (layers) or meat production (broilers) so male chicks from layer breeders grow slower, develop less breast muscle, and are of lower slaughter quality than broilers [4]. Conversely, males are more desired in the broiler industry because females have a higher fat deposition and lower weight gain, thus reducing their body weight at slaughter. If the laying industry could manipulate hens such that a majority of chicks were female and the broiler industry could manipulate hens such that the majority of chicks were male, productivity would dramatically increase while fewer newly hatched chicks were killed, thus reducing waste and increasing profits. The work presented in this dissertation provides the opportunity to move towards intentional sex ratio manipulations by the industry. Understanding that elevations of the reproductive hormone testosterone and the stress hormone corticosterone just a few hours prior to ovulation can stimulate females to produce more male offspring, and that the timing and magnitude of these elevations are critical, is an important step in the process of determining the mechanism controlling these changes and developing a non-hormonal treatment for the industry.

Similarly, a better understanding of the role of hormones and other factors in sex ratio manipulation can have a profound impact on captive breeding and conservation programs. A more complete knowledge of how several factors, such as diet, body condition, territory, mate attractiveness, and hormone concentrations, work independently or cooperatively to bias offspring sex could improve the effectiveness of breeding and management programs. Scientists
could avoid unintentional sex ratio manipulations, stemming from a lack of knowledge of the factors influencing sex ratios in the species, that impede population growth [2]. With a better understanding of the influential factors, and eventually the proximate mechanisms involved, it could be possible to intentionally bias offspring sex ratios to promote population growth [5, 9, 14].
References


