

HORMONE CONTENT OF ROOSTER SEMINAL PLASMA AND EFFECTS ON SPERM QUALITY AND FERTILITY

by

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ABSTRACT

It has been demonstrated that female birds allocate hormones to eggs to enhance offspring quality, but the hormone content of semen contributed by males has received less attention. We measured the concentrations of steroid hormones in seminal and blood plasma from White Leghorn roosters. Progesterone was the most abundant hormone in seminal plasma. To determine the effect of progesterone on fertility, we inseminated hens with semen supplemented with either a high physiological dose of progesterone or a control vehicle, and evaluated fertility using a perivitelline sperm penetration assay. Progesterone inhibited sperm penetration. We then explored how progesterone related to semen quality to pinpoint where progesterone exerts an effect. Progesterone concentrations in circulation and seminal plasma were inversely related to sperm mobility, indicating that males producing more progesterone have poorer quality sperm. These results suggest that steroid hormones exist in avian seminal plasma and may influence sperm performance.

INDEX WORDS: progesterone, sperm quality, seminal plasma, leghorn, fertility

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

For male birds, the ultimate measure of reproductive quality is how many offspring they can sire. Male birds invest in reproduction by producing vast numbers of sperm capable of fertilizing eggs. At first glance, the function of sperm appears simple. Sperm are self-propelled DNA vessels whose primary task is to successfully reach a female egg and fertilize it. Spermatozoa deliver the male component of the genome, restoring a diploid state to the oocyte and allowing the development of the zygote and ultimately viable offspring. However, this seemingly simple function belies the complexity that accompanies fertilization. The different investments that males and females make in their gametes drive different options for maximizing individual reproductive success, including numbers of mating opportunities and how best to invest in quality offspring. Females have numerous ways to protect their investments in large, energetically expensive eggs, while males invest by finding a balance between producing sperm and inseminating a maximum number of females, ultimately achieving higher reproductive success.

Sperm performance is not solely an attribute of sperm cells themselves, but is determined by interactions with seminal fluid, which are likely to have important effects on fertilization success (Poiani, 2006). The ability of sperm to move through the reproductive tract of the female is crucial to the process of fertilization. Timing of

insemination may or may not coincide with the availability of fertilizable eggs, so sperm may need to survive for long periods of time outside the male's body, which could affect the number of viable sperm in the female tract. Females may mate with more than one male, resulting in sperm competition and a whole mass of competitive traits such as sperm selection, storage time and swimming speed that allow sperm to outcompete one another post-insemination in an attempt to fertilizing an egg. Consequently, sperm and seminal fluid are remarkably variable in composition, morphology, function and behavior across and even within bird species. The variation in sperm characteristics far outweighs those seen for eggs, suggesting that there has been considerable evolution of sperm traits. While we often focus on the sperm themselves in discussions of semen quality, there are many factors that influence sperm quality and overall reproductive success. This review focuses on many of these complexities of sperm function with particular respect to avian species.

Steps to fertilization success

1. Mobility

Sperm must possess several inherent characteristics which allow them to succeed at fertilizing an egg. A prerequisite to natural fertilization is that sperm cells must be capable of movement (motility) and more specifically, forward, progressive motion (mobility). The role of sperm mobility in oviductal sperm storage and transfer from the male to the female was recently reviewed in detail by Froman et al. (2011). During sperm maturation, sperm are released into the seminiferous tubules, suspended in seminiferous fluid and carried to the cloaca through a series of excurrent ducts (Kirby and Froman, 2000). Sperm in the seminiferous tubules, epididymis, and deferent ducts in

chickens are essentially immotile (Ashizawa and Sano, 1990), but as they are transported along the excurrent ducts, they acquire motility due to the influence of factors that are secreted into the seminal fluids along the way (Ashizawa and Sano, 1990; Ashizawa et al., 2004; Froman and Feltmann, 2005). Specifically, Froman and Kirby (2005) proposed that sperm mobility phenotypes for each individual rooster depend upon the extent to which glutamate induces mitochondrial calcium uptake in the sperm prior to ejaculation. In the rooster, flagellar movement is temperature-dependent and sperm motility is thought to be acquired by a drop in temperature at the time of ejaculation and ejaculate transfer into the hen (Ashizawa and Sano, 1990; Ashizawa et al., 2004). Sperm motion also depends upon genetic factors that influence mitochondrial morphology and function (Froman and Kirby, 2005). To date, sperm mobility has been described as the primary determinant of fertility and male fitness in birds (Birkhead et al., 1999; Donoghue et al., 1998, 1999; Froman and Feltmann, 1998, 2000; Froman et al., 1997, 1999, 2003). Gaining mobility is only the first of many steps that sperm must achieve before fertilizing an egg.

2. Sperm transfer to the hen

After ejaculation, sperm must be able to overcome a variety of challenges inside the hen's reproductive tract. Spermatozoa must first gain entry into the hen's vagina. As reviewed by Bakst et al. (1994), the fate of sperm within the hen's vagina is a fundamental determinant of fertility in the domestic fowl. Upon deposition into the cloaca, there are two obstacles that sperm encounter in order to enter the vagina and migrate toward the sperm storage tubules (SSTs). First, a mechanical barrier limits sperm migration to those sperm that are capable of progressive motility by impairing the

passage and providing storage of immobile spermatozoa (Allen and Grigg, 1957; Bakst et al., 1994). Second, the vagina selects sperm based on surface characteristics (Takeda, 1974; Wishart and Steele, 1990). Specifically, a study in chickens reported the presence of IgA or IgG on the surface of the sperm head in over 80% of the sperm population recovered from the vaginal portion of the oviduct, demonstrating that an immunological identification of sperm migrating toward the SSTs had taken place (Steele and Wishart, 1992a). Due to the physical and immunological barriers that sperm face intravaginally, less than 2% of the initial inseminated sperm population ever reach the sperm storage tubules (Brillard and Bakst, 1990; Steele and Wishart, 1992b; Brillard, 1993; Bakst et al., 1994), leaving behind unselected sperm which become imbedded in mucosal secretions on the surface of the vaginal epithelium and are evacuated towards the cloaca (Blesbois and Brillard, 2007). After overcoming these obstacles in the hen's vagina, the select subset of sperm passes into the oviduct.

3. Sperm storage

After gaining entry and passage through the hen's vagina post-insemination, sperm may either travel directly toward the ovulated oocyte or enter the hen's SSTs, which are crypts that store sperm in the hen for extended periods of time. Sperm storage is a reproductive strategy practiced by multiparous species, such as birds, that may help maximize fertility when copulation is infrequent (Bakst et al., 1994). Sperm introduced by copulation or artificial insemination are stored in two locations within the female reproductive tract: the infundibulum and the utero-vaginal junction (Bakst et al., 1994). These sperm reservoirs help ensure that sperm are available between inseminations and secure sustained fertilization over a period of time, from a few days to up to three weeks

in chickens (Etches, 1996; Donoghue, 1999). A series of studies have been conducted attempting to explain the mechanisms by which sperm storage and sperm survivability are achieved in the avian oviduct; however, little is known about the precise mechanism(s) involved and the process is yet to be fully understood.

In order for sperm to be stored into the SSTs, sperm must first be mobile and able to survive the environment of the vagina to reach the SSTs (Donoghue, 1999; Froman et al., 1999). It is likely that residence in SSTs depends upon the ability of sperm to move against the fluid current generated by the epithelial cells within the SSTs (Froman, 2003; Froman and Feltmann, 2005). In support of this theory, a study of retention rate of sperm in the SSTs found that highly mobile sperm were lost at a significantly slower rate than low mobile sperm, emphasizing that mobility elongates the fertile period and increases the fertilizing efficiency of the ejaculate (Froman et al., 2002). Optimal SST filling and release are crucial to fertilization, and it appears as though sperm mobility is a fundamental quality that allows for this to occur.

Immunological mechanisms inside the hen oviduct also play an important role in sperm selection at the level of the SSTs. The innate and acquired immune systems are well developed in the hen oviduct and anti-sperm immune responses may be induced in the vagina of the oviduct, as mentioned previously. Although the immune system in the oviduct is essential to the prevention of infection by various microorganisms, it may also adversely affect the reproductive performance in birds. Das et al. (2005) noticed lower fertility in hens that were repeatedly artificially inseminated compared to those that were not. They attributed this low fertility rate to an increase in the population of lymphocytes in the SSTs paired with antigen-presenting cells expressing MHC class II in the stroma of

the uterovaginal junction. Given that hens have inherent immune responses to protect themselves from foreign invasion of cells, sperm cells must overcome this attack by suppressing these immune responses. They do so by expressing transforming growth factor β (TGF β), which is responsible for suppressing the proliferation of T- and B-lymphocytes in birds (Quere and Thorbecke, 1990; Das et al., 2008). The interaction of sperm and SST cells may stimulate sperm cell TGF β expression protecting sperm from immune responses of the hen.

4. Release of SST sperm and sperm:egg binding

Once released from the SSTs, sperm traverse the oviduct in an attempt to ultimately fuse and fertilize the newly ovulated oocyte. The mechanism(s) of sperm release from the SSTs has been the focus of speculation for years (see Bakst et al., 1994 for review). Some have suggested that release from the SST is a female induced neural-mediated mechanism that initiates contraction of the SST epithelium (Freedman et al., 2001), thus ejecting sperm from the lumen of the SST. More recently, however, it has been suggested that sperm inside the SST lumen are subjected to a fluid current moving toward the exit of the SSTs and sperm remain in the lumen as long as their swimming speed exceeds the flow rate of the luminal fluid (Froman et al., 2011). Sperm released from the SSTs are activated by exposure to calcium-rich uterine fluids and ascend to the infundibulum (Bakst, 2010), the site of the upper oviduct where fertilization takes place. It was first thought that sperm transport beyond utero-vaginal sperm storage sites was transported by the action of cilia to the infundibulum (Wentworth and Mellen, 1964; Brillard, 1993). Now, it is accepted that within the oviduct, sperm cells are transported by antiperistaltic movement of the oviduct itself (Mimura 1939; Allen and Grigg, 1957).

At the time of fertilization, the plasma membrane of the oocyte is surrounded by the inner perivitelline layer (IPVL) and avian spermatozoa must bind to and penetrate this layer before gaining access to the plasma membrane of the oocyte. Sperm bind to the germinal disc region of the oocyte, which contains the female pronucleus (Romanoff, 1960), and more sperm are associated with this region than any other area of the ovum (Bakst and Howarth, 1977; Bramwell and Howarth, 1992; Birkhead et al., 1994). This seems appropriate, as spermatozoa penetrating other regions are unlikely to enter the germinal disc and be incapable of fertilization. It was initially theorized that sperm were able to simply pass through the fibrous meshwork of the IPVL (Bellairs et al., 1963), but subsequent research established that sperm utilize acrosomal enzymes to hydrolyze holes in the protein fibers of this layer in order to gain entry into the ovum (Bakst and Howarth, 1977; Okamura and Nishiyama, 1978). The number of sperm holes found in the IPVL can be assessed and has shown to be highly correlated with fertility (Wishart, 1987; Bramwell et al., 1995). Following sperm penetration, the outer perivitelline layer (OPVL) is deposited around the IPVL, covering sperm binding sites and thus preventing further penetrations of the IPVL by spermatozoa. Polyspermy is typical of avian fertilization and appears to increase the probability of syngamy; however, the likelihood of pathological polyspermy and successive loss of zygote viability in birds are minimized by the deposition of the OPVL (Bakst and Howarth, 1977).

5. Competition with rival sperm

Sperm must not only be capable of several basic functions in order to fertilize an egg, but must also have the ability to outcompete rival sperm. As male birds typically invest less in offspring than females, their mating frequency is high and their overall

reproductive success is mainly determined by the number of females they are able to fertilize; consequently, competition between males to mate with females is strong and has led to the evolution of traits to outcompete one another. Parker (1970) introduced the concept of 'sperm competition', which occurs when a female mates with more than one male in a single reproductive period and paternity of the clutch can be shared among males. After copulation, ejaculates from different males may compete with one another in an attempt to fertilize an ovum. This is particularly common for promiscuous species as males can become depleted of both sperm and seminal fluid (Pizzari et al., 2003) and must learn to allocate their ejaculates to maximize reproductive success. Female birds store sperm for several days or weeks in SSTs, which may result in competition between several males with which they mated (Briskie and Montgomerie, 1993). The concept of competitive ejaculates is thought to be a major force driving the evolution of several sperm quality traits (Birkhead, 1998; Pizzari and Parker, 2009).

There are multiple ways males selectively alter their ejaculate quality in response to social cues. The size of testes is arguably the most universal male adaptation to sperm competition (Birkhead, 1998). Testes are larger relative to body mass in species in which sperm competition is more intense (Møller, 1991). Sperm production is energetically costly (Dewsbury, 1982), and the number of sperm males ejaculate can be limited. Larger testes enable males to increase sperm production, in turn increasing their likelihood of fertilizing a female. Spermatozoa can be allocated to either many small ejaculates or a few large ejaculates. In the case of red jungle fowl, males can produce different size ejaculates depending on the number of females nearby (Pizzari et al., 2003). Over successive copulations, male fowl progressively reduced their sperm investment in

a female, and when a new female was presented, the number of sperm inseminated increased, indicating that male fowl respond to the threat of sperm depletion under sperm competition through strategic sperm allocation to different females (Pizzari et al., 2003). Males also selectively alter their ejaculate quality based on their social status. Cornwallis and Birkhead (2006) reported that dominant males will differentially allocate sperm in favor of a higher quality female, when given the choice between two females of differing quality. These dominant males have preferential access to females and can afford to invest less initially in a low quality female to see if a better one comes along. On the contrary, subordinate males will invest the most sperm in the female that he mates with first, regardless of quality, as they are limited in their access to females and must invest heavily in the first female available. Although it is well established that males adjust the number of sperm they ejaculate according to social status and female attractiveness, less is known about how males increase their fertilization success through adjusting the fertilizing ability of their sperm, or semen quality.

Males may adjust the fertilizing competency of their sperm directly, by allocating sperm of different quality or numbers to ejaculates, or indirectly, by allocating non-sperm components (seminal fluid) to ejaculates that in turn influence sperm performance by changing the resources available to sperm and the environmental conditions sperm experience (Cornwallis and O'Connor, 2009). Males can become exhausted of seminal fluid even when ample sperm are available for ejaculation and therefore males are predicted to allocate seminal fluid according to the reproductive benefits they gain from copulations (Cameron et al., 2007; Wigby et al., 2009). Male birds may also fertilize more eggs by increasing sperm mobility or longevity in the

female reproductive tract; for example, in a competitive situation, male chickens with highest sperm mobility sired more progeny than those of low mobility (Birkhead et al., 1999). The importance of increasing sperm mobility was emphasized by Brillard (1993) who demonstrated that sperm mobility positively affected the rate of sperm loss from the SSTs, with sperm from high mobility ejaculates being lost more slowly and hence maintaining their fertilizing power over a longer period of time. Extending the duration of sperm's fertilizing ability may be a reason as to why male birds increase the mobility, or swimming velocity, of their ejaculates. Cornwallis and O'Connor (2009) reported that seminal fluid alone had a positive effect on sperm velocity, increasing the competitiveness of the ejaculate and highlighting the importance of seminal fluid on avian sperm performance.

Semen quality and fertility

Male birds differ substantially in the quality of their ejaculates (e.g. Wishart, 1995; Ward, 1998; Simmons and Kotiaho, 2002; Birkhead et al., 2005). Methods used to assess these qualitative differences, both by humans and conspecifics, range from observations of external appearance to specific functional tests. The ability to evaluate males on the basis of their fertilizing ability could prove useful in both natural and artificial contexts. Male mallards with brighter bill coloration during pairing produced sperm with accelerated swimming speeds during mating several months later (Peters et al., 2004), a similar relationship to those documented in guppies. In the poultry industry, broiler breeder roosters are selected at hen-house placement on the basis of their physical characteristics such as comb size and color as well as overall body size; however, according to Wilson et al. (1979) these physical characteristics are not predictive of male

fertility. On the contrary, Navara et al. (in review) showed a correlation between comb size and color and sperm performance, indicating that males with smaller, redder combs had better quality ejaculates, and McGary and colleagues (2003) showed a strain-specific relationship between comb size and fertilizing ability of sperm. Many conflicting reports examine how physical characteristics are indicative of reproductive potential (Birkhead and Fletcher, 1995; Birkhead et al., 1997; Blount et al., 2001; Pizzari et al., 2004), highlighting the importance of evaluating semen quality to predict the fertility of an individual male.

The most accurate way to assess the quality of semen is to conduct analyses of individual functional qualities of sperm. Variation in paternity after insemination is often explained by the fertilization efficiency of an ejaculate, or sperm quality (e.g. Birkhead et al., 1999; Gage et al., 2004; García-González and Simmons, 2005). Sperm quality encompasses an assorted range of traits including sperm size, morphology, swimming velocity, metabolic performance, longevity and seminal fluid effects (Birkhead and Pizzari, 2002; Pizzari and Birkhead, 2002; Snook, 2005). Semen quality characteristics that are routinely measured by researchers consist of semen volume and concentration, sperm viability, motility and mobility (McDaniel et al., 1998; Donoghue, 1999; Parker et al., 2000). Measures of semen quality are often used in mating systems to assess the potential fertilizing ability of an ejaculate or pooled ejaculates. Evaluating semen samples from males provides a chance to eliminate low sperm-quality producing males, and increase overall reproductive success (Sexton, 1983).

It is necessary to not only test the ejaculate quality but also link these characteristics to fertilizing ability. Different methods and specific protocols for semen

analysis are reviewed in Bakst and Cecil (1997). These tests are useful for understanding sperm function, but the ultimate question concerns how different semen quality measurements relate to overall fertilizing ability of the ejaculate. Several aspects of semen quality have been correlated with fertilizing ability of male chickens such as sperm motility, sperm metabolism and percentage abnormal or dead sperm (McDaniel and Craig, 1959; Wilson et al., 1979). More recently, measures of sperm mobility (Birkhead et al., 1999; Donoghue, 1999; Froman et al., 1999) and sperm ATP content (Wishart and Palmer, 1986) also correlate strongly with fertility. There are conflicting reports linking sperm concentration to fertility. Some have claimed that sperm number in an ejaculate reflects situation-specific male reproductive allocation and cannot be used as a measure of semen quality (Pizzari et al., 2003) or have indicated no relationship between fertility and concentration (McCartney, 1956; Cooper and Rowell, 1957, 1958). On the other hand, Wilson et al. (1979) found a negative correlation between sperm concentration and fertility; while others established concentration of spermatozoa is positively related to fertility (Kamar, 1960; Boone, 1968).

While many sperm performance tests evaluate the associations between sperm traits and fertilization, female effects acting inside the genital tract after sperm transfer may also be key for the outcome of copulations. The process of fertility involves sperm transport, storage in the hen's oviduct, sperm binding and penetration of the ovum (Bakst et al., 1994). Sperm penetrate the inner perivitelline layer of a newly ovulated ovum (Romanoff, 1960) and use their acrosomal enzymes to hydrolyze holes in the protein fibers of this layer and gain entry to the female pronucleus (Baskt and Howarth, 1977). The quantitative relationship between fertility in chickens and the numbers of

spermatozoa that interact with the egg was first demonstrated by Wishart (1987), who correlated the numbers of spermatozoa trapped in the outer perivitelline layer with egg fertility and length of fertile period. These points of hydrolysis made by sperm can also be quantified in laid eggs. The perivitelline sperm penetration assay is often used as an indicator of overall fertility and sperm performance (Bramwell et al., 1995), because in order to penetrate the egg, the sperm may enter the sperm storage tubules, effectively travel through the reproductive tract, and penetrate the perivitelline layer. Therefore, a high number of sperm holes in the perivitelline layer have been correlated with higher fertility (Wishart, 1987; Bramwell et al., 1995). Assays like these paired with standard semen quality testing has allowed researchers to assess sperm function at multiple steps along the path to fertilization and gain a better understanding of sperm performance pre and post-insemination.

Components of Avian Seminal Fluid

While we often focus of the function of sperm in discussions of semen quality, there are many factors within semen that affect sperm quality and overall reproductive success. Semen is composed of spermatozoa suspended in the surrounding seminal fluid which is a complex medium made up of a great variety of molecules mainly produced by secretions from male reproductive organs. Seminal fluid provides optimal conditions for sperm survival and performance during reproduction. The biochemical content of the seminal fluid is complex, containing numerous substances with a wide variety of actions. In a number of species, including birds, seminal fluid has been shown to contain a complex mixture of molecules that are both energetically costly to produce and influence sperm performance (Lake, 1984; Fujihara, 1992; Poiani, 2006).

Seminal fluid provides a means of transport for male gametes, while also providing vital nutrients for sperm survivability. Seminal plasma contains the electrolytes sodium, calcium, potassium, and chloride (Hammond et al., 1965; Lake et al., 1958; Takeda, 1959; Samour et al., 1986), which are vital to homeostasis, as both osmotic pressure within the sperm cells and pH are greatly affected when these electrolytes are not in balance (Hammond et al., 1965). The energy metabolites available to sperm vary between species, but they generally consist of fatty acids (Scott et al., 1962) and sugars (McIndoe and Lake, 1973; Sexton, 1974). Glucose is the main carbohydrate found in avian seminal plasma with small amounts of fructose (Hammond et al., 1965; Samour et al., 1986). The presence of alkaline and acid phosphatase was evaluated by Bell and Lake (1962) and Wilcox (1961), and these were reported to be involved in the hydrolysis of carbohydrates (Hammond et al., 1965). Other components found in mammalian seminal plasma include prostaglandins, growth factors, proteins, steroids and leukocytes (Pohanka et al., 2002). These components have a multitude of effects in mammalian species in which they have been studied. For example, prostaglandins play a role in primate sperm immunity and may be necessary for the survival of sperm under adverse conditions, such as in the presence of infection (Kelly, 1997), and proteins aid in sperm protection and membrane integrity in Holstein bulls (Moura et al., 2010). Much of these semen components are widely studied in mammalian systems, but the existence and/or function of these have not been extensively studied in avian semen.

Antioxidant Activity of Seminal Fluid

Seminal fluid must also provide a way to protect sperm cells from oxidative stress during storage in the male as well as storage in the hen's oviduct. Avian sperm are

rich in polyunsaturated fatty acids (PUFAs) and display high rates of metabolic activity, and consequent free radical production, which renders them susceptible to oxidation by free radicals (Wishart, 1984; Blount et al., 2001). Excess oxygen causes peroxidative damage, destroying the structure of the sperm lipid matrix which results in membrane instability (Hammerstedt, 1993). Poultry sperm exposed to oxidative attack exhibit a variety of problems including morphological defects, reduced motility and poor fertilizing ability (Long and Kramer, 2003). Seminal plasma has an array of antioxidants that can protect sperm membrane integrity against these negative impacts of oxidants by maintaining membrane integrity, motility, and fertilizing ability (Donoghue and Donoghue, 1997; see review Khan, 2011). Among the well-known biological antioxidants are superoxide dismutase and glutathione peroxidase as well as natural antioxidants such as vitamin E and ascorbic acid that create an integral system in avian semen, capable of protecting them against the toxic products of metabolism (Froman and Thurston, 1981; Donoghue and Donoghue, 1997; Lin et al., 2005; Eid et al., 2006). Carotenoids were also found in small quantities in avian seminal plasma (Rowe and McGraw, 2008; Helfenstein et al., 2010) and may serve as antioxidant protectors of sperm as well.

It is suggested that the antioxidant system in seminal plasma must only exert protection over a relatively short period from ejaculation to sperm transfer to the hen, whereas the antioxidant system in sperm membranes must maintain its activity over prolonged periods during sperm storage within the female (Brèque et al., 2003). Sperm cells may spend a majority of their time in the SSTs of the oviduct; therefore these specialized sites must circumvent the negative effects of peroxidation products in sperm

membranes during in vivo storage (Brèque et al., 2003). After insemination, seminal plasma is quickly eliminated and replaced by fluid secreted by the oviduct, but the stability of the sperm membrane must be maintained throughout the extended periods of fertilization. Currently, work is being done to establish the role of the antioxidant system at the utero-vaginal junction and at the SST level. Specifically, Brèque et al. (2003) found that antioxidant enzymatic activity is more active in the avian SSTs than in the liver, indicating that this system has adapted to the role of providing protection for residing spermatozoa against peroxidation.

Seminal fluid signaling in the female reproductive tract

Historically seminal fluid was thought of as a survival and transport medium for male gametes, but now its role is understood to extend beyond sperm transfer and target female tissues after insemination. In insects, seminal products induce well-documented changes in the behavior and physiology of the female, such as aiding in sperm transport, triggering ovulation or oviposition and induction of resistance to further insemination by other males (Eberhard, 1995). Human seminal plasma contains several glycoprotein signaling substances such as cytokines and growth factors as well as sex hormones and several prostaglandins, all of which bind to receptors in the target tissues of the female reproductive tract and alter function of female reproduction (Mann, 1964; Aumüller and Riva, 1992; review by Robertson, 2005). Studies in pigs also demonstrated the effects of seminal plasma in females, suggesting that uterine exposure to seminal plasma influences immune ovarian cell trafficking and enhances steroidogenesis in early pregnancy, potentially contributing to reproductive success (O'Leary et al., 2006). The underlying

molecular and cellular mechanisms involved in seminal fluid signaling are, for the most part, still a mystery.

Due to the vast differences between avian and mammalian reproduction, it would be beneficial to study these effects in the avian system. Seminal plasma can no longer be considered as only a sperm transport and survival medium but instead recognized as a means of communication between male and female reproductive tissues. Improvement in this ability to communicate and induce female responses via seminal fluid could result in increased reproductive success for males.

Hormones in Seminal Plasma

Since male reproduction is primarily under endocrine control, hormones play a crucial role in sperm production and performance. Steroid hormone receptors are present on mammalian sperm, having a multitude of effects on sperm function (Baldi et al., 1995; Sabeur et al., 1996; Luconi et al., 1999; Shah et al., 2005; Solakidi et al., 2005), but similar receptors have yet been identified on avian sperm. Sexton (1974) showed that chicken spermatozoa metabolism is influenced by various steroid hormones like androstanediol, estradiol, progesterone and testosterone. This ability of sperm to respond to steroid hormones suggests that hormones deposited into seminal plasma could potentially influence the performance of avian sperm. Testosterone is a clear hormone of interest because it exists in measurable and variable quantities in seminal plasma (Anderson and Navara, 2011) and the process of spermatogenesis relies substantially on testosterone (Sharp, 1975; Tan et al., 2005). Circulating androgen concentrations have been correlated with fertility in poultry species (Rosenstrach et al., 1998; Biswas et al., 2007); however, the function of testosterone in seminal plasma is unclear, due to a lack of

evidence linking testosterone in blood or seminal plasma to sperm function (Zeman et al., 1983; Cecil and Bakst, 1986; Cecil and Bakst, 1988).

Although progesterone and estradiol are generally associated with female reproduction, there is evidence that these hormones also exist in measurable quantities in male chickens and influence the male reproductive system (Tanabe et al., 1986; Weil et al., 1999). Progesterone is involved in sexual behaviors in addition to regulation of ovulation in female birds (Johnson et al., 1985), but in males, its role is not very well understood. Steroid hormone receptors including estrogen and progesterone have been localized in the SSTs of the oviduct, and studies suggest that these sex steroids play significant roles in the formation and maintenance of the SST structure as well as in the regulation of sperm storage function (Yoshimura et al., 1999). Progesterone receptors have been detected in the testes of chicken embryos, but the actions of progesterone on testicular cells and the expression of its receptors in adult chickens have not been described (González-Morán et al., 2008). In mammals, progesterone induces sperm capacitation, motility, and can potentiate the acrosome reaction (Osman et al., 1989; Sabeur et al., 1996; Cheng et al., 1998; Wu et al., 2006; Baldi et al., 2009). However, Lemoine et al. (2008) showed that the progesterone dose that induces the acrosome reaction in mammalian species had no effect in chickens. Further research is needed to determine the role of progesterone on avian sperm function.

Thesis Objectives

Most research to date has focused on the hormonal constituents of mammalian seminal fluid, but the hormone content of avian seminal fluid is not fully understood. Both testosterone and progesterone are produced by the male in the vicinity of sperm

maturation and also regulate important sexual behaviors when they pass into circulation. Understanding the concentrations of hormones present in the reproductive fluids of the male chicken, along with the impacts of those hormones on sperm performance, will provide a better understanding of the endocrine regulation of male reproduction.

Here, I tested for the presence and concentrations of various reproductive steroid hormones in rooster seminal plasma and blood plasma. To determine the potential effect of progesterone, the most prevalent hormone in seminal plasma, we elevated the progesterone content of semen within the physiological range and evaluated the ability of those sperm to hydrolyze the perivitelline membrane of the avian egg. I then tested whether natural concentrations of both progesterone and testosterone in seminal plasma relate to different measures of sperm quality, in an effort to understand where these steroid hormones may exert their effect on sperm cell function. Results from these studies shed light on the influence of steroid hormones on male reproduction.

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CHAPTER 2

STEROID HORMONE CONTENT OF SEMINAL PLASMA INFLUENCES
FERTILIZING ABILITY OF SPERM IN WHITE LEGHORNS¹

¹ Anderson, E.M. and K.J. Navara. Accepted for publication in *Poultry Science* May 9, 2011, 90:2035-2040. Reprinted here with permission of publisher, 12/05/11.

Abstract Maternal and paternal influences prior to fertilization can significantly affect the ultimate reproductive output. In avian species, previous studies have shown that concentrations of testosterone 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), testosterone (T), dihydrotestosterone (DHT), and estrogen (E) within seminal and blood plasma collected from White Leghorn roosters. Progesterone was the most abundant hormone compared to all others measured, and concentrations of progesterone in seminal plasma were significantly higher than concentrations found in circulation. Given the relatively high concentration of seminal plasma progesterone, 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 progesterone 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 progesterone into seminal plasma may have a decreased capability to fertilize an egg.

Key words: progesterone, sperm penetration, fertility, reproduction, rooster

Introduction

Maternal and paternal influences on avian reproduction prior to 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 last two decades (reviewed in Groothuis and Schwabl, 1997). While 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 also 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 s within the utero-vaginal junction of the hen, which may play a role in sperm survival in the sperm storage tubules (SSTs) (Das et al., 2010). Unlike mammals where 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 in order to fertilize an ovum (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 (Buckett and Lewis-Jones, 2002; Hammond et al., 1965). 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 (Vanha-Perttula et al., 1990; Hammond et al., 1965). 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 exist on mammalian sperm and have multiple effects on sperm function (Sabeur et al., 1996; Luconi et al., 1999; Baldi et al., 1995; Solakidi et al., 2005; Shah et al., 2005). Sexton (1974) showed that the metabolism of chicken spermatozoa is influenced by various steroid hormones such as androstanediol, estradiol, progesterone and testosterone. 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 sperm storage tubules (**SSTs**), 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., 1999). If hormones in seminal plasma reach the SSTs, 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 hens' SSTs, binding and penetrating the inner perivitelline layer (**IPVL**), as well as 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 four reproductive steroid hormones, including progesterone (**P4**), testosterone (**T**), dihydrotestosterone (**DHT**) and 17 β estradiol (**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 progesterone, the most prevalent hormone in seminal plasma, to determine the effects of a physiologically high level of progesterone 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 (Hyline International – West Des Moines, IA USA) 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 (14hL: 10hD). Egg-laying was monitored and recorded daily.

Experiment 1- Concentrations of Hormones in Seminal Plasma and Circulation

We measured concentrations of P4, T, DHT and E in both seminal plasma and in circulation. Semen samples were collected from 18 White Leghorn roosters via the abdominal massage technique for quantification of hormone content (Burrows and Quinn, 1937). A blood sample was also taken from the brachial vein directly before semen collection for each of the roosters so hormonal content of seminal and blood

plasma could be compared. All semen and blood samples were centrifuged (at 5°C, 10 min, 20,800 x g) and the supernatant was retained for hormone analysis. To ensure that measurements reflect baseline hormone concentrations without handling stress, blood and semen samples were taken within three minutes of capture (Wingfield et al., 1982; Romero and Romero, 2002).

Radioimmunoassay

We first extracted P4, DHT, T, and E from both seminal and blood plasma using diethyl ether and separated the hormones by liquid column chromatography according to methods described by Schwabl (1993). Prior to extraction, we added 1000 counts per minute (cpm) each of tritiated androstenedione, dihydrotestosterone, testosterone, and estradiol for later calculation of recovery efficiencies. In previous studies, we have found that radiolabelled progesterone breaks down quickly, and does not allow for accurate recovery calculations (unpublished data). Because androstenedione is similar in structure and elutes in the same column fraction as progesterone, androstenedione was used to calculate average progesterone recoveries from columns. Concentrations of testosterone were determined using a standard competitive binding radioimmunoassay (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 300ul phosphate buffered saline (**PBS**) gel, added duplicate aliquots of 100ul of each sample to assay tubes, and used an additional 50ul sample to determine extraction efficiencies. To each assay tube and to additional tubes containing a graduated curve of the target hormone, we added 50ul of tritiated testosterone (approximately 10,000cpm) and 50ul of rabbit-derived anti-testosterone

antibody (MP Biomedicals, Solon, OH USA, cat# 07-189016). After an incubation time of 16h, we added 500ul of a dextran-coated charcoal solution to each tube, incubated tubes for 10min, and centrifuged tubes at 4,063 x g for 10min at 23 degrees Celsius to separate bound and free fractions. Supernatant was decanted into scintillation vials followed by the addition of 4ml of scintillation fluid and radioactivity was counted.

We quantified concentrations of progesterone and dihydrotestosterone in extracted samples using standard I-125 labeled radioimmunoassay kits (Diagnostic Systems Laboratories, Webster, TX USA, P: DSL-3900, DHT: DSL-9600). Seminal plasma estradiol concentration was evaluated in a single assay and was not found in detectable amounts, so estradiol concentrations were not quantified in blood plasma. All hormone values were corrected based on recovery efficiencies. Intra-assay 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 for both blood and seminal plasmas.

Experiment 2 – Progesterone-treated Semen

Progesterone was the predominant steroid hormone found in seminal plasma, which led us to test the effects of progesterone 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 (n=24). We vortexed each semen sample briefly to mix and then pipetted two aliquots of semen from every individual sample, 45ul each, into different tubes. One aliquot was treated with 0.4ng of P4 in 5ul diluent (Progesterone, Sigma-Aldrich, Inc, prod# P0130; 6h SemAid, PHL Associates, Inc, Davis, CA USA, ser#02112010) while the other was treated with

5ul of diluent only, resulting in a 50ul 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 50ul 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, as 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 progesterone treated or control treated sperm. Eggs laid prior to 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 for paired analyses (see additional details below).

Inner Perivitelline Layer Sperm Penetration Assay

The IPVL 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 may enter the SSTs, effectively travel through the reproductive tract, and penetrate the IPVL. Therefore, a high number of sperm holes in the IPVL indicates higher fertility (Bramwell et al., 1995; Christensen et al., 2006). For our study, the assay was modified from procedures described by Bramwell and colleagues (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 IPVL by blotting with a Kimwipe[®]. 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 IPVL was then placed on a microscope slide followed by the addition of 3-4 drops of 3% formaldehyde directly on the membrane and immediately decanted. Finally, the IPVL was stained with Schiff's reagent (Medical Chemical Corp., Torrance, CA USA, cat# 804A) and set to dry. The IPVL holes were counted using a Zeiss Axio Observer (Carl Zeiss Inc, Thornwood, NY USA) at a magnification of 100x. The blastoderm area was located on each slide and centered in the field of vision (area=785 μm^2) and each hole within this area was counted (Fig. 3A, 3B).

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 only conducted for hormones that were found in seminal plasma in measurable amounts (including P4, T, and DHT).

To determine whether progesterone elevation 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. While we would have liked to utilize a design to control for hen quality by treating each hen with each of our two treatments, we chose not to do so because we do not know the long-lasting effects of seminal plasma progesterone on the hen reproductive tract. All analyses were performed

using Statview Statistical Software (SAS Institute, Cary, NC USA), and significance values were assigned at $P < 0.05$.

Results

Progesterone was the most predominant hormone of the four reproductive steroid hormones quantified in rooster seminal plasma, and ranged from 1.85 to 4.89 ng/ml ($\bar{x} = 3.38 \pm 0.18$). Testosterone and dihydrotestosterone were present in lower concentrations ranging from 0.01 to 3.71 ng/ml ($\bar{x} = 1.05 \pm 0.24$) and 0.43 to 1.13 ng/ml ($\bar{x} = 0.64 \pm 0.04$) respectively (Fig. 1.3). 17β -Estradiol was undetectable in seminal plasma and was therefore removed from further analyses. In addition, progesterone in seminal plasma was significantly greater than concentrations in the blood ($t = 8.78$, $p < 0.0001$). Testosterone and dihydrotestosterone differed from progesterone in that blood plasma concentrations significantly exceeded those of seminal plasma (T: $t = -5.07$, $p < 0.0001$; DHT: $t = -3.56$, $p = 0.002$)(Fig. 1.3).

Progesterone concentrations in blood plasma were positively correlated to semen progesterone concentrations ($p = 0.037$, $r^2 = 0.24$)(Fig. 1.2). Neither blood plasma testosterone nor dihydrotestosterone were correlated to those of seminal plasma (T: $p = 0.571$, $r^2 = 0.02$; DHT: $p = 0.927$, $r^2 = 0.001$).

Progesterone treatment significantly impaired the ability of sperm to penetrate the IPVL. Hens inseminated with progesterone-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 ($\bar{x} = 210.3 \pm 45.8$)($p = 0.034$) (Fig. 1.1).

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 progesterone in seminal and blood plasma were positively correlated. In addition, progesterone concentrations were significantly higher in seminal plasma when compared to blood plasma, which contrasts findings in mammals where concentrations of all steroids so far detected in seminal plasma in humans were significantly lower than corresponding blood levels; these include testosterone, dihydrotestosterone, androstenedione, estradiol, progesterone, and cortisol among others (Pohanka et al., 2002). To our knowledge, this is the first study to measure progesterone content of avian seminal plasma, and it is unclear why progesterone 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 dihydrotestosterone were also present in seminal plasma in lower amounts than progesterone, 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 there was no correlation between blood or seminal plasma testosterone concentration and sperm quality in turkeys; however a study with Leghorns cockerels showed a correlation between the testosterone levels in seminal plasma and ejaculate volume and sperm concentration (Zeman et al., 1983). Liu and colleagues (2008) proposed the use of a

balanced ratio of testosterone and estradiol levels as an indicator for evaluating the reproductive potential for individual ganders. Further research is needed to determine whether any of the three hormones we measured in seminal plasma could be used as indicators of reproductive potential in a similar manner. Although concentrations of 17β -estradiol in rooster seminal plasma were below detection limits in this study, previous work has shown that P450 aromatase converts androgens to estrogen in testicular germ cells as well as in the epididymis of roosters (Kwon et al. 1995). The absence of seminal plasma estrogen may be due to a down regulation of aromatase activity in our birds or due to the fact that males may produce and use estrogens rather than aromatase estrogen for ejaculation.

We also showed that experimentally elevated concentrations of progesterone in seminal plasma inhibited inner IPVL sperm penetration. The mechanism by which progesterone acts to impair sperm function in roosters remains to be determined. In mammals, progesterone induces sperm capacitation, motility, and can potentiate the acrosome reaction (Osman et al., 1989; Baldi et al., 2009; Cheng et al., 1998; Wu et al., 2006; Sabeur et al., 1996). However, Lemoine et al. (2008) showed that the progesterone dose that induces the acrosome reaction in mammalian species had no effect in chickens, and here we show that a lower dose of progesterone (0.08ug/ml compared to doses of 3-10ug/ml in human studies) instead exerts detrimental effects. The reason for this decline in fertilization capability is still unclear, though there are multiple sites where progesterone may exert its effect.

Since the IPVL sperm hole penetration measurement occurs at the final stage of the fertilization process, progesterone could have acted by affecting mobility and/or

overall viability of sperm during our study. First, progesterone may affect fertility at the level of individual sperm cells. Progesterone is known to act on human spermatozoa through an unidentified membrane receptor (Shah et al., 2005), so it is possible that avian sperm may have a similar receptor; however, the presence of this receptor and its effects when activated by progesterone in avian systems remains untested. Additionally, the presence of progesterone receptors in the SSTs in the female may allow for regulation of sperm storage function if seminal plasma progesterone reaches the SSTs (Yoshimura et al., 1999). Whether progesterone in seminal plasma reaches the SSTs needs to be tested to determine whether this potential mechanism is plausible. If progesterone in seminal plasma does interact with receptors in the hen, the actions of seminal fluid could also reach beyond the site of insemination and exert effects on other reproductive functions in the female body, such as at the point of gamete fusion or even during embryonic development as has been suggested in mammals by Robertson (2006) and O'Leary et al. (2006).

There are numerous factors that influence the fertility of avian eggs. A full understanding of how hormones in seminal plasma affect fertility may provide insight into flock maintenance strategies that could optimize fertility. For example, based on our results, keeping progesterone concentrations low in roosters could potentially improve flock fertility. Additional tests need to be done to confirm whether semen collected from roosters with naturally high concentrations of progesterone exhibit similar inhibitions in the ability to penetrate the egg compared to roosters with naturally low concentrations of progesterone. In addition, while we show that progesterone in seminal plasma influences

sperm hole penetration, additional studies must be done to test whether this effect translates into a progesterone-mediated decline in the number of viable chicks produced.

Acknowledgements

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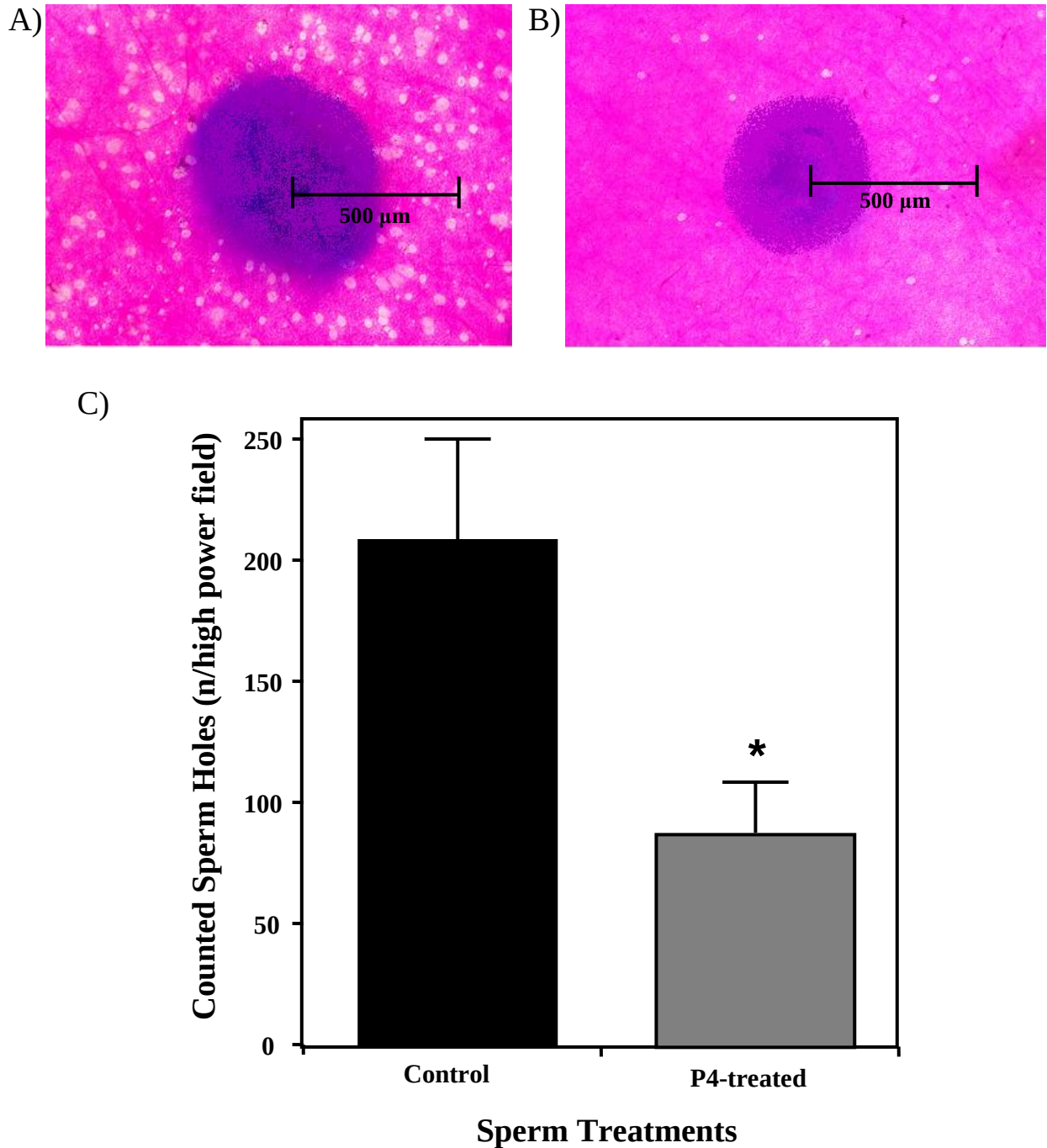


Figure 1.1. Penetration holes in the perivitelline layer surrounding the germinal disc of the chicken egg. A) Representative picture from a control-treated sperm insemination with many points of hydrolysis on the inner perivitelline layer. B) Representative picture from a progesterone-treated sperm insemination with few points of hydrolysis in the inner perivitelline layer. C) The number of sperm holes on the perivitelline layer created by sperm in semen treated with either 0.4 mg of progesterone (P4) in 5 μ l of semen diluent or a control diluent vehicle (n=15). High power field area = 785 μ m². The asterisk indicates a significant difference between treatment groups ($P < 0.05$).

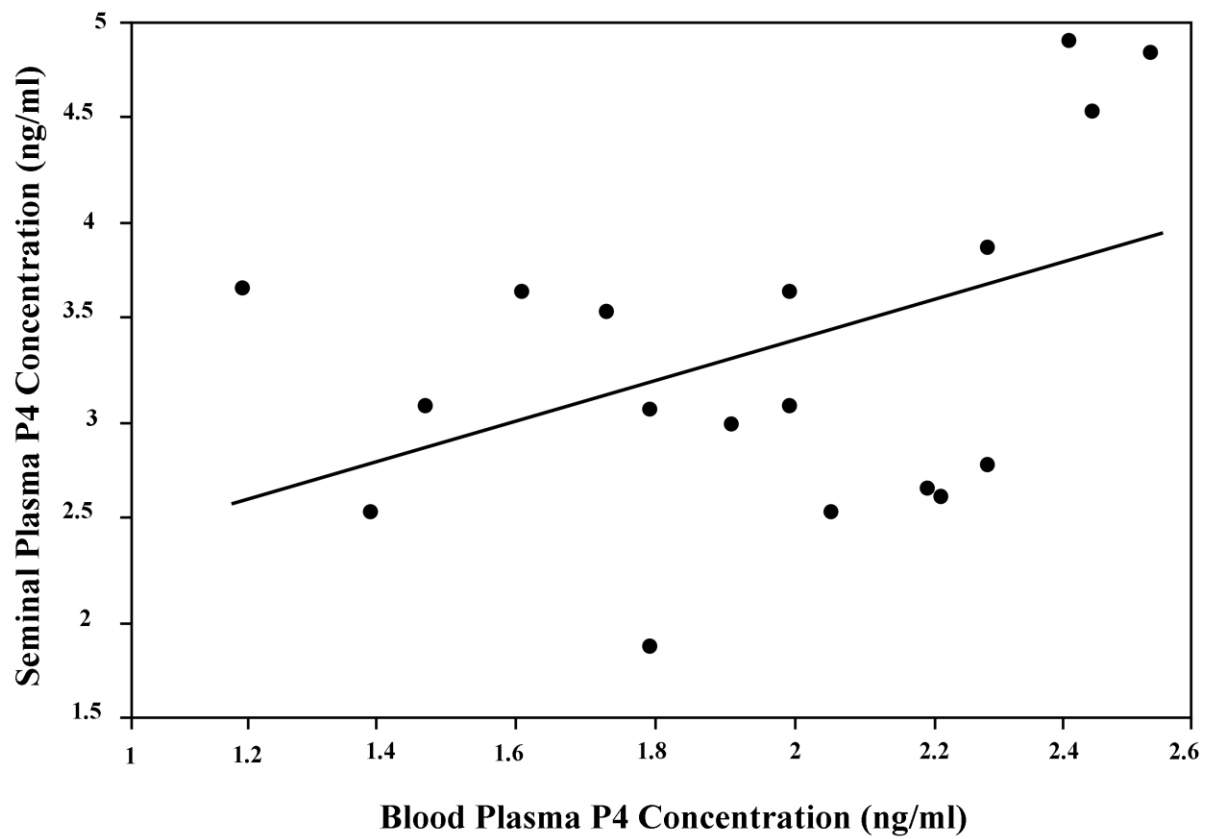


Figure 1.2. Comparison of blood and seminal plasma progesterone (P4) concentrations using a simple regression analysis. Progesterone concentrations in seminal and blood plasma were significantly correlated.

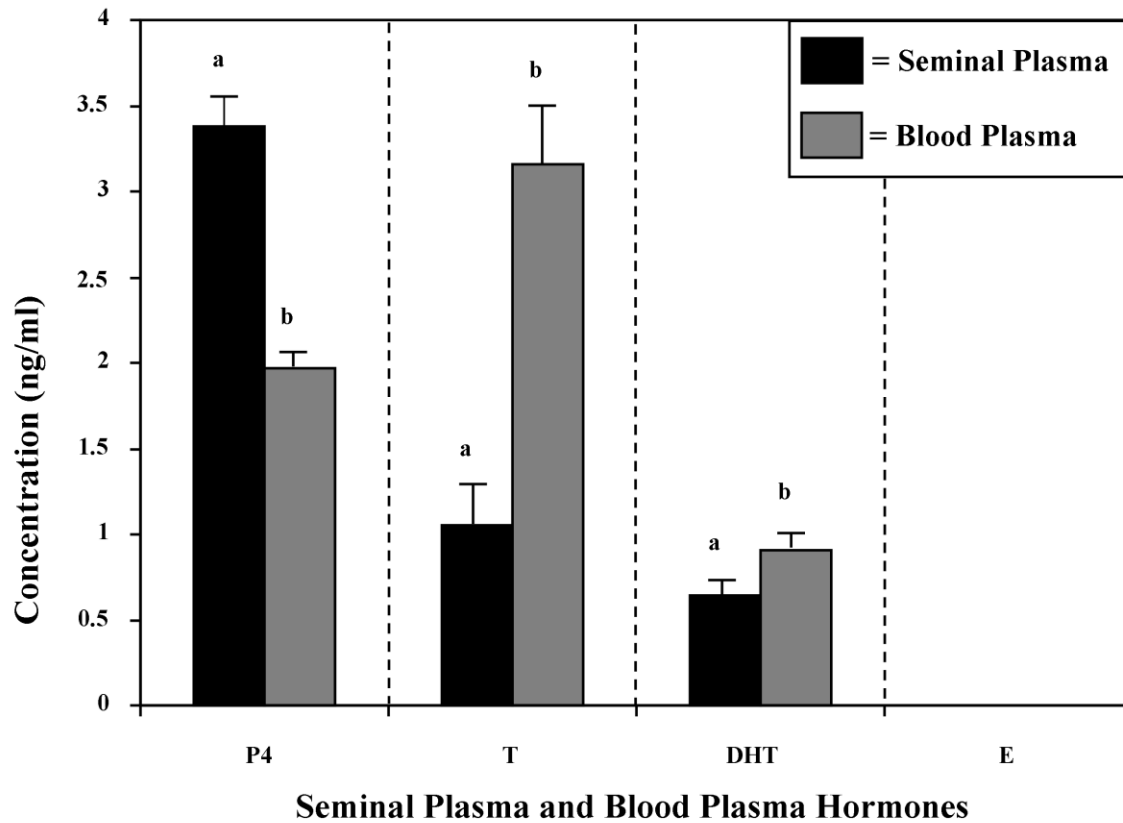


Figure 1.3. The mean ($\pm$ SE) concentrations of progesterone (P4), testosterone (T), and dihydrotestosterone (DHT) measured in both seminal and blood plasma. Different letters above the error bars denote statistical differences in concentrations of hormones between seminal and blood plasmas ($P < 0.05$). Estradiol (E) was undetectable in blood and was therefore not measured in seminal plasma.

CHAPTER 3

HORMONE CONTENT OF ROOSTER SEMINAL PLASMA PREDICTS SPERM FUNCTION¹

¹ Anderson, E.A., A.V. Goedeke, J.L. Wilson, and K.J. Navara. Submitted to *Physiological and Biochemical Zoology*, October 17, 2011.

Abstract A large body of research has focused on identifying the presence and function of hormonal contributions passed along with gametes by female birds, but the hormonal content passed along with gametes by males continues to be relatively unexplored. In previous work, we found that avian semen contains quantifiable concentrations of reproductive steroids, and that progesterone, in particular, exists at high concentrations and exerts an inhibitory effect on the fertilization process. Such an effect could occur either through influences on sperm function or through a functional change in the hen triggered by exposure to seminal plasma progesterone. In the current study, we examined how concentrations of progesterone, as well as testosterone, relate to functional characteristics of sperm, including sperm mobility, concentration, and viability. Progesterone concentrations in both blood and seminal plasma were inversely related to sperm mobility. In addition, seminal plasma testosterone concentrations were inversely related to sperm concentration. Thus, hormones deposited into seminal plasma may have potent influences on sperm function, which could ultimately impair fertility. Implications from this research provide insight to the function and potential adaptive value of steroid hormones in male reproduction.

Key words: progesterone, testosterone, rooster, semen quality

Introduction

A large body of research currently focuses on the idea that female birds may adaptively allocate hormones and other physiologically relevant substances to eggs to enhance offspring quality and survival (reviewed in Groothuis and Schwabl 2007; Gil 2008; Navara and Mendonça 2008). However, the content of fluids contributed with gametes by the male has received less attention.

Semen contains spermatozoa along with the surrounding seminal fluid that has all the components necessary for the survival of sperm cells. Semen provides optimal conditions for sperm survival as it contains antioxidant and immunosuppressive substances that protect spermatozoa from damage in the hen reproductive tract (Pohanka et al. 2002). It is known that male birds can vary the concentrations of antioxidants and immunomodulators they deposit into semen (Khan 2011), and it has been suggested that these substances may provide a link with male ornamentation to help maintain the honesty of sexual signals (Blount et al. 2001; Helfensten et al. 2010). However, the hormonal content of avian semen as well as the potential adaptive value of adjusting hormone content of seminal plasma remains relatively unstudied.

Previous studies have shown that roosters deposit significant quantities of reproductive steroid hormones in seminal plasma. Testosterone is an obvious hormone of interest because it exists in measurable and variable quantities in seminal plasma (Anderson and Navara 2011) and the process of spermatogenesis relies heavily on testosterone (Sharp 1975; Tan et al. 2005). Additionally, androgen concentrations in blood have been used in poultry to estimate fertility (Rosenstrach et al. 1988; Biswas et al. 2007). However, the function of testosterone in seminal plasma is less clear, because previous studies in turkeys and chickens failed to find relationships

between testosterone in blood or seminal plasma and sperm function (Zeman et al. 1983; Cecil and Bakst 1986; Cecil and Bakst 1988).

Progesterone is another hormone of interest because it exists at the highest concentrations among all hormones studied in rooster seminal plasma (Anderson and Navara 2011). Elevation of progesterone concentrations in semen within the physiological range significantly impaired the ability of sperm to penetrate the egg (Anderson and Navara 2011). These results suggest an inhibitory role of progesterone on the fertilization process, though the mechanism by which progesterone acts during reproduction in roosters remains unclear.

To fully understand the adaptive function of reproductive hormones to birds, further knowledge of the mechanism by which these hormones act is crucial. Both testosterone and progesterone are produced by the male in the vicinity of sperm maturation and also regulate important behaviors and sexual signals when they pass into circulation. Steroid hormone receptors exist on mammalian sperm and have multiple effects on sperm function in mammals (Baldi et al. 1995; Sabeur et al. 1996; Luconi et al. 1999; Shah et al. 2005; Solakidi et al. 2005). Similar receptors have not yet been identified on avian sperm, however Sexton (1974) showed that the metabolism of chicken spermatozoa is influenced by various steroid hormones such as androstanediol, estradiol, progesterone and testosterone. This ability of sperm to respond to steroid hormones suggests that hormones that exist in seminal plasma could influence the performance of avian sperm. In the current study, we tested whether natural concentrations of testosterone and progesterone in seminal plasma relate to three measures of sperm quality, including sperm concentration, mobility, and viability. Based on the previous finding that treatment of avian sperm with physiological concentrations of progesterone decreases the number of sperm hydrolyzing the egg, we predicted that progesterone would negatively correlate

to semen quality measures. Since testosterone has been used as an indicator of avian fertility and plays an important role in sperm maturation, we predicted that testosterone would positively correlate to measures of semen quality.

Materials and Methods

Experimental Birds

Sixteen Single Comb White Leghorn roosters of reproductive age were housed in individual cages and given *ad libitum* access to food and water. Birds were fed an industry standard diet and maintained on a standard breeding light schedule (14hL: 10hD). A blood sample was taken from the brachial vein directly before semen collection for each of the roosters so that hormonal content of seminal and blood plasma could be compared. Both blood and semen samples were collected from each bird within three minutes of initial handling to ensure that measurements reflect baseline hormone concentrations without handling stress (Wingfield et al. 1982; Romero and Romero 2002). Alliquots of semen were subsequently used for measures of sperm quality and hormone quantification.

Sperm Quality Measurements

A clean semen sample, free of contaminants, was collected from each rooster via abdominal massage for use in both sperm quality measurements and quantification of hormone content. Both semen concentration and mobility, a measure of progressive forward motion, were measured using an assay outlined in Froman and Feltmann (1998). Only one ejaculate was collected at a time and was evaluated within 7 minutes. This fast processing of sperm prevented the samples from deteriorating over time. Immediately after collection, the fresh semen sample was held in a prewarmed test tube (41°C). Sperm concentration was then determined by diluting 10ul of neat semen in 2ml of NaCl solution (3% w:v) in a standard polystyrene cuvette and

measured with a Barnsted-Turner SP-830 Spectrophotometer at 550 nm. The unknown concentration was then calculated by comparing to a standard curve. Subsequently, mobility was determined using a modified version of the Accudenz assay© designed by Froman (1996). Although this technique was initially validated using Accudenz©, we utilized Nycodenz, which is equivalent in composition (Sbracia et al. 1996) and produces similar results to those obtained by Accudenz© (E. Anderson, unpublished data). A 50ul volume of the semen sample was diluted to 6×10^6 sperm per milliliter with 50mM *N*-tris-[hydroxymethyl]methyl-2-amino-ethanesulfonic acid, pH 7.4 containing 128mM NaCl and 2mM CaCl₂ (Animal Reproduction Systems, cat# PMB-OF-02). A 60ul volume of this diluted sperm solution was overlaid onto 600ul pre-warmed Nycodenz (6%) in a semi-micro cuvette and incubated at 41°C for 5 minutes after which we measured the optical density of the Nycodenz layer; this absorbance value is representative of the sperm mobility score for that individual as it reflects the amount of sperm that actively penetrated the viscous medium. This procedure was repeated for each of the 16 roosters. Following measurement of concentration and mobility, sperm viability was determined using a nigrosin/eosin (N/E) stain for determining live/dead and abnormal sperm counts (Dott and Foster 1972; Bakst and Cecil 1997). Neat semen was diluted 1:2 in semen diluent (6h SemAid, PHL Associates, Inc, Davis, CA USA, ser#02112010). Diluted semen was then combined with 375ul N/E stain and mixed thoroughly by gentle inversion for 2 minutes. Using a clean pipette tip, 20ul of the sperm-stain mixture was smeared along a microscope slide and 300 sperm cells per slide were counted under light microscopy with an oil immersion lens (magnification 100X). The N/E stain procedure is based upon the principle that viable sperm have intact cell membranes that are able to exclude the eosin stain; therefore sperm were considered live, or viable, when they were not stained. Magenta-stained sperm cells were

indicative of dead, or nonviable, sperm that possess permeable cell membranes and permit eosin to enter and stain the sperm cell. A percentage of live:dead sperm cells was counted for the first 300 sperm cells observed in semen samples from each of the roosters (n=16).

Hormone Assays

All semen and blood samples were centrifuged and the supernatant was retained for hormone analysis. We measured concentrations of progesterone and testosterone in both seminal plasma and in circulation. We first extracted each hormone from both seminal and blood plasma using diethyl ether and separated the hormones by liquid column chromatography according to methods described by Schwabl (1993). Prior to extraction, we added 1000cpm each of tritiated androstenedione and testosterone for later calculation of recovery efficiencies. In previous studies, we have found that radiolabelled progesterone 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 progesterone, androstenedione was used to calculate average progesterone recoveries from columns. Concentrations of testosterone were determined using a standard competitive binding radioimmunoassay (RIA) as described by Wingfield and Farner (1975) and Etches (1976) and validated on seminal plasma (E. Anderson, unpublished data). Blood plasma and seminal plasma hormones were quantified together in a single assay for each hormone. Briefly, we resuspended extracted samples with 300ul phosphate buffered saline (PBS) gel, added duplicate aliquots of 100ul of each sample to assay tubes, and used an additional 50ul sample to determine extraction efficiencies. To each assay tube and to additional tubes containing a graduated curve of the target hormone, we added 50ul of tritiated testosterone (approximately 10,000cpm) and 50ul of rabbit-derived anti-testosterone antibody (MP Biomedicals, Solon, OH USA, cat# 07-189016). After an incubation time of 16h, we added

500ul of a dextran-coated charcoal solution to each tube, incubated tubes for 10min, and centrifuged tubes at 5,200 rpm for 10min to separate bound and free fractions. Supernatant was decanted into scintillation vials followed by the addition of 4ml of scintillation fluid and radioactivity was counted.

We quantified concentrations of progesterone in extracted samples using an Enzyme Immunoassay kit (Cayman Chemical, Ann Arbor, MI USA, #582601), which we validated in both blood and seminal plasma (E. Anderson, unpublished data). All hormone values were corrected based on recovery efficiencies. Intra-assay variations for progesterone and testosterone were 3.63 and 3.80 respectively. Average recoveries were 33.7% for progesterone and 59.5% for testosterone.

Statistical Analyses

We compared mean concentrations of each hormone between blood and seminal plasma using paired t-tests. We then tested for correlations between blood and seminal plasma hormone concentrations by performing a simple regression analysis for each hormone. Because sperm viability is calculated as a percentage, sperm viability data were arcsin transformed. Distributions of all variables were tested for normality using the Shapiro-Wilk test. Testosterone in seminal plasma and progesterone in blood plasma were non-normally distributed and logarithmically transformed.

To determine whether steroid hormones influenced semen quality we performed stepwise regressions for each semen quality measure to determine which seminal and blood plasma hormones best explained variation in semen quality measures. Statistics presented are the results of subsequent simple regressions conducted for each significant contributor. All analyses were

performed using Statview Statistical Software (SAS Institute, Cary, NC USA), and significance values were assigned at $p < 0.05$.

Results

As in previous studies, progesterone was the most predominant hormone of the two steroid hormones quantified in rooster seminal plasma, ranging from 1.09 to 4.18 ng/ml ($\bar{x} = 2.63 \pm 0.25$) while seminal testosterone was present in lower concentrations ranging from 0.00 to 1.67 ng/ml ($\bar{x} = 0.73 \pm 0.16$). In circulation, however, testosterone concentration exceeded progesterone ranging from 0.02 to 10.68 ng/ml ($\bar{x} = 4.45 \pm 0.69$) compared to progesterone ranging from 1.04 to 4.54 ng/ml ($\bar{x} = 2.35 \pm 0.31$)(Figure 1). In addition, progesterone concentrations in seminal and blood plasma were similar ($t=0.81$, $p=0.43$) while testosterone concentrations in blood plasma significantly exceeded those in seminal plasma ($t=-5.187$, $p=0.0001$)(Figure 1). Neither blood plasma progesterone nor testosterone were correlated to concentrations in seminal plasma (progesterone: $p=0.33$, $r^2=0.07$; testosterone: $p=0.97$, $r^2=8.38 \times 10^{-5}$).

Stepwise regressions showed that hormone concentrations in blood and seminal plasma significantly predicted sperm quality. Progesterone concentrations in both blood plasma and seminal plasma were inversely related to sperm mobility ($p=0.009$, $r^2=0.39$; $p=0.03$, $r^2=0.28$ respectively)(Figure 2a,b). No significant relationships were found between blood progesterone to either sperm concentration ($p=0.98$, $r^2=6.58 \times 10^{-5}$) or sperm viability ($p=0.98$, $r^2=6.47 \times 10^{-5}$). Likewise, seminal progesterone did not relate to either concentration ($p=0.85$, $r^2=0.003$) or viability ($p=0.49$, $r^2=0.03$).

Testosterone in seminal plasma was inversely related to sperm concentration ($p=0.04$, $r^2=0.25$)(Figure 3); however this relationship did not hold true when comparing blood

testosterone to sperm concentration ($p=0.89$, $r^2=0.001$). There were no further relationships found between blood or seminal plasma testosterone to either sperm mobility or viability (blood: mobility, ($p=0.79$, $r^2=0.005$), viability, $p=0.79$, $r^2=0.005$, semen: mobility, $p=0.07$, $r^2=0.21$, viability $p=0.57$, $r^2=0.02$).

Discussion

We have shown that avian seminal and blood plasma contain measurable quantities of the reproductive steroid hormones, progesterone and testosterone, and that these hormones were significant predictors of semen quality measures. Male roosters with elevated progesterone in either their semen or blood produce sperm with lower mobility, and birds with elevated testosterone concentrations in seminal plasma produce ejaculates with lower sperm concentration.

The effects of progesterone in this study are in the predicted direction, given that progesterone-treated sperm were less likely to penetrate the egg in the hen (Anderson and Navara 2011). Precisely how progesterone inhibits sperm mobility is still unclear. Progesterone is known to act on human spermatozoa through an unidentified membrane receptor and participates in various reproductive functions such as acrosome reaction, capacitation and hyperactive motility of spermatozoa (Shah et al. 2005), so it is possible that avian sperm may have a similar receptor; however, the presence of this receptor and its effects when activated by progesterone in avian systems remains untested. Another explanation for the inhibitory effect of progesterone on sperm mobility may be due to oxidative stress. Both spermatozoa and seminal plasma contain reactive oxygen species that may cause cell damage including ATP depletion and loss of motility and viability (Lamirande et al. 1997). Glucocorticoids such as testosterone and progesterone are known to impair antioxidant defenses or directly induce oxidative stress in various tissues (Von

Shantz et al. 1999). Specifically, Zhu et al. (1997) found that progesterone and testosterone promoted damaging lipoprotein oxidation on placental cells. Perhaps progesterone is acting as a cytotoxin, preventing sperm cells from forming or functioning as they should. Sperm mobility has been shown to be the primary determinant of fertility in the domestic fowl (Froman et al. 1999). Birkhead et al. (1999) also found that more progeny were sired by males with higher sperm mobility in relation to males with lower mobility. As a result, the effects of progesterone shown here can have substantial impacts on reproductive success.

The negative relationship between testosterone concentrations in seminal plasma and sperm concentration was unexpected. Given the value of androgens to male reproduction we predicted a positive relationship between testosterone and semen quality. However, there is much variation and controversy as to the function of testosterone in male reproduction. While testosterone is necessary for spermatogenesis and for processes of sperm maturation, it has been shown in humans that pharmacological doses of androgens resulted in lower sperm quality (Torres-Caleja et al. 2001). A study with red deer mating systems found a significant relationship between circulating testosterone and proportion of normal spermatozoa (Gomendio et al. 2007). Thongtip et al. (2008) found that Asian elephants with higher concentrations of testosterone in seminal plasma compared to blood plasma had lower-motile ejaculates compared to bulls with higher testosterone in their blood. Based on these variable findings, it appears testosterone may have different effects on reproduction in different species and warrants future research to understand the mechanism by which testosterone influences fertility. There are also discrepancies as to the relationship between sperm concentration and fertility. Kamar (1960) and Boone (1968) found significant positive correlation coefficients between concentration of spermatozoa and fertility following artificial insemination; however, Bilcik et al. (2005) detected

broiler breeder males with high sperm concentration had lower fertility. Due to the lack of strong evidence one way or another, it remains difficult to draw conclusions as to the effect of testosterone on fertility in our study. In addition, it remains necessary to examine the effects of testosterone and progesterone on sperm mobility and concentration in an experimental rather than a correlative context to ensure that hormones are, in fact, exerting effects at this level, rather than reacting with sperm function to another unknown variable.

Multiple studies have addressed the hormonal constituents of seminal plasma in the reproductive system of mammals (Killian et al. 1993; Pohanka et al. 2002; Poiani 2006), but to our knowledge, ours is the first to examine the relationship between hormone content of seminal plasma and sperm function in birds. Given the negative effects of progesterone on sperm function and fertility and the fact that androgens are largely responsible for maintaining spermatogenesis in the testes (Tan et al. 2005), it is still unclear why progesterone exists in such high concentrations in seminal plasma. Conventionally, seminal fluid is thought to function simply as a means of transport and survival medium for spermatozoa traversing from the male to the female reproductive tract, but much more is now known about the influences of seminal fluid on reproduction. It is well established that male birds have the ability to promote fertilization success by adjusting the fertilizing ability of their sperm, though the mechanisms underlying such an adjustment remain unknown (Cornwallis and Birkhead 2007). Males adjust the quality of their sperm either (i) directly, by distributing sperm to different quality ejaculates and/or (ii) indirectly, by allotting seminal fluid (non-sperm) components to ejaculates that sequentially influence sperm performance by changing the resources and environmental conditions available for sperm to use (Poiani 2006; Cornwallis and O'Connor 2009). In promiscuous mating systems, reproductive success is restricted by the costly production of ejaculates (Dewsbury 1982).

Perhaps birds maintain hormones and metabolites in the seminal fluid at a level of least energetic cost until a proper social cue arises which would change allocation of varying hormone concentrations. Alternatively, given that concentrations of blood plasma were also negative predictors of sperm mobility, it is possible that progesterone represents a link with sexual signals that could help maintain signal honesty. Additional work in this area would help to better understand the underlying mechanisms that enable ejaculate modulation and to determine how hormonal control of male ejaculation can have long term effects on reproductive fitness. Future studies should examine how seminal plasma hormones vary in relation to male and female quality, as well as potential costs and benefits to the rooster when depositing hormones into seminal plasma.

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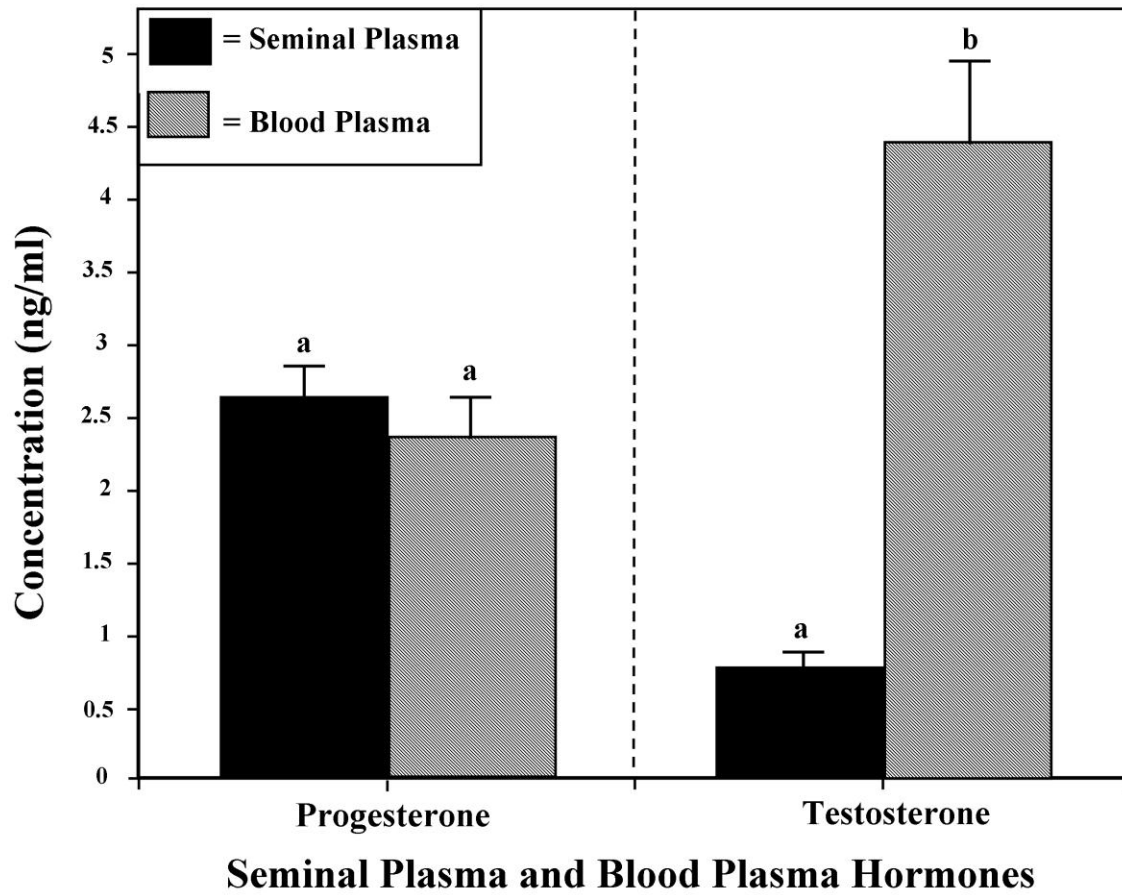


Figure 2.1. Concentrations of progesterone and testosterone in seminal and blood plasma. Depicted are the mean ($\pm$ SE) concentrations of progesterone and testosterone measured in both seminal and blood plasma. Different letters above the error bars denote statistical differences between concentrations of hormones between seminal and blood plasma ($P < 0.05$).

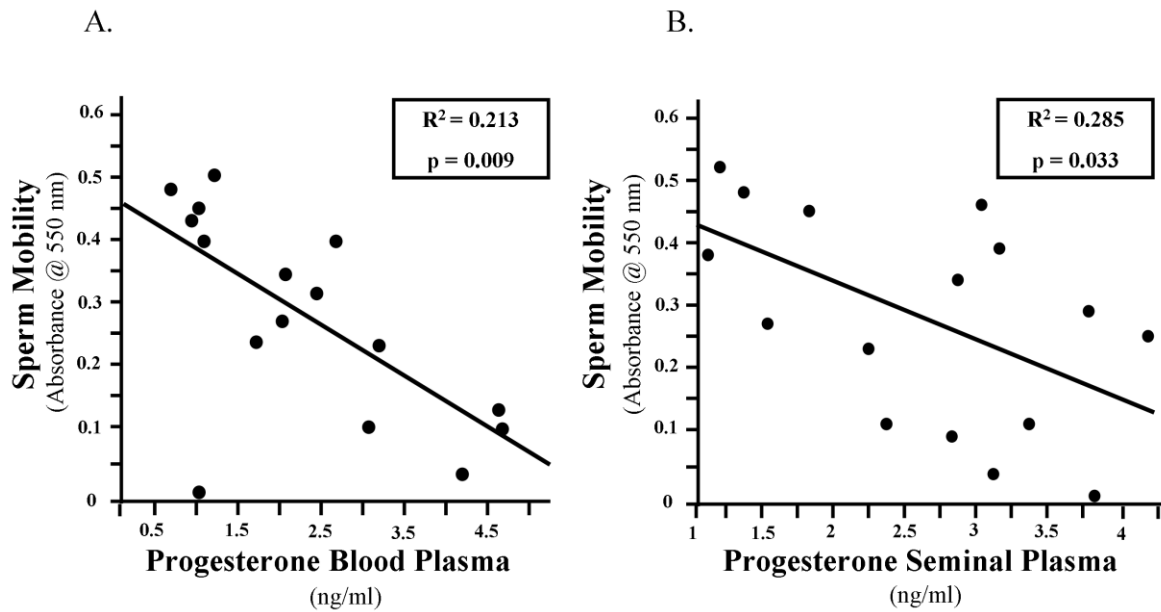


Figure 2.2. Progesterone relationship to sperm mobility. Shown here are the relationships between progesterone and sperm mobility using simple regression analyses. A) Progesterone in rooster circulation is inversely related to sperm mobility ($p=0.009$, $r^2=0.39$). B) Likewise, progesterone concentration in rooster seminal plasma is inversely related to mobility ($p=0.03$, $r^2=0.28$). Non-log transformed values are presented here for ease of interpretation; log-transformed values were used for statistical purposes.

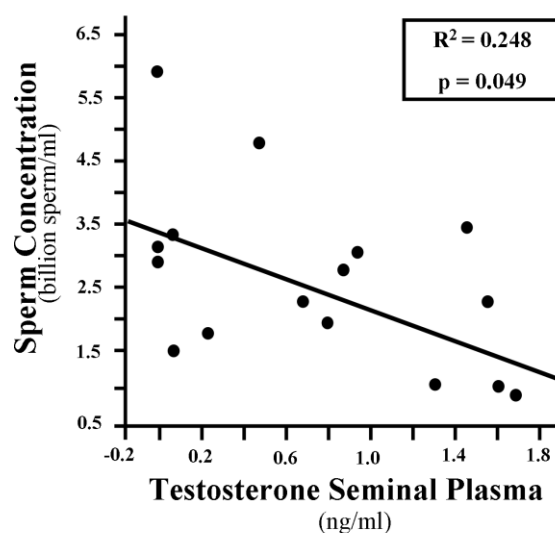


Figure 2.3. Testosterone relationship to sperm concentration. Included here is the relationship between testosterone and sperm concentration using simple regression analyses. Blood testosterone is inversely related to sperm concentration ($p=0.04$, $r^2=0.25$). Non-log transformed values are presented here for ease of interpretation; log-transformed values were used for statistical purposes.

CONCLUSIONS

Understanding the role of steroid hormones in avian male reproduction would make an important contribution to improving the success of mating systems, both in commercial livestock industries, as with poultry, or with natural systems. There are multiple factors that influence the fertility of avian eggs. While a wealth of literature has focused on maternal contributions to reproduction, the reproductive contributions and role of those contributions from males have yet to be fully explored. Much research addressing hormonal constituents of seminal fluid have dealt with androgens in relation to functions of the male reproductive system, but the role of progesterone in male reproduction has not been extensively examined, especially in birds. My research focused on evaluating the concentrations of various steroid hormones in rooster seminal plasma, and further, elucidating the role of these hormones on sperm function and fertility.

Our initial study examined the presence of reproductive steroid hormones (testosterone, dihydrotestosterone, progesterone, and estrogen) in rooster seminal plasma and in circulation, and we showed that experimentally elevated concentrations of progesterone in seminal plasma inhibited inner perivitelline layer sperm penetration. Progesterone was the most abundant reproductive steroid hormones in rooster seminal plasma, but the reason for this is unclear, particularly given that androgens are largely responsible for regulating functions involved with male reproduction. Results from this study revealed that progesterone acts to impair sperm performance in some way, but given that the perivitelline layer sperm hole penetration measurement occurs at the final stage of the fertilization process, there are several sites where progesterone may exert its effect.

In an attempt to further elucidate where progesterone acts to impair sperm function, we investigated how natural concentrations of progesterone related to different measures of semen quality. We found that roosters with elevated progesterone in either their semen or blood produced sperm with lower mobility. This was not surprising, given that progesterone-treated sperm were less likely to penetrate the egg in the hen in our first study. In addition, birds with elevated concentrations of testosterone in seminal plasma produce ejaculates with lower sperm concentration, which was unexpected. Though there is much controversy as to the function of testosterone in male reproduction, we predicted a positive relationship between testosterone and semen quality, given the impact of androgens on male reproduction.

Precisely how progesterone inhibits sperm mobility remains uncertain. It is possible that progesterone may act on avian sperm through an unidentified membrane receptor, which has been documented only in human spermatozoa and has yet to be even tested for in birds (Shah et al., 2005). Alternatively, testosterone and progesterone are known to impair the antioxidant defenses in various tissues (Von Shantz et al., 1999). Since both spermatozoa and seminal plasma contain reactive oxygen species that may cause cell damage such as loss of motility and viability (Lamirande et al., 1997), progesterone may act as a cytotoxin, preventing sperm cells from functioning as they should. The importance of progesterone's inhibitory effect on sperm mobility may have substantial impacts on reproductive success given that sperm mobility has been shown to be a primary determinant of fertility in the domestic fowl.

The reason for the negative relationship between testosterone and sperm concentration is even less clear, given that testosterone is critical for the process of spermatogenesis (Sharp, 1975; Tan et al., 2005). There are numerous discrepancies as to the relationship between testosterone and sperm quality. Blood androgen concentrations have been used in poultry to

estimate fertility (Rosenstrach et al., 1988; Biswas et al., 2007), but this may not be a reliable predictor of fertility as work in turkeys and chickens failed to find relationships between testosterone and sperm function (Zeman et al., 1983; Cecil and Bakst, 1986; Cecil and Bakst, 1988). In humans, however, it has been shown that pharmacological doses of androgens resulted in lower sperm quality (Torres-Caleja et al., 2001). Further, a study with red deer mating systems found testosterone to be closely associated with testes size and sperm production, but only weakly associated with other variables of semen quality (Gomendio et al., 2007). There are also inconsistencies defining the relationship between sperm concentration and fertility. Based on these variable findings, it appears testosterone may have different effects on reproduction in different species and warrants future research to understand the mechanism by which testosterone influences fertility.

While we have examined the relationships between hormones and sperm function, there are many unanswered questions. We found correlations between hormones and semen quality measures; however, correlation does not necessarily mean causation. It would benefit to study the effects of progesterone and testosterone on sperm mobility and concentration in an experimental situation rather than a correlative one to verify that hormones are, in fact, exerting effects at this level. Just because we observed relationships between semen quality and hormones, this may not be the mechanism preventing fertilization. We must examine other possibilities such as female influences as well. It has been demonstrated in mammals that the actions of seminal fluid exert effects on reproductive functions in the female body, such as during ovulation, gamete fusion or even embryonic development (O'Leary et al., 2006; Robertson, 2006). The presence of progesterone receptors in the SSTs of the hen may allow for regulation of sperm storage function if seminal plasma reaches the SSTs (Yoshimura et al.,

1999); therefore, the possibility of seminal plasma reaching the SSTs needs to be tested to determine whether this potential mechanism is plausible. If hormones in seminal plasma in fact interact with receptors in the hen, the actions of seminal fluid could also reach beyond the site of insemination.

Multiple studies have addressed the hormonal constituents of seminal plasma in the mammalian reproductive system (Killian et al., 1993; Pohanka et al., 2002; Poiani, 2006), but to our knowledge, ours is the first to examine the relationship between hormone content of seminal plasma and sperm function in birds. While we show that progesterone in seminal plasma influences sperm hole penetration, additional studies should be done to test whether this effect translates into a progesterone-mediated decline in the number of viable offspring hatched. It is also well-known that male birds are capable of promoting fertilization success by adjusting the fertilizing ability of their sperm, though the mechanisms underlying such an adjustment remain unknown (Cornwallis and Birkhead, 2007). Additional work in this area would help us to better understand the underlying mechanisms that permit ejaculate modulation and to determine how hormonal control of male ejaculation can have long term effects on reproductive fitness.

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