INTRODUCTION

Maternal and paternal influences on avian reproduction before and at the site of fertilization can affect survival and overall fitness of avian offspring. Female birds deposit key nutrients and modulators into eggs that influence the survival and quality of offspring. In particular, contributions of yolk hormones by female birds have received much attention over the past 2 decades (reviewed in Groothuis and Schwabl, 2008). Whereas it is known that male birds contribute important antioxidants and immunomodulators to aid in sperm survival, the hormonal content of avian semen remains relatively unstudied.

Semen contains spermatozoa along with the surrounding seminal fluid that has all the components necessary for the survival of sperm cells. Semen fulfils a dual role in that it provides optimal conditions for fertilization and contains immunosuppressive substances that protect spermatozoa from damage in the hen reproductive tract (Pohanka et al., 2002). Chicken semen has also been shown to upregulate transforming growth factor β within the uterovaginal junction of the hen, which may play a role in sperm survival in the sperm storage tubules (SST; Das et al., 2010). Unlike mammals, in which sperm gain the ability to penetrate the ovum within the female, avian spermatozoa are unique in that they do not require a period of capacitation within the hen’s reproductive tract to fertilize an ova (Howarth, 1971). In mammals, sugars such as glucose and fructose are essential for the nourishment and metabolism of sperm and are secreted into seminal fluid by seminal vesicles, and although birds lack seminal vesicles their seminal plasma also contains such sugars for sperm survival (Hammond et al., 1965; Buckett and Lewis-Jones, 2002). Because secondary sexual organs do not exist in domestic birds, the seminal fluid is derived entirely from the testes and excurrent ducts (Froman, 1995). Enzymes and electrolytes also play important roles in the metabolism of fowl spermatozoa, which provides energy for many sperm functions, mainly motility (Hammond et al., 1965; Vanha-Perttula et al., 1990). Such seminal fluid components are widely known and studied, but the hormonal contributions from the male reproductive tract to the seminal fluid along with the effects of these hormones on fertility remain unclear.

The secretory activity of the testes as well as other tissues associated with the reproductive system is under endocrine control. Receptors for steroid hormones

Steroid hormone content of seminal plasma influences fertilizing ability of sperm in White Leghorns

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ABSTRACT Maternal and paternal influences before fertilization can significantly affect the ultimate reproductive output. In avian species, previous studies have shown that concentrations of testosterone (T) in seminal plasma vary greatly and are related to sperm quality. To our knowledge, the presence of other reproductive hormones in avian seminal plasma and their potential influences on fertility remain unstudied. We measured the concentrations of progesterone (P4), T, dihydrotestosterone, and estrogen in seminal and blood plasma collected from White Leghorn roosters. Progesterone was the most abundant hormone compared with all others measured, and concentrations of P4 in seminal plasma were significantly higher than concentrations found in circulation. Given the relatively high concentration of seminal plasma P4, we then attempted to determine its effect on fertility. Hens were inseminated with semen samples that were supplemented with either a high physiological dose of P4 or a control vehicle. Fertilization ability of all semen samples was then characterized using a perivitelline sperm hole penetration assay. Progesterone treatment significantly decreased the ability of sperm to reach and penetrate the egg, suggesting that males that deposit more P4 into seminal plasma may have a decreased capability to fertilize an egg.

Key words: progesterone, sperm penetration, fertility, reproduction, rooster
exist on mammalian sperm and have multiple effects on sperm function (Baldi et al., 1995; Sabeur et al., 1996; Luconi et al., 1999; Shah et al., 2005; Solakidi et al., 2005). Sexton (1974) showed that the metabolism of chicken spermatozoa is influenced by various steroid hormones such as androstanediol, estradiol (E), progesterone (P4), and testosterone (T). The ability of sperm to respond to steroid hormones suggests that hormones that exist in seminal plasma could influence the maturation and performance of avian sperm.

Additionally, steroid hormone receptors exist in the avian female reproductive tract. Specifically, estrogen and progesterone receptors have been localized to the female SST, and studies suggest that these sex steroids may play significant roles in the formation and maintenance of the SST structure as well as in the regulation of the sperm storage function (Yoshimura et al., 2000). If hormones in seminal plasma reach the SST, they may affect sperm function at the level of the female as well. To successfully fertilize an egg, sperm must complete all the steps of the fertilization process including movement, storage in the hen SST, binding and penetrating the inner perivitelline layer (PVL), and fusion with the ovum (Donoghue, 1999). Exposure to hormones in seminal plasma could potentially influence the ability of sperm to function at any one of these stages.

To our knowledge, the hormonal content of seminal plasma in roosters remains unstudied. We measured the concentrations of 4 reproductive steroid hormones, including P4, T, dihydrotestosterone (DHT), and 17β E, in both avian seminal plasma and blood plasma. To determine potential effects of seminal plasma hormones on fertility, we then treated seminal plasma with P4, the most prevalent hormone in seminal plasma, to determine the effects of a physiologically high level of P4 on the ability of sperm to hydrolyze the perivitelline membrane of the avian egg.

MATERIALS AND METHODS

Experimental Birds

Single Comb White Leghorn hens and roosters (Hy-Line International, West Des Moines, IA) in peak production were housed in individual cages in a climate-controlled room and were given ad libitum access to food and water. Birds were fed an industry standard diet and maintained on a standard breeding light schedule (14L:10D). Egg laying was monitored and recorded daily.

Experiment 1: Concentrations of Hormones in Seminal Plasma and Circulation

Concentrations of P4, T, DHT, and E were measured in both seminal plasma and in circulation. Semen samples were collected from 18 White Leghorn roosters via abdominal massage for quantification of hormone concentration. A blood sample was taken from the brachial vein at the same time as semen collection for each of the roosters so that hormonal content of seminal and blood plasma could be compared. All semen and blood samples were centrifuged at 20,800 × g for 10 min at 5°C and the supernatant was retained for hormone analysis. To ensure that measurements reflected baseline hormone concentrations without handling stress, blood and semen samples were taken within 3 min of capture (Wingfield et al., 1982; Romero and Romero, 2002).

RIA

Progesterone, DHT, T, and E from both seminal and blood plasma were extracted using diethyl ether and the hormones were separated by liquid column chromatography according to methods described by Schwabl (1993). Prior to extraction, we added 1,000 counts per minute each of tritiated androstenedione, DHT, T, and E for later calculation of recovery efficiencies. In previous studies, we found that radiolabelled P4 breaks down quickly and does not allow for accurate recovery calculations. Because androstenedione is similar in structure and elutes in the same column fraction as P4, androstenedione was used to calculate average P4 recoveries from columns. Concentrations of T were determined using a standard competitive binding RIA as described by Wingfield and Farner (1975) and Etches (1976). Blood plasma and seminal plasma hormones were quantified together in a single assay for each hormone. Briefly, we resuspended extracted samples with 300 μL of PBS gel, added duplicate aliquots of 100 μL of each sample to assay tubes, and used an additional 50-μL sample to determine extraction efficiencies. To each assay tube and to additional tubes containing a graduated curve of the target hormone, we added 50 μL of tritiated T (approximately 10,000 counts per minute) and 50 μL of rabbit-derived anti-T antibody (cat. no. 07-189016; MP Biomedicals, Solon, OH). After an incubation time of 16 h, we added 500 μL of a dextran-coated charcoal solution to each tube, incubated tubes for 10 min, and centrifuged tubes at 4,063 × g for 10 min at 23°C to separate bound and free fractions. Supernatant was decanted into scintillation vials followed by the addition of 4 mL of scintillation fluid and radioactivity was counted.

We quantified concentrations of P4 and DHT in extracted samples using standard I-125 labeled RIA kits (P: DSL-3900, DHT: DSL-9600; Diagnostic Systems Laboratories, Webster, TX). Seminal plasma E concentration was evaluated in a single assay and was not found in detectable amounts, so E concentrations were not quantified in blood plasma. All hormone values were corrected based on recovery efficiencies. Intraassay variations for P4, T, DHT, and E were 1.17, 2.30, 2.60, and 2.03, respectively. Average recoveries were 66.9% for P4, 82.7% for T, 58.6% for DHT, and 53.9% for E.
**Experiment 2: P4-Treated Semen**

Progesterone was the predominant steroid hormone found in seminal plasma (see results below), which led us to test the effects of P4 on the ability of sperm to fertilize the egg. We collected individual semen samples via abdominal massage from the same 18 roosters as above as well as an additional 6 roosters from the same flock (total n = 24). We vortexed each semen sample briefly to mix and then pipetted 2 aliquots of semen from every individual sample, 45 μL each, into different tubes. One aliquot was treated with 0.4 ng of P4 in 5 μL of diluent (P4, product no. P0130, Sigma-Aldrich Inc., St. Louis, MO; 6 h SemAid, ser. no. 02112010, PHL Associates Inc., Davis, CA) and the other was treated with 5 μL of diluent only, resulting in a 50-μL sample for insemination for each group; in this way, each rooster served as a control for itself. This dose was chosen to encompass the high physiological range of mean basal seminal plasma concentrations. We then introduced each treated 50-μL semen aliquot intracloacally to an individual hen, resulting in 24 hens that received P4-treated aliquots and 24 hens that received control-treated aliquots (a total of 48 hens). Eggs were collected on the second day following insemination because previous studies suggest that sperm penetration numbers decrease logarithmically on consecutive days after insemination (Wishart, 1987). This protocol maximized the chance of fertilization of the collected egg by our P4-treated or control-treated sperm. Eggs laid before this collection time may have been in the oviduct at the time of treatment. Of the 48 hens initially inseminated, 15 from each treatment group laid an egg on the target day, providing a sample size of 15 roosters in each group; in this way, each hen served as a control for itself. This design allowed each rooster to act as a control for itself. We would have liked to use a design to control for hen quality by treating each hen with each of our 2 treatments; however, we chose not to do so because we do not know the long-lasting effects of seminal plasma P4 on the hen reproductive tract. All analyses were performed using Statview statistical software (SAS Institute, Cary, NC), and significance values were assigned at P < 0.05.

**Perivitelline Layer Sperm Penetration Assay**

The PVL sperm penetration assay is often used as an indicator of overall fertility and sperm performance (Bramwell et al., 1995) because to penetrate the egg the sperm must be able to enter the SST, effectively travel through the reproductive tract, and penetrate the PVL. Therefore, a high number of sperm holes in the PVL indicates higher fertility (Bramwell et al., 1995; Christensen et al., 2006). For our study, the assay was modified from procedures described by Bramwell et al. (1995). Each egg was opened and the albumen was separated from the yolk. The yolk was then placed in a weigh boat with the germinal disc positioned on the top. Excess albumen was removed from the PVL by blotting with a Kimwipe (Kimberly-Clark, Irving, TX). After the addition of 2% NaCl to wet the yolk, the germinal disc (blastoderm area) was removed with scissors and immediately rinsed in Krebs-Ringer bicarbonate buffer solution to remove excess yolk from the membrane. The PVL was then placed on a microscope slide followed by the addition of 3 to 4 drops of 3% formaldehyde directly on the membrane and immediately decanted. Finally, the PVL was stained with Schiff’s reagent (cat. no. 804A, Medical Chemical Corp., Torrance, CA) and set to dry. The inner PVL holes were counted using a Zeiss Axio Observer (Carl Zeiss Inc., Thornwood, NY) at a magnification of 100×. The blastoderm area was located on each slide and centered in the field of vision (area = 785 μm²) and each hole within this area was counted (Figure 1A and B).

**Statistical Analysis**

We compared mean concentrations of each hormone between blood and seminal plasma using t-tests. We then tested for correlations between blood and seminal plasma hormone concentrations by performing a simple regression analysis for each hormone. These analyses were conducted only for hormones that were found in seminal plasma in measurable amounts (including P4, T, and DHT).

To determine whether increased P4 in semen influenced the ability of sperm to penetrate the egg, we compared the number of sperm holes produced by sperm from P4-treated versus control aliquots of semen from the same rooster using a paired t-test. This design allowed each rooster to act as a control for itself. We would have liked to use a design to control for hen quality by treating each hen with each of our 2 treatments; however, we chose not to do so because we do not know the long-lasting effects of seminal plasma P4 on the hen reproductive tract. All analyses were performed using Statview statistical software (SAS Institute, Cary, NC), and significance values were assigned at P < 0.05.

**RESULTS**

Progesterone was the most predominant hormone of the 4 reproductive steroid hormones quantified in rooster seminal plasma and ranged from 1.85 to 4.89 ng/mL (X = 3.38 ± 0.18). Testosterone and DHT were present in lower concentrations and ranged from 0.01 to 3.71 ng/mL (X = 1.05 ± 0.24) and 0.43 to 1.13 ng/mL (X = 0.64 ± 0.04), respectively (Figure 2). 173-Estradiol was undetectable in seminal plasma and was therefore removed from further analyses. In addition, P4 in seminal plasma was significantly greater than in the blood (t = 8.78, P < 0.001). Testosterone and DHT differed from P4 in that blood plasma concentrations significantly exceeded those of seminal plasma (T: t = −5.07, P < 0.001; DHT: t = −3.56, P = 0.002; Figure 2).

Progesterone concentrations in blood plasma were positively correlated to semen P4 concentrations (P = 0.037; r² = 0.24; Figure 3). Neither blood plasma T nor DHT were correlated to those of seminal plasma (T: P = 0.571, r² = 0.02; DHT: P = 0.927, r² = 0.001).

Progesterone treatment significantly impaired the ability of sperm to penetrate the PVL. Hens inseminated with P4-treated semen aliquots laid eggs with a
lower number of sperm penetration holes ($\bar{x} = 88.1 \pm 25.0$) than those inseminated with control-treated semen aliquots ($\bar{x} = 210.3 \pm 45.8$; $P = 0.034$; Figure 1C).

**DISCUSSION**

We have shown that avian seminal plasma contains measurable quantities of reproductive steroid hormones and that at least one of these hormones may exert effects on sperm function and fertility at high physiological doses. Progesterone was the most abundant reproductive steroid hormone in rooster seminal plasma, and concentrations of P4 in seminal and blood plasma were positively correlated. In addition, P4 concentrations were significantly higher in seminal plasma when compared with blood plasma, which contrasts findings in mammals in which concentrations of all steroids so far detected in seminal plasma in humans were significantly lower than corresponding blood levels; these include T, DHT, androstenedione, E, P4, and cortisol, among others (Pohanka et al., 2002). To our knowledge, this is the first study to measure P4 content of avian seminal plasma, and it is unclear why P4 was the most abundant hormone found, particularly given that androgens are largely responsible for maintaining spermatogenesis in the testes (Tan et al., 2005).

Testosterone and DHT were also present in seminal plasma in lower amounts, and concentrations in blood and seminal plasma were not correlated for either hormone. The potential function of these hormones in avian seminal plasma remains unclear. Cecil and Bakst (1986, 1988) demonstrated that no correlation existed between blood or seminal plasma T concentration and sperm quality in turkeys; however, a study with Leghorn cockerels showed a correlation between the T levels in seminal plasma and ejaculate volume and...
progesterone is known to act on human spermatozoa at the level of individual sperm cells. First, P4 could have acted by affecting mobility or over-


