STRATEGIES FOR IMPROVING BROILER BREEDER ROOSTER FERTILITY

by

ZACHERY RYAN JARRELL

(Under the Direction of Andrew P. Benson)

ABSTRACT

Broiler breeder rooster fertility has been gradually declining as a result of intense selection for bodyweight gain in broiler chickens. The nature of this issue is multifaceted, stemming from genetic, physiologic and behavioral influencers resultant from selection focused on growth rate. The goal of this work was to investigate strategies for determining and improving broiler breeder rooster fertility.

First, sperm-associated antigen 6 (SPAG6) was characterized for its utility as a biomarker of rooster sperm mobility, due to its importance in sperm flagellar structure and previously being documented as associated with decreased rooster fertility. SPAG6 was found to be expressed throughout the male reproductive tract and to increase in abundance as sperm mature, localizing mainly in the flagellar axoneme but also in the midpiece and surrounding the acrosomal region of the sperm head. SPAG6 abundance was not found to be a biomarker suitable as a stand-alone predictor of sperm mobility.

Next, sperm protamination was investigated as an indicator of rooster sperm quality. In order to assess rooster sperm protamination, a combined method of sperm protamine extraction and heparin-stabilized gold nanoparticle-based quantification of sperm protamination was optimized. Sperm protamination was not found to be a strong indicator of sperm quality or

rooster fertility, despite its recent implication in determining fertility of various mammalian species.

Last, glyphosate-based herbicide (GBH) residues were studied for their effects as a reproductive toxicant in broiler breeder roosters. Residues GBH ingredients like those found in animal feeds produced from conventionally grown sources were shown to negatively impact rooster testis morphology and sperm mobility. Additionally, humic acids incorporated into rooster feeds remedied the effect of the glyphosate present in the feed. Through adsorption of glyphosate found on feeds, humic acids improved rooster testis morphology and sperm mobility, thereby improving the overall reproductive health of roosters fed conventionally grown feed crops.

The findings of factors influencing rooster fertility explored in this work may be applied in future broiler breeding and broiler breeder management programs in order to improve the productive efficiency of the broiler industry.

INDEX WORDS: broiler breeder rooster, male fertility, sperm physiology, sperm mobility, sperm protamination, glyphosate-based herbicide, reproductive toxicology

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DEDICATION

This dissertation is dedicated in memory of Johnny W. Goolsby, a man who cared far more for my success and fulfillment in life than I will ever be capable of comprehending.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

AN INTRODUCTION TO THE REPRODUCTIVE PHYSIOLOGY OF THE ROOSTER

Gross Reproductive Anatomy

In avian species, the male's reproductive organs are entirely inside the body cavity, with the testes attached to the dorsal wall and flanking the vertebrae. The location of the testes inside the body cavity means that sperm production in aves must occur at a body temperature of 41ºC, much higher than the 32-35 ºC scrotal temperature at which mammals produce sperm (Fronda, 1921; Wang et al., 1997). As a result, sperm cells die much more rapidly in the avian system, and the reproductive system must compensate for this with a higher capacity for sperm production (McDaniel et al., 1995). Birds accomplish this in two ways. First, avian testes have a much higher rate of synthesis of sperm cells per gram of testis weight when compared to non-avian species. In any given day, a mature rooster produces 80-120 x 10^6 spermatozoa per gram of testis, more than four times the production rates reported in rats and ten times the production rate of a healthy man in the 1980s (de Reviers & Williams, 1981; Johnson et al., 1980). Second, avian species typically have a higher testes-to-bodyweight ratio, allowing for more space for sperm production relative to overall bodyweight.

The testes are composed mainly of interstitial tissue and seminiferous epithelium. The interstitial tissue houses blood and lymphatic vessels, nerves and Leydig cells, the hormonally active cells of the interstitial tissue. The interstitial tissues are separated from the seminiferous epithelium by a thin layer of connective tissues which define the shape of the seminiferous

tubules (Rothwell & Tingari, 1973). The seminiferous tubules are a network of ducts which are lined with seminiferous epithelium for the production of sperm and the passage of sperm out of the testes. The seminiferous epithelium consists of Sertoli cells and spermatogonia, which together are responsible for spermatogenesis, the production of male gametes. Sertoli cells are large support cells which maintain an ideal environment for spermatogenesis and seize testosterone and nutrients for sperm cells during development, forming the blood-testis barrier. Sertoli cells support the growth and maintenance of spermatogonia, spermatocytes and maturing spermatids throughout the processes of spermatogenesis and spermiogenesis (Rothwell & Tingari, 1973; Walker & Cheng, 2005).

Sperm produced within each testis exit at the rete testis and begin to traverse the excurrent ducts which make up the epididymis and the vas deferens, or deferent duct. The rete testis marks the beginning of the epididymis, which further consists of the efferent ducts, connecting ducts and epididymal ducts (Tingari, 1971). These ducts are distinguished by their degree of mucosal folding and the ciliation of their epithelial cells, with the efferent ducts having a high degree of folding and epithelial cell ciliation and the epididymal ducts having a low degree of folding and epithelial cell ciliation (Aire & Josling, 2000; Bakst, 1980).

Sperm leave the epididymal ducts to enter the deferent ducts, which run the length from the epididymis towards the cloaca down the dorsal wall of the body cavity. The deferent ducts differ from the epididymal ducts in that they possess a layer of smooth muscle which surrounds the mucosa of the duct (Tingari 1971). The distal ends of the deferent ducts open at the cloaca through papillae which are found on each side of the phallus, which is nonintromittent in gallinaceous birds (Briskie & Montgomerie, 2001). When erected, the phallus fills with

lymphatic fluid and everts outside of the cloaca in order to engage in a “cloacal kiss” for mating (Briskie & Montgomerie, 2001).

The Hypothalamic-Pituitary-Gonadal Axis

Testosterone and its derivative, estrogen, drive the process of spermatogenesis, engagement in mating behavior and the upkeep of reproductive tissues. The availability of testosterone, however, is controlled by complex, coordinated action between the hypothalamus, anterior pituitary gland and gonads, known as the hypothalamic-pituitary-gonadal (HPG) axis.

Control of sex hormones begins in the hypothalamus, where gonadotropin-releasing hormone (GnRH) is produced. GnRH is a ten amino acid peptide which is expressed in two isoforms in the chicken and acts on two GnRH receptors (GnRHR) (Katz et al., 1990; King & Millar, 1982; Joseph et al., 2009; Millar, 2005). The type I GnRHR is expressed in the anterior pituitary gland, whereas the type II GnRHR is expressed in both the anterior pituitary and in the gonads (Joseph et al., 2009; Sun et al., 2001). The two chicken GnRH isoforms are both expected to contribute to gonadotropin release (Katz et al., 1990).

GnRH synthesis and release are controlled by a combination of behavioral, sensory and seasonal cues. Engagement in mating behavior has been documented to effect GnRH synthesis in Passeriformes and Columbiformes (Mantei et al., 2008; Stevenson & Ball, 2009). For most seasonal breeders, Galliformes included, GnRH release is regulated by photoperiod through control of thyroid stimulating hormone (TSH) (Nakane & Yoshimura, 2014). Whenever these species are exposed to a long-day photoperiod, either naturally or artificially, TSH in the pars tuberalis of the anterior pituitary upregulates the activity of type II deiodinase in the hypothalamus. This increases local conversion of available thyroxine (T_4) to active triiodothyronine (T_3) which then upregulates release of GnRH (Ikegami et al., 2015; Nakane &

Yoshimura, 2014). During exposure to a short-day photoperiod, not only is TSH activity downregulated, but lower temperatures lead to an increase in dietary needs, which increase conversion of T_4 in the liver. This results in shrinkage of the testes during a non-breeding season. The increase in circulating T_3 signals activation of genes responsible for directing meiotic arrest and germ cell apoptosis (Ikegami et al., 2015). A short-day period also limits hypothalamic release of GnRH indirectly through increased melatonin levels (Zhang et al., 2016).

Released GnRH acts on the pars distalis of the anterior pituitary to stimulate the synthesis and release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) by gonadotrophs. LH and FSH, when released into circulation, act on the gonads (Ishii & Furuya, 1975). The release of LH and FSH, as well as the release of GnRH, can be inhibited by release of gonadotropin-inhibitory hormone (GnIH), which is a 12 amino acid peptide synthesized in the hypothalamus and controlled by melatonin levels, furthering the influence of photoperiod on the activity of the reproductive system (Bentley et al., 2008; Tsutsui et al., 2000). GnIH is also likely to be the effector through which melatonin levels inhibit release of GnRH, as melatonin has been found to increase GnIH levels (Ubuka et al., 2005).

LH acts on the Leydig cells found in the interstitial tissues of the testes (Ishii & Furuya, 1975). Pulses in LH stimulate the Leydig cells to produce testosterone and androstenedione (Yu & Wang, 1987). High levels of these androgens provide negative feedback to the hypothalamus and anterior pituitary, creating a normal cycle of pulsatile shifts in LH release (Bacon et al., 1991). FSH controls the proliferation and activity of Sertoli cells (Ishii & Furuya, 1975; Walker & Cheng, 2005). Circulation of FSH stimulates the production of androgen binding protein, which allows the Sertoli cells to sequester testosterone to support spermatogenesis through activation of genes essential to sperm production (Vizcarra et al., 2010). The sequestered

testosterone promotes production of inhibin in the Sertoli cells, which is secreted to provide additional negative feedback on the hypothalamus and anterior pituitary, regulating the release of FSH (Davis & Johnson, 1998; Thurston & Korn, 2000).

Spermatocytogenesis & Spermatidogenesis

Most extensive research on spermatogenesis in galliform birds has occurred in the Japanese quail, with similar function expected in other Galliformes (Lin & Jones, 1992; Lin et al., 1990). In the seminiferous epithelium, spermatogenesis begins in the spermatogonia with spermatocytogenesis, the mitotic stage of spermatogenesis. The spermatogonia can be subdivided into one of four types: A_d, A_{p1}, A_{p2} and B. The cells are differentiated by the intensity of toluidine blue stain taken up by the cell, indicating low condensation of chromatin for the A_d (or "dark stained") cells and higher condensation of chromatin for the A_p (or "pale stained") cells (Aire, 2014; Lin & Jones, 1992; Lin et al., 1990). The cells of the A_d spermatogonia subtype represent the true spermatogonial stem cells. A_d spermatogonia undergo mitosis to produce one daughter cell which remains an A_d spermatogonia while the other daughter cell becomes an A_{p1} spermatogonia, the cell which will undergo further division. Each A_{p1} spermatogonia undergoes mitosis to produce two A_{p2} spermatogonia, and the pattern continues with each A_{p2} spermatogonia producing two B type spermatogonia and each B type spermatogonia producing two primary spermatocytes through mitosis (Lin & Jones 1992, 1993). Through this process, production of one A_{p1} spermatogonia results in production of eight diploid primary spermatocytes.

The genesis of the primary spermatocytes marks the end of spermatocytogenesis and the beginning of spermatidogenesis, the meiotic stage of spermatogenesis. Spermatidogenesis requires an estimated 6-7 days in the chicken, and roughly 96% of that time is spent in meiosis I

(Lin et al., 1990). In galliform males, meiosis I is divided into eight phases: preleptone, leptone, zygotene, pachytene, diplotene, diakinesis, metaphase and anaphase (Lin et al., 1990). At the end of the anaphase of meiosis I, there is a short period of time where a secondary spermatocyte is discernible before it proceeds into meiosis II (Lin et al., 1990). Meiosis II occurs rapidly and is completed in approximately half a day, resulting in four haploid round spermatids for each one primary spermatocyte to undergo the process of spermatidogenesis.

Spermiogenesis

Spermiogenesis is the third and final stage of spermatogenesis and concerns the stages of sperm development through which a round spermatid transforms into a mature, elongated spermatozoa ready to detach from the seminiferous epithelium (Aire, 2014; Gunawardana & Scott, 1977). The number of distinct steps of spermiogenesis differs considerably between species, but non-passerine birds are typically agreed to exhibit 10-12 steps (Aire, 2014; Gunawardana, 1977; Lin & Jones, 1992). Regardless of the species, the steps of spermiogenesis in mammalian and avian systems are classified into four phases: the Golgi phase, the cap phase, the acrosome phase and the maturation phase. In the 12-step interpretation of non-passerine avian spermiogenesis, as reviewed by Aire (2014), these phases are comprised of steps 1-3, 4-6, 7-9 and 10-12, respectively.

The Golgi phase of spermiogenesis is characterized by the accumulation of proacrosomal granules inside the Golgi complex. It starts with the step 1 spermatid, which is a round spermatid characterized by its enlarging Golgi complex filling with proacrosomal granules. The centrioles are freely associated with each other in the cytoplasm, and mitochondria are somewhat concentrated near the Golgi complex. The step 1 spermatid's chromatin is partially condensed (Aire, 2014; Nagano, 1962). As a spermatid develops into step 2, the chromatin decondenses and

fills the nucleoplasm. Proacrosomal granules accumulating inside the Golgi complex join to form one large acrosomal vesicle (Aire, 2003, 2014). The distal centriole makes contact with the cell membrane. At the point of contact, an annulus develops, and the fibrous sheath that will envelop the flagellum begins to grow out of the annulus (Aire, 2014). The step 3 spermatid is characterized by almost entirely decondensed chromatin with few small granules formed and the disappearance of the Golgi complex. The acrosomal vesicle becomes pronounced and settles near the nucleus, and the nuclear membrane thickens at the point of contact with the acrosomal vesicle (Aire, 2014). The centriolar complex typically settles near the nuclear membrane as the fibrous sheath continues to grow out of the annulus, and striations resembling an axoneme begin to appear (Aire, 2014).

Step 4 marks the beginning of the cap phase, where the nucleus begins to reshape and condense (Aire, 2014). The step 4 spermatid's chromatin begins to form granules and takes on a granulofilamentous form. The acrosomal vesicle elongates and invaginates into the nucleus, and the nuclear membrane thickens more at this point of contact. This invagination is a precursor to the perforatorium (Nagano, 1962). The proximal centriole appears to attach to the nuclear membrane as microtubules begin to develop (Aire, 2014). The step 5 spermatid develops more granular chromatin as the nucleus and acrosome elongate, and the perforatorium further develops between the acrosome and nucleus (Aire, 2003, 2014; Nagano, 1962). By this point, the centriolar complex has migrated to the caudal end of the nucleus (Aire, 2014). The step 6 spermatid's nucleus elongates into an ovular shape as its chromatin transitions to a uniform granulofilamentous form (Aire, 2003). Circular manchette microtubules begin to form in clumps along the length of the nucleus, setting up an architecture around it, inside of which the nucleus reshapes into its final, drastically elongated shape. These circular manchette microtubules are

found around the nucleus from its cranial end to the point of contact of the proximal centriole (Aire, 2014).

The acrosome phase is marked by rapid elongation of the spermatid nucleus (Aire, 2014). This begins at the step 7 spermatid, where the spermatid's nucleus begins to stretch and thin as the circular manchette develops fully (Aire, 2014). Mitochondria begin migrating to the caudal end of the nucleus near the centrioles; the mitochondria begin to elongate as well, and their cristae take a longitudinal conformation. The proximal centriole attaches fully to the caudal end of the nucleus, and the distal centriole remains perpendicular to the proximal centriole. This complex forms the base of the developing midpiece (Aire, 2014). By this point, the distal centriole has become much longer than the proximal (Aire, 2003). The spermatid shifts to step 8 as the granulofilamentous chromatin begins to develop larger, intermittent, tightly-condensed granules (Aire, 2003, 2014). It is during this step that the longitudinal manchette can be observed at the same time as the circular manchette. Similar to the circular manchette, the longitudinal manchette is an architecture of microtubules surrounding the reshaping nucleus. The longitudinal manchette, as the name suggests, runs longitudinally along the length of the nucleus. The longitudinal manchette is formed from a rearrangement of the circular manchette (Aire, 2014). The step 9 spermatid is characterized by a completed longitudinal manchette and complete disappearance of the circular manchette. By step 9, mitochondria have completely aggregated at the caudal end of the spermatid nucleus (Aire, 2014).

The last phase of spermiogenesis is the maturation phase, where the elongating spermatid becomes recognizable as a mature, vermiform spermatid. The step 10 spermatid's nucleus and mitochondria continue to elongate, and its chromatin continues to pack even more tightly into granules (Aire, 2014). The step 11 spermatid's chromatin reaches the point of maximum

chromatin condensation, and the spermatid head develops a slight curvature at the cranial end (Aire, 2014; Nagano, 1962). The acrosome begins to stretch dramatically over the cranial end of the nucleus, and the cranial end of the nucleus is situated into the subacrosomal space (Nagano, 1962). The mitochondria begin to surround the axoneme of the flagellum in a linear formation (Aire, 2003, 2014). In the step 12 spermatid, the mitochondrial sheath is completely formed around the axoneme at the midpiece as the longitudinal manchette disappears (Aire, 2003). The acrosome fully surrounds the nucleus at this step as well as the proximal and distal centrioles, with its caudal end reaching the cranial end of the mitochondrial sheath (Aire, 2014). The spermatid's extra cytoplasm has collected into a segregated droplet to be later absorbed by the adjacent Sertoli cells. The nearby Sertoli cells hold weak contact with the step 12 spermatid up until the moment of spermiation (Aire, 2003, 2014).

The process of spermiogenesis contains in it several cellular rearrangements and manufactures of new structures that occur simultaneously. These include migration of the centrioles and manufacture of the flagellum, chromatin reprogramming and condensation, migration and restructuring of mitochondria, creation of the acrosome and disposal of unnecessary cellular components (Aire, 2014). The overall process of spermiogenesis is typically summarized in a chronological fashion with concurrent discussion of the many cellular processes, as described above; however, for the purpose of this review, sperm flagellar development and structure and sperm chromatin condensation during spermiogenesis must be discussed in detail.

Sperm flagellar development & structure

The development of sperm flagella and the mechanisms involved in their assembly have not been widely investigated in vertebrates, however the structure of the flagella is highly

conserved between kingdoms (Pereira et al., 2017; Teves et al, 2016). Specific to avian sperm flagella, development begins in step 2 of spermiogenesis, with a fibrous sheath developing out of an annulus in the cell membrane. The step 5 spermatid has a centriolar complex which has migrated to the caudal end of the nucleus, and by step 7, the distal centriole has elongated significantly, with the flagellar axoneme rapidly synthesizing at its end. The longitudinal columns of the fibrous sheath are clearly visible at this step, and the axoneme and outer fibers continue to grow throughout spermatid elongation (Aire, 2014).

Flagellar structure in non-passerine birds is similar to that found in mammals (Aire, 2014). In an avian or mammalian spermatozoon, the flagella consists of an axoneme, outer dense fibers and a fibrous sheath containing two longitudinal columns (Aire, 2014; Eddy et al, 2003; Inaba, 2003). The axoneme is composed of two central microtubules surrounded by nine microtubule doublets, with each doublet containing an A and B subfiber. The nine peripheral microtubule doublets are anchored to the central microtubules by radial spokes connected to microtubule A, and nexin links serve as anchors between adjacent peripheral microtubules. Microtubule A also has an outer dynein arm and three types of inner dynein arms which engage in ATP-dependent binding with the adjacent microtubule B, causing sliding between tubules which, when coordinated between all nine peripheral microtubules, propagates thrust and progressive motility (Inaba, 2003).

Outer dense fibers and a fibrous sheath are observed in the sperm flagella of all animal species which engage in internal fertilization (Aire, 2014; Eddy et al., 2003; Inaba, 2003). An outer dense fiber is found associated with each of the nine peripheral microtubule doublets, and these fibers are expected to provide an elastic nature to the flagella (Eddy et al., 2003). The fibrous sheath is composed of two sets of three protein-based longitudinal columns associated

with two microtubule doublets on opposite sides of the axoneme. The opposing positioning of these two columns provide a natural plane of movement for the flagella (Eddy et al., 2003). These structures are all present in the flagellum at the point of spermiation (Aire, 2014).

Sperm chromatin condensation

Sperm chromatin condensation has not been extensively studied in Aves, however the general pattern of chromatin condensation and decondensation observed is consistent with what is known about the mammalian mechanism of sperm chromatin condensation (Aire, 2014; Hoghoughi et al., 2018). In non-passerine Aves, a round spermatid's chromatin decondenses until it reaches step 3, condenses to a granulofilamentous form through steps 4-6 and finally begins to dramatically condense at and after step 8 (Aire, 2014). In mammals, overall expression of histones decreases at the beginning of the elongation of the nucleus, and the histones are temporarily replaced by basic, non-histone transition proteins (TPs). These TPs disappear shortly after, as they are permanently replaced by strongly polycationic protamines (Balhorn, 2007; Hoghoughi et al., 2018).

The replacement of histones with protamine is imperative for development of normal, mature sperm. Inadequate protamination in a spermatozoon directly results in inadequate condensation of the sperm nuclear chromatin. Inadequate condensation of chromatin in a mature spermatozoon typically results in increased DNA damage and fragmentation and abnormal morphology of the sperm head, making the cell less hydrodynamic (Balhorn, 2007; Boe-Hansen et al., 2018; Dogan et al., 2015). There is evidence that degree of protamination of a sperm cell dictates the rate of decondensation of chromatin in the sperm pronucleus, influencing the success of fusion of genetic material (Balhorn, 2007; Perreault et al., 1988). Replacement of histones with protamine also accomplishes epigenetic reprogramming of most of the sperm haploid

genome, with only a small percentage of histones remaining to transfer epigenetic modifications to the next generation (Hoghoughi et al., 2018; Meistrich et al., 2003; Zalensky et al., 2002).

Chromatin condenses much more tightly when bound to protamine, compared to histones, due to the unique chemistry of the protein (Balhorn, 2007). In the chicken, the mass of protamine is 58% that of a single H2B subunit of a histone octamer. In addition to its smaller size, protamine is much more strongly basic than a histone. Chicken protamine contains 36 arginine residues out of its total 62 amino acids, and it has a pI of 13.3. These arginine residues are mostly found in groupings of 3-7 in sequence together. This is compared to histones which typically consist of only around 5-15% arginine residues, 10-15% lysine residues and 1-5% histidine residues, and histones are already considered basic proteins (UniProt, 2019). This much higher concentration of arginine residues in protamine results in much tighter packing of chromatin. Chicken protamine also has 10 serine residues and 1 threonine residue, and all of these, save one of the serine residues, are located adjacent to an arginine. These residues are expected to serve as phosphorylation sites. The purpose of these phosphorylation sites is not yet confirmed, but it has been proposed that these sites prevent the region from interacting with DNA (Balhorn, 2007; UniProt, 2019).

In mammalian studies, protamine synthesis has been shown to occur through translation in free ribosomes, and protamine mRNA is only found in developing spermatozoa (Kleene, 1996). The chicken protamine gene, so far unnamed and found at two distinct loci on chromosome 4, is intronless, unlike most mammals in which protamine has been studied (Oliva & Dixon, 1989). Another interesting difference in chicken and other avian protamines, when compared to mammalian models, is that they lack cysteine residues (Balhorn, 2007; UniProt, 2019). The presence of cysteine residues typically allows for disulfide linkages to be formed

between DNA-protamine complexes, further tightening the chromatin packing (Vilfan et al., 2004). The lack of cysteine residues in chicken protamine suggests that these disulfide bonds between protamines do not occur in chicken sperm. This may explain the observation that in avian sperm, fully condensed chromatin is not homogenous, as in mammals, but is found still in tightly packaged but distinct granules (Aire, 2014). Protamine sequences vary highly between species, and the only highly conserved characteristics are the high arginine concentration and the presence of serine and threonine phosphorylation sites. Despite several sequence variations, the function and effect of protamine in the sperm genome are expected to be the same across vertebrates (Balhorn, 2007; UniProt, 2019).

Spermiation & Extragonadal Sperm Maturation

Spermiation is the release of elongated spermatozoa from their association with the Sertoli cells and into the seminiferous tubule lumen. Upon release, the Sertoli cell breaks contact with the spermatozoon and phagocytoses the residual cytoplasmic droplet which the spermatozoon leaves behind (Aire, 2003). Released spermatozoa are suspended in fluid which is secreted from the Sertoli cells and flow through the seminiferous tubule lumen through the rete testis and into the excurrent ducts (Rothwell & Tingari, 1973).

As sperm travel through the efferent ducts, the seminiferous tubule fluid is reabsorbed, and it is exchanged for a smaller volume of seminal plasma as the sperm travel through the epididymis, resulting in concentration of spermatozoa and a complete change of their aqueous environment (Clulow & Jones, 1988). This plasma differs in its ion composition and also contains several sperm maturation proteins excreted from the epididymal epithelium which incorporate into the sperm cells (Ahammad et al., 2011a, 2011b).

Despite the ability of testicular sperm to fertilize an oocyte, incorporation of epididymal sperm maturation proteins is necessary for fully developed sperm which are capable of traversing the female reproductive tract to fertilize an egg (Ahammad et al., 2011a, 2011b; Howarth, 1983). The mechanism of incorporation of the various maturation proteins is yet to be discovered, however the effects of their incorporation are well established. As sperm mature through the epididymis and the vas deferens, they increase in their survivability, motility, acrosomal proteolytic activity and penetrability of the inner perivitelline layer (IPVL) of the egg (Ahammad et al., 2011a, 2011b).

In an unejaculated rooster, the distal vas deferens contains the majority of sperm in the reproductive tract. It is the primary site of extragonadal sperm storage, usually containing 95% of the extragonadal sperm (Clulow & Jones, 1982). Contrary to the mammalian model, where there is a need for sperm to undergo capacitation in the female reproductive tract, the sperm found in an avian distal vas deferens are completely matured, and rooster sperm are fully capable of traveling to, penetrating and fertilizing an egg once ejaculated (Howarth, 1971).

Fertilization & Polyspermy

Upon reaching a newly ovulated follicle in the infundibulum of the oviduct, sperm have a narrow window of time to bind with the egg IPVL before the outer perivitelline layer coats the egg and blocks further sperm binding (Bellairs et al., 1963; Olsen, 1942). Sperm must bind to the IPVL through interaction between sperm IPVL-binding proteins and IPVL, or zona pellucida (ZP) as the proteins are named by their mammalian homologues, sperm-binding proteins (Benson et al., 2009). Successful binding of the sperm with the IPVL surface by way of ZP proteins induces the acrosome reaction, and the sperm releases its acrosomal contents to locally degrade the IPVL. This degradation is achieved through release of sperm proteases within the

acrosome and results in a hole in the IPVL through which the sperm penetrates the egg (Sasanami et al., 2011; Takeuchi et al., 2001).

Avian ova are penetrated by multiple sperm and undergo polyspermic fertilization, unlike in mammalian reproduction where only one sperm penetrates the egg surface (Snook et al., 2011). In the case of the chicken, typically 20 or more sperm penetrate the egg at the germinal disc, with 62 being the most sperm ever found in the germinal disc region of the egg (Nakanishi et al., 1990). Despite the high number of sperm entering the cytoplasm of the germinal disc, still only one sperm pronucleus fuses with the egg pronucleus (Hemmings & Birkhead, 2015). Penetration of the avian egg by multiple sperm is not needed in artificial fertilization, but it is necessary in natural fertilization (Bramwell et al., 1995; Mizushima et al., 2014). Originally, polyspermy was thought necessary in order to compensate for the size of the egg, increasing the number of sperm present in order to increase the likelihood that one might locate the egg pronucleus. The finding that it is actually concentration of sperm protein, not sperm pronuclei, present which matters has led to the popular hypothesis that several sperm are needed inside the egg cytoplasm in order to deliver enough sperm egg-activating proteins to reactivate meiosis in the egg (Mizushima et al., 2014; Shimada et al, 2014). After the one sperm pronucleus fuses with the egg pronucleus, several supernumerary sperm are left. Many of these become pronuclei and decondense their chromatin. These extra pronuclei migrate to the edge of the germinal disc, undergo a few rounds of mitosis and eventually degrade early into development of the blastoderm (Perry, 1987).

Summary

Spermatogenesis in the rooster is an intricate process of the reproductive tract regulated by the response of the HPG axis to a variety of stimuli. Spermatogenesis relies on mitotic and

meiotic processes to produce haploid gametes from spermatogonial stem cells. The subsequent coordination of several, synchronous cell rearrangements followed by extragonadal development results in mature spermatozoa capable of delivering their haploid genome through the hen's oviduct. Upon reaching the infundibulum, polyspermic fertilization of an egg is the product of a joint effort formed by several IPVL-binding and egg-penetrating sperm.

CHAPTER 2

ADDRESSING BROILER BREEDER ROOSTER FERTILITY

The Broiler Breeder Paradox

"Broiler breeders" are the genetic stock used to produce the broiler chicks grown for meat production, making broiler breeder roosters the sires of broiler chickens. These genetic lines have been subject to intense artificial selection in recent decades with the goal of increasing the efficiency of poultry meat production. In light of the goal, the breeding programs used to produce these lines have been extremely successful, resulting in chickens with a voracious appetite and capable of reaching market weight in 7 to 8 weeks (Decuypere et al., 2010; Pollock, 1999). Despite the positive outcomes of any breeding programs, intense selection towards one trait or set of traits often results in unintended negative effects as well (Hocking, 2014; Rauw et al., 1998). In the case of broiler breeders, the negative effects have come in the form of impairment of fertility, or the ability of an individual to produce offspring and pass on their genes to the next generation (Decuypere et al., 2010; Hocking, 2014).

In general, in poultry, body weight and fertility have a long history of strong negative correlation, and as body weight increases with each year, the impacts on fertility become more noticeable (Decuypere et al., 2010; Jaap & Muir, 1968; Pollock, 1999; Reddy & Siegel, 1977). This negative correlation and the reasons for it were originally described in 1954 by Lerner's Theory of Genetic Homeostasis, an explanation that stabilizing selection and the probable tendency towards heterozygosity provided an adaptive buffer for organisms. This explanation

has fallen out of favor over the last 60 years due to lack of evidence for advantages of heterozygosity and the overall complexity of the explanation (Hall, 2005). Another popular explanation is the Resource Allocation Theory. This theory states that characteristics of an animal are sometimes driven to compete for resources. In a situation where metabolic resources do not allow for optimal fueling of all characteristics of the animal to be adequately expressed, the response is to allocate suboptimum resources to all characteristics. In the case of broiler breeders, this theory would suggest that artificial selection has tilted the scales in favor of traits pertaining to bodyweight gain, and resources are shunted away from adequate expression of other unrelated traits such as those pertaining to reproduction (Decuypere et al., 2010; Rauw, 2008). The issue as explained by the Resource Allocation Theory is somewhat ameliorated by the effective lack of resource scarcity typically experienced by domesticated animals, however it may be the case that a physiological ceiling on the metabolic activity of an animal impose logistical limits on resource allocation within the animal's system. In addition to this, there is the reality that the voracious appetite exhibited in broiler breeders necessitates feed restriction as a management practice, thereby creating external resource scarcity through the current feeding practices.

Whatever the mechanism behind the negative correlation, the relationship between selection for body weight and loss of fertility is clear (Decuypere et al., 2010; Hocking, 2014). While special management practices may be used with broiler breeders to reduce the impact of the problem, long-term solutions must consider inherited reproductive characteristics in the decision making in order to generate a sustainably fertile genetic stock (Decuypere et al., 2010; Froman et al., 2002; Wolc et al., 2009).

Determinants of Rooster Fertility

The characteristics which influence rooster fertility are many. These characteristics can generally be grouped into either those influencing mating ability or those influencing sperm production and quality. Both groups are expected to have genetic and environmental factors controlling them (Campo et al., 2005; Millman et al., 2000; Wolc et al., 2009).

Characteristics influencing mating ability

For the purpose of this review, fertility-related characteristics influencing mating ability are any of those which might encourage or discourage a rooster from engaging in mating and depositing semen into a hen. These include aggression, dominance, libido and the ability to mount a hen and produce a cloacal kiss.

Aggression and dominance are social disruptors to mating. All roosters will exhibit some level of aggression; however, broiler breeder roosters have been noted for their exceptional level of aggression towards females when compared with layer strains and even compared to birds selected for cockfighting (Campo et al., 2005; Millman et al., 2000). In commercial flocks, this high aggression can result in isolation of females, injury and even death, which ultimately negatively effects the flock fertility (Millman et al., 2000). Male-male dominance behavior is often less violent in the long-term, as physical altercations diminish when hierarchy is set and undisturbed; however, there are short-term risks to the health of males engaged in establishing a dominance hierarchy (Grigor et al., 1995; Rushen, 1984). Hierarchies between males and hierarchies between females are established independently of each other, and broiler breeder males do exhibit more male-male aggression compared to layer males but less compared to male game birds (Millman & Duncan, 2000; Millman et al., 2000). The male hierarchy in roosters can negatively affect the fertility of a flock, as dominant males typically have more opportunities to

mate, despite the fact that dominance may be negatively correlated with sperm quality. Subdominant roosters have previously been reported to have much higher sperm mobility than dominant roosters (Froman et al., 2002).

Beyond social behavior, the libido and ability to mate of an individual rooster will also play into its effective fertility. Roosters experience a loss of libido with age, the mechanism of which is well studied in quails. Roosters eventually undergo reproductive senescence, which is the loss of endocrine and behavioral functions necessary for reproduction. Much of this is through alteration of circuitries involved in GnRH release (Ottinger et al., 1997). Ability to physically mate with a hen typically becomes an issue before loss of libido in broiler breeder roosters in cases where weight is not adequately controlled. As roosters become too large, they experience foot and leg issues which decrease their mobility, and roosters eventually grow too big to make cloacal contact with a hen (Robinson et al., 1993). The issue of large body size and its impact on fertility in broiler breeders is usually managed through feed restriction, although feed restriction introduces new stressors to broiler breeders, negatively impacting fertility through stress (Decuypere et al., 2010; Robinson et al., 1993).

Characteristics influencing sperm quality

Sperm quality can be assessed by a variety of sperm characteristics in the rooster. These include sperm count, viability, morphology, inner perivitelline layer (IPVL)-binding ability, motility, mobility, metabolism, acrosomal proteolytic activity, membrane lipid composition and DNA quality. Those that are most commonly assessed in broiler breeder roosters are sperm count, viability, IPVL-binding ability, mobility and/or motility and metabolism (Ahammad et al., 2011a; Barbato et al., 1998; Donoghue et al., 1996; Froman et al., 1999; Haunshi et al., 2011; Jones & Wilson, 1967; Korn et al., 2002; McDaniel et al., 1998). These qualities may be

assessed with less resolution by a general measure of sperm fertility, artificial insemination of a hen followed by assessing eggs for percent fertility (Soler et al., 2016; Wolc et al., 2019).

Sperm count, or concentration, and sperm viability are very simple characteristics to measure. Sperm count is a measure of the number of sperm cells in a semen sample, and it thereby has potential to impact fertility by determining, in part, the number of spermatozoa which reach the egg (Jones & Wilson, 1967). The relationship between sperm count and fertility is disputed in poultry, and this is likely due to ability of the hen modify selection within her reproductive tract when available sperm is low (Hemmings & Birkhead, 2015; McCartney, 1956; McDaniel & Craig, 1959; Shaffner & Andrews, 1948). Hens have the ability to reduce selective barriers to migration of sperm in order to ensure an adequate number of sperm reach the egg whenever access to a supply of sperm is limited (Hemmings & Birkhead, 2015). Sperm count is typically measured in roosters by either hemacytometer counting under a microscope or by measuring relative cell concentration via optical density of a semen sample. It can also be measured by automatic cell counters (Bilgili & Renden, 1984; Jones & Wilson, 1967; McDaniel et al., 1998). Sperm viability is a measure of the number of live spermatozoa per dead sperm in a sample, which is why it is often assessed alongside count, as dead sperm in a sample can confound the impact of a high measured sperm count. Viability can be assessed using nucleic acid stains or by fluorometric detection to reveal dead sperm (Bilgili & Renden, 1984; Chalah & Brillard, 1998).

IPVL-binding is the ability of a sperm cell to recognize and bind with a newly ovulated egg's surface. IPVL-binding is essential to fertilization and can be measured through *in vitro* and *in vivo* techniques. It has been shown to have a strong correlation with fertility in poultry (Barbato et al., 1998). IPVL-binding ability can be measured by incubating sperm *in vitro* with

isolated IPVL, fixing and staining the incubated IPVL and then quantifying sperm penetration holes in the IPVL through microscopy (Barbato et al., 1998). This can similarly be assessed by allowing natural mating to occur or performing AI and analyzing laid, fertilized eggs for sperm penetration holes (Bramwell et al., 1995; Hemmings & Birkhead, 2015).

The terms “sperm motility” and “sperm mobility” are sometimes used interchangeably, but the two characteristics, while related, are distinct from one another. Sperm motility is the degree of movement observed in a sperm cell, a qualitative trait which may be assessed as either “motile” or “non-motile” or with any number of descriptive degrees between the two opposing terms. Sperm motility can be assessed by spectrophotometry, videomicroscopy, digital image analysis and sperm migration assays (Froman & McLean, 1996). Sperm mobility is the net movement of an entire population of sperm against resistance when held at body temperature (Froman et al., 1999, 2011). In short, mobile sperm must be motile, but not all motile sperm are mobile, as their movement may lack directionality (Froman et al., 2011). Selection of semen donors by sperm mobility has been shown to increase fertilizing efficiency; however, in selecting breeders for regeneration, sperm mobility appears to be a trait inherited from the hen (Birkhead et al., 1999; Froman et al., 1997, 1999). Sperm mobility is considered a major determinant of fertility among roosters (Froman et al., 1999). Sperm mobility is also more quantifiable than motility, as it can be relatively quantitated using the Accudenz sperm penetration assay. Either a controlled volume of raw semen or a washed sperm sample adjusted for cell concentration is overlaid in a cuvette containing a preheated solution of Accudenz. After being incubated for a short time at body temperature, absorbance of the solution is read to assess relative migration of the sperm population (Froman & Feltmann, 1998; Froman & McLean, 1996). In discussion of sperm mobility, sperm mitochondrial function and metabolic activity are sometimes considered

as well, due to the relationship between cell metabolism, mitochondrial function and mobility (Bowling et al., 2003; Froman et al., 2011; Froman & Kirby, 2005). Measures of mitochondrial function and metabolic activity have been less popular methods for measuring sperm quality when compared to the popularity of sperm mobility measures. This is likely due to the equipment requirements of these measures combined with the convenience of sperm mobility as a fair predictor of these more difficult measures (Froman et al., 1999; Froman & Kirby, 2005).

Inclusion of Sustained Fertility in Breeding Goals

Any breeding program implemented for maintaining and improving a broiler breeder population must have clearly defined breeding goals. Generation of a broiler breeder line with sustained fertility will require correction of fertility related traits as part of the breeding goals, possibly at some expense of the current meat producing capabilities of broiler lines (Decuypere et al., 2010; Thiruvenkadan et al., 2011). An understanding of the genetic, transcriptomic, proteomic and metabolomic factors positively and negatively influencing the fertility-related traits of broiler breeders will support targeting of these factors as biomarkers in marker-assisted selection (MAS) breeding programs (Dekkers, 2004; Kovac et al., 2013). Identification of such biomarkers is already underway, as previous proteomic study has identified three potential protein biomarkers of subfertility in commercial broiler and layer lines: Heat shock protein HSP 90-alpha, sperm-associated antigen 6 (SPAG6) and tektin 3. Of these three potential biomarkers identified, two, SPAG6 and tektin 3, are related with flagellar structure and motility, suggesting that MAS through biomarkers of flagellar function may be most readily applied (Soler et al., 2016). As the technologies required for MAS become more widely available and as more biomarkers are identified and characterized, they will allow for improvement of these traits in

broiler breeder lines. Rates of improvement will be dependent on the degree of heritability of the differences identified through MAS (Hocking, 2014).

Summary

Overemphasis of growth-related traits in broiler breeder lines has resulted in impaired fertility of both males and females of the lines, due both to direct negative genetic correlation and indirect negative effects from resulting management practices. A complete understanding of the multiple characteristics affecting rooster fertility and the factors controlling these traits will aid in the optimization of rooster fertility in future broiler breeder flocks while minimizing the impact on meat producing ability.

CHAPTER 3

GLYPHOSATE-BASED HERBICIDES AS A SOURCE OF REPRODUCTIVE TOXICANTS IN BROILER BREEDERS

Glyphosate-based Herbicides

In modern crop production practices, herbicides have been a revolutionary tool for weed management. Their worldwide uses have increased exponentially because they provide an easy, efficient and cost-effective way of controlling weeds when compared to the alternative methods they have replaced. Their broad-spectrum application for all varieties of weeds helps increase total crop production value (Coupe & Capel, 2016; Franz et al., 1997; Schroder et al., 1984). The most popular and heavily applied, branded, broad-spectrum commercial herbicide in the world is Roundup, first produced by Monsanto Technology LLC in 1974 in the USA (Duke & Powles, 2008). Like many other herbicides, the Roundup formulation is composed of glyphosate [N-(phosphonomethyl) glycine], a primary active ingredient introduced in 1971, and inert ingredients, making it the leader in a group of herbicides known as glyphosate-based herbicides (GBHs) (Baird et al., 1971; Benbrook, 2016; Guyton et al, 2015).

GBHs typically consist of glyphosate concentrated between 356 and 540 g acid equivalent/L at time of use and various additional adjuvants and surfactants (Mertens et al., 2018). Glyphosate, a derivative of glycine, is a weak acid whose water solubility is low (Farmer, 2010). In typical formulations of commercially available GBH products, glyphosate acid is incorporated in the form of either isopropylamine (IPA), potassium, monoammonium,

diammonium, trimethylsulfonium or sesquisodium salt to enhance its water solubility and stabilization and to make the product easier to handle. The isopropylamine salt of glyphosate is the most commonly used active ingredient in the formulation of GBHs (Mertens et al., 2018). Upon entering into and transportation throughout the plant the glyphosate is separated from its cation, and its herbicidal acid is eventually absorbed by the plant to inhibit biosynthesis of aromatic amino acids required for construction of proteins (Mertens et al., 2018). This effect is achieved by blocking the activity of 5-enolpyruvylshikimate-3-phosphate synthase in the shikimate pathway, which is essential to the production of phenylalanine, tryptophan and tyrosine (Gomes et al., 2014; Mertens et al., 2018).

In order to function, GBH formulations inevitably require adjuvants (surfactants, spreader stickers, crop oils, anti-foaming materials, buffering agents, and compatibility agents) to facilitate adequate plant coverage with glyphosate salts and the penetration of the salts through the waxy coverings of leaves and stems so they may be transported within the plants without losing the toxic effect of glyphosate, thereby increasing bioavailability of GBH (Stock & Holloway, 1993). By nature of their function, the adjuvants are typically considered by the manufacturer to be inert ingredients, meaning that they are physically, chemically and biologically inactive, and the contents of inert ingredients are generally not declared on product labels for the proprietary reasons (Mertens et al., 2018). Although the effect of glyphosate has been tested exclusively and very extensively, a complete toxicity risk assessment for GBH formulations is often hindered by the lack of adequate product-specific information on the inert ingredients (Cox & Surgan, 2006). Studies have demonstrated that polyethoxylated tallow amine (POEA), the most common of the identified surfactants stated on product labels of common GBHs, increases phytotoxicity of herbicide formulations and exerts toxic effects on humans,

animals, and microorganisms (Cox & Surgan, 2006; Defarge et al., 2016; Mann & Bidwell, 1999; Moore et al., 2012; Tsui & Chu, 2003; Tush & Meyer, 2016). As such, it is increasingly well documented that the chemical mixtures in the formulations exhibit far more toxicity than glyphosate alone (Benachour et al., 2007; Benachour & Seralini, 2009; Clair et al., 2012; Frontera et al., 2011; Gasnier et al., 2010; Gasnier et al., 2011; Moore et al., 2012; Mesnage et al., 2014; Peixoto, 2005).

Glyphosate-tolerant Crops

In industrial agriculture, the commercial success of GBHs has been largely due to the introduction of transgenic crops, known as genetically engineered (GE) crops (Fernandez-Cornejo et al., 2014; Qaim & Traxler, 2005; Qaim & Zilberman, 2003). This includes the Monsanto Company's 1996 introduction of Roundup Ready cultivars aimed at an increased level of crop protection through increased tolerance towards exposure to GBHs during the entire growth season of the crops (Benbrook, 2012; Powles, 2014; USDA, 2013; Zilberman et al., 2018). The US has approved 165 unique GE events in 19 plant species, although only a few have been widely grown commercially since the late 1990s, these being soybean, corn, alfalfa, cotton, sugar beet and canola (Brookes & Barfoot, 2015; James, 2013).

The widespread adoption of GE glyphosate-tolerant (GT) crops contributed to global use of GBHs (Benbrook, 2012; Coupe & Capel, 2016; Fernandez-Cornejo et al., 2014; Osteen & Fernandez-Cornejo, 2013). The application of GBHs as a pre-harvest herbicide or desiccant has also elevated the number of exposures to GBH ingredients during the crop growing cycle. In addition, for over a decade, with the emergence of glyphosate-resistant weeds as a result of increased usage of GBHs, the concentration and frequency of GBH applications have been on rise (Coupe & Capel, 2016; Cruz-Hipolito et al., 2011; Green, 2018; Heap, 2014; Powles &

Preston, 2006; Preston et al., 2009). This assures an increased accumulation of glyphosate residues in GT crops, as GT crops have been shown to be capable of absorbing and translocating applied glyphosate at high levels in the entire plant (Arregui et al., 2004; Cuhra, 2015; Cuhra et al., 2016; Duke et al, 2003; Feng et al., 2003; Hetherington et al., 1999; Reddy et al., 2004; Satchivi et al., 2000). The presence of post-application glyphosate and/or its notable metabolite, aminomethylphosphonic acid (AMPA), in GE crops has been well documented (Arregui et al., 2004; Bai et al., 2016; Bøhn et al., 2014; Duke, 2011). Depending on the frequency of GBH application and stage of growth, the GE crops have been shown to contain glyphosate and/or its metabolites at a wide range of concentrations (Arregui et al., 2004; Duke et al., 2003).

Residues of GBHs in GM Crops

Testing for glyphosate and/or AMPA residues in crops and food products has been a topic of interest in industrial food production, however, very few large-scale studies of crops have been performed. Among these few studies, grains and legumes have been primary focuses (Arregui et al., 2004; Bøhn et al., 2014; Cetin et al., 2017; Cuhra, 2015; Tarazona et al., 2017; USDA, 2013). Of the crops studied for presence of glyphosate and AMPA, soy has exhibited the highest levels, displaying residue levels as high as 18.5 and 20.0 ppm and averaging at levels closer to 2.0 and 3.5 ppm, respectively (Arregui et al., 2004; Bøhn et al., 2014; Duke et al., 2003; USDA, 2013). A 2011 USDA (2013) study of soy crops found glyphosate residues in 90.3% of samples and AMPA in 95.7% of samples. Residue levels are generally increased with higher frequency of application and/or application closer to time of harvest (Duke et al., 2003). There is no data on corn products and their levels of glyphosate and AMPA levels, however, no large-scale studies of glyphosate and AMPA residues in corn have taken place.

The United Nations Food and Agriculture Organization (FAO) agreed on maximum residue levels (MRL) for glyphosate in corn, soybean, cereal grains, cotton seed, sorghum straw, wheat, wheat straw, alfalfa and hay set at 5.0, 20, 30, 40, 50, 200, 300, 500 and 500 ppm, respectively (WHO, 1994). The FAO suggests that total glyphosate residues should be calculated as the sum of the amount of glyphosate residues and 1.5 times the amount of AMPA residues (Bøhn et al., 2014).

Effects of GBH Residues on Animal Nutrition

Livestock, worldwide, are the largest consumers of GT crops. Livestock in the US consumes feed comprised 95% of GE ingredients, and livestock outside of the US consumes feed which consists 70-90% of GE ingredients (Flachowsky et al., 2012). Corn and soybean are two primary components in poultry feed, with diets consisting of as much as 35% soybean meal and 65% corn grain (Van Eenennaam & Young, 2014). Broiler and turkey flocks, consuming 44% of all US soybean meal produced, are the single largest domestic consumer of soybean meal (USDA, 2015; Van Eenennaam & Young, 2014; Waldroup & Smith, 2018). Approximately 8% of US corn production is used by the broiler industry (National Chicken Council, 2019; National Corn Growers Association, 2018). In 2018, cultivars genetically modified for herbicide resistance were used for production of 94% of soybean and 90% of corn grown in the United States (USDA, 2018a, 2018b). Specific to GT cultivars, roughly 50% of US corn and soy grown are GT (Kniss, 2018).

As for nutrient composition and nutritive value, GT crops have been shown to be equivalent to their non-GT counterparts (Cheng et al., 2008; García-Villalba et al., 2008; Herman & Price, 2013; Hollingworth et al., 2003). Studies have found no difference in the productive performance or health of any swine, beef or dairy cattle, broiler or layer chicken, or quail fed on

GE-based feedstuffs (Aulrich et al., 1998; Erickson et al., 2003; Flachowsky & Aulrich, 2001; Flachowsky et al., 2005a, 2005b, 2007, 2012; Guertler et al., 2010; Sartowska et al., 2015; Taylor et al., 2007a, 2007b, 2007c; Van Eenennaam & Young, 2014; Walsh et al., 2011, 2012).

These investigations, however, have only focused on animal performance and nutrition, and few have included the tissue specific effects of exposure to GBH ingredients in livestock. This is despite evidence for accumulation of GBH ingredients in tissues of animals fed GT crops. In commercial broilers and dairy cows that were fed on GT crop-based diets, glyphosate residues have been detected in the liver, spleen, lungs, intestines, heart, muscles and kidneys (Krüger et al., 2014, Shehata et al., 2014). The residue has also been found in human blood and in the urine of humans, dairy cows, rats, and rabbits (Acquavella et al., 2004; Conrad et al., 2017; Curwin et al., 2007; Krüger et al., 2013; Mills et al., 2017; Panzacchi et al., 2018; von Soosten et al., 2016; Zouaoui et al., 2013). Of the studies which have investigated GE crops fed to livestock and included measures of reproductive success in their assessment, none have included GT crops in their study (Flachowsky et al., 2007; Snell et al., 2012).

Effects of GBH Ingredients on Animal Male Reproductive Health

A body of research has been produced in the past two decades suggesting that GBH formulations have toxic effects on the function of male and female reproductive tracts at relatively low dosages, with the most evidence being from studies of male reproductive toxicity. Most studies of reproductive toxicity have focused on mammalian models, with the only avian study being performed on male ducks (Oliveira et al., 2007). The effects shown across all animal models include disruption of the endocrine system, damage to reproductive tissues and negative effects on spermatogenesis.

Endocrine disruption

Glyphosate and GBHs are well documented endocrine disruptors. GBHs are reported to inhibit aromatase activity and transcription in human cells at levels as low as 10 ppm, well below the no-observed adverse effect level (Defarge et al., 2016, 2018; Gasnier et al., 2010). At an even lower dose of 1 ppm, aromatase transcription is shown to increase in male murine cells (Clair et al., 2012). Aromatase activity in human placental cell lines were observed to have a reduction in aromatase activity at a glyphosate level of 2000 ppm (Richard et al., 2005).

In the male system, estrogen levels are shown to increase with GBH dosage of 50 mg/kg bodyweight in rats but to decrease with dosage of 5 mg/kg bodyweight in ducks (Oliveira et al., 2007; Romano et al., 2012). *In vitro* treatment of murine Leydig cells with GBH at 25 ppm displayed a decrease in progesterone levels (Walsh et al., 2000). In studies of both duck and rat males, decreases in androgen levels were observed with treatments of GBH as low 5 mg/kg bodyweight, with magnitudes of effect in a dose-dependent manner (Abarikwu et al., 2015; Clair et al., 2012; Dallegrave et al., 2007; Nardi et al., 2017; Oliveira et al., 2007; Owagboriaye et al., 2017; Pandey & Rudraiah, 2015; Romano et al., 2010). One study reported an increase in androgen levels at a GBH treatment of 50 mg/kg bodyweight (Romano et al., 2012). Another recent study of male rats treated with either oral gavage of glyphosate alone or GBH at 25 mg/kg bodyweight displayed no significant changes to androgen levels, but the GBH formulation resulted in a small effect on steroidogenic gene expression (Johansson et al., 2018). Male ducks have shown a decrease in androgen receptors with GBH treatment at 5 mg/kg bodyweight (Oliveira et al., 2007). In a human liver cell line, HepG2, treatment with GBH at a concentration of 2 ppm results in an anti-estrogenic effect and disrupts transcriptional activities on both estrogen receptors, ERα and ERβ (Gasnier et al., 2010).

Romano et al. (2012) reported increases in FSH and LH transcription levels at treatment with 50 mg/kg bodyweight in rats, while a decrease in FSH and LH levels were observed in albino rats treated with a much lower dose of 3.6 mg/kg bodyweight (Owagboriaye et al., 2017). Male albino rats treated with GBH at doses as low as 3.6 mg/kg bodyweight saw an increase in prolactin levels in a dose dependent manner (Owagboriaye et al., 2017). Excess of prolactin disrupts release of gonadotropin-releasing hormone, which leads to decreased testosterone levels (Zeitlin & Rajfer, 2000). High levels of prolactin in males tends to impede gonadal development, further decreasing reproductive function (Corsello et al., 2003).

The results tying glyphosate and GBHs to endocrine disruption are somewhat conflicting, as many findings differ significantly between dosage levels. Different results in endocrine responses with respect to differing dosage levels are fairly common in toxicological studies, and differences between animal models studied must also be taken into account. Despite this and the many claims listed above, the endocrine disrupting effects of GBHs have been subject to speculation. The European Food Safety Authority (EFSA) performed a peer review (2017) of studies investigating endocrine disruption caused by glyphosate. The EFSA concluded that the glyphosate does not cause any endocrine disruption, criticizing the experimental design of many studies.

Reproductive tissues

In males, a variety of effects on testicular and epididymal tissues have been observed with respect to glyphosate and GBH treatment at levels as low as 5 mg/kg bodyweight. An increase in seminiferous tubule lumen diameter was observed in ducks and rats treated with GBH through oral gavage (Oliveira et al., 2007; Romano et al., 2010). With regard to seminiferous tubule epithelia, differing results have been noted, these being both increases and decreases in

epithelium height, degeneration of the epithelium, vacuolation of the tubule and separation of the tubule epithelium from interstitial cells (Dallegrave et al., 2007; Ikpeme et al., 2012; Oliveira et al., 2007; Owagboriaye et al., 2017; Romano et al., 2010, 2012). Rat studies have reported decreases in testis interstitial cells with GBH treatment at 50.4 mg/kg bodyweight but increases in interstitial space at 250 mg/kg bodyweight treatment of GBH due to invasion by inflammatory cells (Ikpeme et al., 2012; Owagboriaye et al., 2017). Recently, a study of male rats exposed by oral gavage to either glyphosate or a GBH formulation at 25 mg/kg bodyweight revealed no significant changes to the histology of testes (Johansson et al., 2018). Reduction in epididymal tissues was observed in rat and duck studies at treatments with glyphosate and GBH as low as 5 mg/kg bodyweight (Abarikwu et al., 2015; Ikpeme et al., 2012; Oliveira et al., 2007).

Clair et al. (2012) reported death of murine Sertoli, Leydig and germ cells with *in vitro* treatment of GBH at 1000 ppm. Other murine studies have reported death of Sertoli cells with *in vitro* treatment of GBH and POEA at 500 ppm and death of Leydig cells with *in vivo* treatment with glyphosate at levels as low as 3.6 mg/kg bodyweight (Ikpeme et al., 2012; Owagboriaye et al., 2017; Vanlaeys et al., 2018). One study on mice exposed to GT soybeans *in vivo* throughout development from fetal to adult stages found no significant differences in testicular cell kinetics (Brake & Evenson, 2004).

Spermatogenesis

Spermatogenesis has been a large topic of study. Murine studies of males treated with oral gavage of glyphosate at levels as low as 3.6 mg/kg bodyweight have reported a reduction in sperm count as well as an increase in sperm morphological abnormalities (Abarikwu et al., 2015; Ikpeme et al., 2012; Owagboriaye et al., 2017). Dallegrave et al. (2007) investigated prenatal exposure to GBH at a concentration of 50 mg/kg bodyweight of gestating rat mothers and

reported decreased sperm count and increased sperm morphological abnormalities in male offspring. Sperm viability has been reported to decrease in murine systems with doses of glyphosate as low as 5 mg/kg bodyweight (Abarikwu et al., 2015; Ikpeme et al., 2012). Reduction in sperm DNA integrity has been shown in zebrafish treated in an aquatic environment containing glyphosate at 5 ppm (Lopes et al., 2014). Oral gavage of rats with glyphosate doses as low as 3.6 mg/kg bodyweight has resulted in reduction of sperm motility (Abarikwu et al., 2015; Ikpeme et al., 2012; Owagboriaye et al., 2017). Similarly, treatment of zebrafish in an aquatic environment containing glyphosate at 5 ppm resulted in decreased sperm motility and decreased mitochondrial function, as did *in vitro* treatment of human sperm cells with GBH at 1 ppm and glyphosate at 0.36 ppm (Anifandis et al., 2017, 2018; Lopes et al., 2014).

Summary

The ingredients of GBHs, both active and inert, appear to act as reproductive toxicants, having a wide range of effects on the male reproductive system, including endocrine disruption, tissue damage and dysfunction of spermatogenesis. GBH ingredient residues are present in measurable levels on harvested crops grown from GT cultivars, and much of this produce is used in animal production, broiler breeder rearing included. Further study is needed of the effects of GBHs and their ingredients on the long-term reproductive health of broiler breeders. More large-scale analyses of GBH residues on livestock feeds are needed, as are investigation of the absorption of GBH residues from feed consumed by poultry. Should the minimum level of GBH exposure required to produce negative effects on the reproductive health of broiler breeders prove to be lower than typical GBH residue levels found in feed, attention should be given to

investigation of potential methods for minimizing the effects of GBH exposures to breeder populations.

CHAPTER 4

STATEMENT OF PURPOSE

Interventions are needed to reduce or remove the gradual decline in fertility exhibited in broiler breeder roosters. This decline in fertility is multifaceted in nature and will therefore require a multifaceted solution. Many areas affecting broiler breeder rooster fertility have remained uninvestigated. Sperm mobility is widely accepted as a primary determinant of rooster fertility; however, no biomarkers of sperm mobility have been characterized, limiting selection based on sperm mobility to direct evaluations of sperm mobility. This is despite previous identification of sperm-associated antigen 6 (SPAG6) as a biomarker of subfertility which is closely linked with flagellar structure. Sperm protamination has been virtually unstudied in Aves, even though there is a known correlation between sperm protamination and fertility in mammals. Last, despite a body of research demonstrating the male reproductive toxicity of glyphosate-based herbicides (GBHs) at low dosages in several animal models and a clear route of exposure to GBH ingredients in broiler breeder diets, the reproductive toxicity of GBHs in broiler breeder roosters has not been assessed. The objectives of this dissertation, therefore, are 1) to characterize SPAG6 for its use as a biomarker of rooster sperm mobility, 2) to optimize a method of assessing rooster sperm protamination and explore the relationship between rooster sperm protamination, classical measures of sperm quality and fertility, and 3) to assess the male reproductive toxicity of GBHs in broiler breeders and explore a strategy for counteracting the effect.

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CHAPTER 5

CHARACTERIZATION OF SPERM-ASSOCIATED ANTIGEN 6 EXPRESSION IN THE REPRODUCTIVE TRACT OF THE DOMESTIC ROOSTER (*GALLUS DOMESTICUS*) AND ITS IMPACT ON SPERM MOBILITY[1]

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Abstract

Sperm mobility is a major determinant of sperm quality in the domesticated chicken (*Gallus domesticus*) and is therefore an area of interest for improving fertility. Sperm-associated antigen 6 (SPAG6) is an important flagellar protein necessary for flagellar function but negatively associated with rooster fertility. This study was aimed to characterize the expression of SPAG6 and investigate its utility as a protein biomarker of sperm mobility. By western analysis, relative SPAG6 abundances were compared between the testicular, epididymal and vasal tissues and in sequentially maturing sperm. Immunocytochemistry techniques were used to detect localization of SPAG6 in chicken sperm. Last, western analysis was used to compare relative SPAG6 abundances in sperm of differing mobility. SPAG6 was found in higher abundance in epididymal tissues and in highest abundance in vasal tissues, relative to that of the testis. SPAG6 was also found to sequentially increase in abundance in maturing sperm. SPAG6 localizes between the axonemal central pair of microtubules in the sperm flagella, but it is also found in lower concentration in the acrosomal region. SPAG6 abundance, alone, is not a strong predictor of sperm mobility. Its impact on rooster fertility is likely unrelated to its impact on sperm mobility.

Key words: sperm-associated antigen 6, sperm mobility, chicken, flagellar protein, protein abundance

Introduction

Successful internal fertilization of an egg is dependent on the ability of sperm to migrate to the egg at its site of fertilization (Birkhead et al., 1999; Froman et al., 1997, 1999; Mortimer, 1997). Sperm mobility, or the directional, progressive movement of a sperm population, is a quantifiable and heritable trait which may be measured to determine the success of this sperm migration in chickens (*Gallus domesticus*) (Froman & Feltmann, 1998; Froman et al., 2002). Not only is a minimum level of sperm mobility necessary for delivery of sperm to the egg, but the hen's oviduct also selects for sperm with adequate mobility (Bakst et al., 1994). Only 1-2% of sperm reach the sperm storage tubules (SSTs) of the uterovaginal junction, and this selection results from barriers exhibited in the distal end of the hen's oviduct, with adequate sperm mobility being necessary for entrance into the SSTs (Bakst et al., 1994; Froman et al., 1999; Steele, 1992). In the domestic chicken, sperm mobility is considered a primary determinant of overall rooster fertility, and selection of semen donors by measures of sperm mobility lead to an increase in fertilization success (Birkhead et al., 1999; Froman et al., 1997, 1999). Due to the intense selection in the hen's oviduct for highly mobile sperm, an understanding of flagellar proteins and their influence on this mobility is important for informing any proteomic efforts towards improving rooster fertility.

Sperm-associated antigen 6 (SPAG6) is the vertebrate orthologue of *Chlamydomonas* PF16, the axonemal central apparatus protein (Sapiro et al., 2000; Teves et al., 2016). PF16 is shown in *Chlamydomonas* and *Plasmodium* studies to be essential for flagellar motility and stabilization of correct structure of the central apparatus in the axoneme (Smith & Lefebvre, 1996; Straschil et al., 2010). Knockout of the *Spag6* gene in mice resulted in decreased male fertility and sperm motility, and epididymal sperm exhibited abnormal twitching and abnormal

flagella (Sapiro et al., 2002). Other, non-sperm tissues where *Spag6* is expressed exhibit a decrease in cilia beat in their epithelia in mouse *Spag6* knockouts. (Teves et al., 2014). SPAG6/PF16 stabilizes the central apparatus by binding to the C1 central microtubule of the axoneme through a series of armadillo repeats, which facilitate protein-protein interactions (Sapiro et al., 2002; Smith & Lefebvre, 1996, 1997). The stability of the central apparatus contributed by SPAG6 is essential for proper development of the sperm flagellum (Sapiro et al., 2002).

SPAG6 was recently identified to be differentially expressed in fertile and sub-fertile layer roosters. Sub-fertile roosters, defined as roosters with fertilizing efficiency below 40%, were found to have sperm exhibiting a 1.1-fold increase in SPAG6 relative to sperm from fertile roosters, defined as those with a fertilizing efficiency above 70% (Soler et al., 2016). Given the previously described relationship between SPAG6 expression and rooster fertility as well as the fact that chicken SPAG6 shares an 86% homology with human SPAG6, an antigen for which polyclonal antibodies are commercially available, SPAG6 made an ideal candidate for investigation as a biomarker of rooster sperm mobility (Hamada et al., 2010). The objectives of this study were to 1) characterize the expression of SPAG6 in rooster sperm and 2) investigate SPAG6 as a biomarker of rooster sperm mobility.

Materials and Methods

Animals

Athens-Canadian random bred (ACRB) roosters were reared in individual cages under photostimulation of 14 hours light per day. ACRB roosters were 65 weeks of age. Aviagen Yield Plus (AYP) broiler breeder roosters were kept in individual cages and photostimulated with 15 hours light per day. AYP roosters were 42 weeks of age at the time of individual sample

collection and 44 weeks of age at collection of pooled samples used to assess sperm quality. All animals were reared with water *ad libitum* and given a standard broiler breeder ration. Roosters were maintained in accordance with the rules set forth by the University of Georgia's Institutional Animal Care and Use Committee.

Collection and preparation of tissues and sequentially maturing sperm

Ejaculated semen samples were collected from 20 ACRB roosters by dorso-abdominal massage method (Burrows & Quinn, 1937) and washed twice by dilution 1:2 in phosphate buffered saline (PBS; pH 7.4) followed by centrifugation at 1000 x *g* for 10 min. Samples were reconstituted 1:5 in lysis buffer (LB: 50mM NaCl, 10mM Tris base, 1mM EGTA, 1mM EDTA and 1% (v/v) Triton X) and sonicated using an Artek Model 150 sonic dismembrator (Thermo Fisher Scientific, Waltham, MA, USA) at medium power for 5 repetitions of 15 s with a 1 min remission period on ice. Sonicated samples were centrifuged at 10000 x g for 30 min at 4°C, and lysate was collected. In groups of 10, sperm lysates were pooled, resulting in 2 pooled samples of ejaculated-sperm lysates.

Three days later, the same ACRB roosters were euthanized by CO_2 gas asphyxiation. Roosters were dissected, and their reproductive tracts were collected and transported in PBS on ice. Vasal sperm were expressed from the vas deferens with tweezers, and vasal tissues were then separated from the epididymis. Epididymal sperm were expressed from the epididymis with tweezers, and then the epididymal tissues were separated from the testes. Testis samples were sliced open longitudinally at the site of epididymal attachment, and testicular sperm were expressed from the testes with manual pressure. Sperm samples were diluted 1:2 in PBS and washed by centrifugation at 1000 x *g* for 10 min. Ten immature testicular, epididymal and vasal sperm samples were pooled in the same fashion as the ejaculated sperm lysates, resulting in 2

pooled samples of each stage of maturation. Pooled sperm samples were diluted 1:5 in LB. Testicular, epididymal and vasal tissue samples were flushed of any residual sperm with LB, placed in 5 times tissue volume of LB and homogenized on ice using a 10 mm X 115 mm saw-tooth generator probe (VWR International, Radnor, PA, USA). Sperm and homogenized tissue samples in LB were sonicated and lysates collected as described above. Tissue lysates were pooled in the same fashion as the sperm samples, resulting in 2 pooled samples of 10 lysates from each tissue type. Protein quantitation was performed on all samples using the Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with bovine serum albumin (BSA) as the standard. The measurements were carried out according to the manufacturer's instructions. Lysates were aliquotted and stored at -80°C.

Collection, characterization and preparation of mature sperm

Uncontaminated semen, devoid of transparent fluid, was collected from 24 randomly selected AYP roosters using the dorso-abdominal massage method (Burrows & Quinn, 1937). Immediately after collection, fresh semen samples were assessed for progressive mobility by the Accudenz assay, as described by Froman & McLean (1996) with the following modifications. In polystyrene cuvettes, 1.00 mL aliquots of Accudenz solution (6% Accudenz (w/v), 0.6 mM KCl, 41.2 mM TES, 88.9 mM NaCl, 20.0 mM glucose, 3.20 mM $CaCl_2$; pH 7.4) were heated to 41ºC and overlaid with 100 µL semen. Cuvettes containing semen overlays were incubated at 41ºC for an additional 10 min, and absorbance read at 550 nm using a DU 530 spectrophotometer (Beckman Coulter, Inc., Brea, CA, USA) for relative measures of sperm progressive mobility. Individual semen samples in 250 µL aliquots were diluted 1:2 with PBS and washed two times by centrifugation at 1000 × *g* for 10 min to discard seminal plasma. After washing, sperm pellets were reconstituted to original volume in mobility buffer (MB: 111 mM NaCl, 25.0 mM glucose,

4.00 mM CaCl2, 50.2 mM TES; pH 7.4). Sperm were centrifuged again at 1000 x *g* for 10 min, and sperm pellets were resuspended 1:5 in lysis buffer LB.

Semen samples were collected from an additional 18 randomly selected AYP roosters by dorso-abdominal massage for analysis by sperm quality, and 3 pooled samples were generated by combining 6 individual samples. Samples were pooled in order to generate enough volume for Percoll density gradient centrifugation (PDGC), as PDGC has been previously validated to separate sperm into distinct quality groups, with higher quality sperm exhibiting higher sperm mobility and viability relative to lower quality groups (Ahammad et al., 2018). For each pooled sample, 3 mL were collected and diluted 1:2 in PBS. Diluted samples were subjected to PDGC as described by Ahammad et al. (2018) in triplicate. After PDGC, low-quality sperm from the top of the gradient and high-quality sperm from the bottom of the gradient were collected, washed as described above in PBS, and resuspended 1:5 in LB. Individual sperm samples, low-quality and high-quality sperm samples were sonicated and lysates quantitated in the same fashion as described above for tissue and sequentially maturing sperm lysates.

SDS-PAGE and western blot analysis

Protein samples were thawed and diluted to 1.2 mg/mL in LB. Samples were diluted further 1:1 with 2x sample buffer (4% SDS, 20% glycerol, 120 mM Tris-HCl, 200 mM dithiothreitol (DTT), 0.02% bromophenol blue, pH 6.8) and denatured at 95 °C for 5 min. In 50 µL sample buffer, 30 µg protein were loaded into each lane of Mini-PROTEAN® TGX Stain-Free™ 10% precast gels (Bio-Rad) alongside Precision Plus Protein™ All Blue Prestained Protein Standards (Bio-Rad). Gels were subjected to SDS-PAGE in 1X Tris-glycine-SDS (25 mM Tris, 192 mM glycine and 0.1% (w/v) SDS, pH 8.3) running buffer under 70 V for 10 min followed by 120 V for 60 min in a Mini-PROTEAN® Tetra Cell system (Bio-Rad). After SDS-

PAGE, gels were UV-activated using a ChemiDoc™ MP Imaging System (Bio-Rad). Proteins were then transferred from the activated gels to Immun-Blot® polyvinylidene difluoride (PVDF) membranes (Bio-Rad) in Towbin transfer buffer (25mM Tris base, 192mM glycine and 20% (v/v) methanol) using a wet-blot transfer system (Bio-Rad) at 100V for 1 hr. The PVDF membranes were washed momentarily in Tris-buffered saline (0.02 M Tris base and 0.15M NaCl, pH 7.4) containing 0.1% Tween 20 (TBST) followed by blocking in 5% (w/v) skim milk in TBST for 30 min. The membranes were then imaged using the ChemiDoc™ MP Imaging System for total protein normalization followed by probing with a polyclonal human anti-SPAG6 antibody (1:200 dilution) produced in rabbit (*Sigma-Aldrich,* St. Louis, MO, USA) in TBST overnight at 4°C under constant slow rocking. After washing in TBST, the membrane was probed with 1:10000 horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (Sigma-Aldrich) in TBST at RT for 1 h and then washed 3 times in TBST for 10 min each. Membrane probed with secondary antibody alone served as a negative control, whereas, membrane probed with the SPAG6 antibody pre-incubated with its corresponding blocking peptide, the SPAG6 antigen (Sigma-Aldrich), for 2 h at RT under constant slow rocking followed by probing with secondary antibody to verify the binding affinity for the primary antibody for its antigen. All membranes were finally subjected to visualization by Clarity™ Western enhanced chemiluminescent (ECL) blotting substrate (Bio-Rad), and the images were acquired on the ChemiDoc™ MP Imaging System. Abundances of SPAG6 protein were quantitated by normalizing the densities of the protein bands to that of the total loaded protein per lane as measured by stain-free imaging technology. Calculations of relative SPAG6 abundances were performed using Image Lab™ Software (Version 5.2.1; Bio-Rad).

Immunocytochemistry

Immunocytochemistry was used to localize the presence of SPAG6 antigen in the sperm, according to the method described previously (Bi et al., 2012). Briefly, a 20 µl aliquot of sperm suspension containing $1x10^5$ sperm cells from ACRB roosters at room temperature was placed on a clean microscope slide; a smear was prepared and allowed to air dry for 40 s. The air-dried slides were fixed with 4% formaldehyde in PBS for 10 min. The sperm cells were subjected to permeabilization with 0.2% Triton-X 100 in PBS for 20 min at room temperature and blocked with 1% skim milk for 30 min. Blocked cells were probed with anti-SPAG6 antibody overnight at 4°C. Probed samples were washed with PBS 5 times for 3 min each and probed with HRP-conjugated goat anti-rabbit Alexa Fluor 488 secondary antibody (Thermo Fisher Scientific) for 1 h at RT. Samples probed with only HRP-conjugated goat anti-rabbit Alexa Fluor 488 secondary antibody served as a negative control. Following incubation, the sections were washed five times with PBS for 3 min each and overlaid with a coverslip. Images were visualized and captured using an TH4 100 fluorescence microscope (Olympus Corp., Tokyo, Japan).

Immunogold transmission electron microscopy

A post embedding immunogold transmission electron microscopy protocol was followed to confirm the localization of SPAG6 antigen in the sperm flagella. Fixation of sperm was carried out with electron microscopy grade 4% formaldehyde, 1% glutaraldehyde (v/v) (Electron Microscopy Sciences, Hatfield, PA, USA) in 1x Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS; pH 7.4) overnight at 4ºC. Following fixation, the samples were dehydrated in graded ethanol solutions (50% ethanol for 15min, 75% ethanol 2 times for 30min each at RT). The samples were then infiltrated with 50% and 75% (v/v) LR White hydrophilic acrylic resin (Polysciences, Inc., Warrington, PA, USA) in 95% ethanol for one hour and overnight,

respectively. The final infiltrations were performed with 100% LR White resin two times, each for 1 hour. The specimens were then embedded in gelatin capsules with fresh LR White resin and heated to 60°C for 24 hours for polymerization. The samples were sliced into ultrathin 70-nm sections on a RMC MT-X ultramicrotome (Boeckeler Instruments, Inc., Tuscon, AZ) and mounted on Formvar-carbon coated nickel grids (Electron Microscopy Sciences). Blocking of sections was performed with 1% (w/v) bovine serum albumin (Sigma-Aldrich) in PBS (BSA-PBS) for 30 min, and grids were then jet washed with 1% BSA-PBS for 1 min. Next, grids were incubated with rabbit anti-human SPAG6 primary antibody (Sigma-Aldrich) diluted 1:50 in 1% BSA-PBS overnight at 4ºC in a moist chamber and then jet washed with 1% BSA-PBS for 1 min. Grids were incubated with 10-nm gold-conjugated goat antiserum against rabbit IgG secondary antibody (BBI Solutions, Crumlin, UK) diluted 1:20 in 1% BSA-PBS for 1hr at RT and then jet washed for 1min with 1% BSA-PBS followed by 1min with deionized (DI) water. The sections were then post-stained with 0.5% uranyl acetate for 5min followed by staining with lead citrate (Sigma-Aldrich) for 5 min at RT; grids were jet washed with DI water for 1min following each staining step. The stained sections were observed and analyzed using a JEOL JEM1011 transmission electron microscope (JEOL, Inc., Peabody, MA). Imaging were captured using a charge-coupled device camera (Advanced Microscopy Techniques, Corp. Woburn, MA).

Statistical analysis

Statistical analyses were performed with R 3.5.1 software (The R Foundation for Statistical Computing, Vienna, Austria). Differences in relative expression of SPAG6 between tissues of the reproductive tract, stages of sperm maturation and high- and low-quality sperm were compared by two-way ANOVA, with experimental replicates considered for blocking variables. The relationship between SPAG6 abundance and sperm progressive mobility was

tested by the Spearman rank correlation coefficient test. Differences were considered significant at $p < 0.05$, and significant differences were compared by Tukey's honest significant difference test post hoc.

Results

Sequential expression of SPAG6

Abundance of SPAG6 was evaluated by Western blotting in tissues of the male reproductive tract and in maturing sperm isolated from each area of the reproductive tract of ACRB roosters. Western analysis of testicular, epididymal and vasal tissues reveals a sequential increase in SPAG6 in the tissues (Figure 5.1). Testicular tissues exhibited the lowest concentration of SPAG6, vasal tissues exhibited the highest, at nearly twofold the concentration of that found in testicular tissues, and epididymal tissues had 21% higher SPAG6 abundance than testicular tissues ($p < 0.05$). Maturing sperm exhibited a similar pattern, with SPAG6 increasing in abundance as sperm progressed from the testis, epididymis and vas deferens to mature, ejaculated sperm (Figure 5.2). Ejaculated sperm had almost threefold the SPAG6 of sperm found in the testis ($p < 0.05$).

Localization of SPAG6

Fluorescence immunocytochemistry and immunogold TEM were used to verify localization of SPAG6 in the sperm flagella of rooster sperm. Fluorescence immunocytochemistry against SPAG6 resulted in strong, consistent fluorescence of the tail regions of spermatozoa, with some strong fluorescence in the midpiece and mild fluorescence in the head region (Figure 5.3). TEM displayed distinct gold-nanoparticle labeling in the space between the central pair of microtubules of the flagellar axoneme but no clear pattern of labeling elsewhere on the sperm cell (Figure 5.4).

Relationship between SPAG6 abundance and sperm mobility

The relative differences in SPAG6 abundance by mobility were tested by Western analysis of low- and high-quality sperm separated by PDGC as well as individual sperm samples characterized by their mobility, all taken from AYP broiler breeder roosters. Sperm separated into low- and high-quality groups by PDGC exhibited no significant differences in SPAG6 abundance (Figure 5.5). When sperm mobilities of individual sperm samples were compared by their SPAG6 abundance, a correlation approaching significance was found (Figure 5.6). Sperm mobility of a sample tended to decrease with increasing SPAG6 abundance ($p = 0.059$).

Discussion

The objectives of this work were to characterize the expression of SPAG6 in rooster sperm and to investigate SPAG6 as a biomarker of rooster sperm mobility. Our results show that SPAG6 is expressed throughout the male reproductive tract, that it increases in abundance as sperm mature, localizes not only into the flagellar axoneme but also in the head and midpiece and that SPAG6 abundance is not a strong predictor of sperm mobility.

The sequential expression of SPAG6 observed indicates that SPAG6 continues to accumulate in sperm after development in the testis. This is despite SPAG6 typically being found in the flagellar axoneme, which completes development within the testis (Lehti & Sironen, 2017; Sapiro et al., 2000). The increased abundance of SPAG6 in the epididymis is likely confounded by the fact that the epididymis contains stereocilia, non-motile modifications to the cell closely related to microvilli (Tingari, 1971). The stereocilia in the epididymis have not been shown to contain SPAG6, but stereocilia found in other tissues have been demonstrated to express the protein (Wang et al., 2015). The possibility of SPAG6 presence in stereocilia of the epididymis does not account for the increased abundance of SPAG6 in the vas deferens or in matured sperm

relative to sperm from the testes. Despite being an important protein in the sperm flagellum, SPAG6 has been found in the acrosomal region of canine sperm in addition to being in the tail and midpiece, which is consistent with our findings from fluorescence microscopy (Phillips & Verstegen, 2009). While an important protein for flagellar structure, SPAG6 may also be secreted onto the surface of the acrosomal region as one of the many sperm maturation proteins secreted from the reproductive tract during extragonadal maturation (Jones, 1998).

SPAG6 is reported to be found in sperm from sub-fertile roosters at 1.1-fold the abundance of that found in sperm from fertile roosters (Soler et al., 2016). Upon investigating the impact of SPAG6 abundance on sperm mobility, given its function in the sperm flagellum, no significant relationship was observed. Degree of abundance of SPAG6 is likely to impact sperm mobility, as knockout studies have shown that SPAG6 is necessary for adequate sperm motility and that mice possessing only one functional copy of the *Spag6* gene had higher sperm motility than those with two functional copies (Sapiro et al., 2002). This considered, SPAG6 abundance alone was not a significant predictor of sperm mobility.

SPAG6, alone, would not function well as a biomarker of sperm mobility; however, it may serve as a biomarker of sperm mobility when used in conjunction with others. SPAG6 has still been clearly demonstrated to be a protein biomarker of sub-fertility in roosters, and SPAG6 appears to localize in areas other than the flagellar axoneme of sperm (Phillips & Verstegen, 2009; Soler et al., 2016). This indicates that the impact of SPAG6 abundance on rooster fertility may not be entirely due to an impact on sperm mobility. More work is needed to elucidate the purpose of SPAG6 localization outside of the sperm tail region. This will help to determine the overall effect of the degree of SPAG6 abundance on rooster fertility and to evaluate the amount

of information which may be inferred from the abundance of SPAG6 in a sperm sample for selection of roosters for breeding.

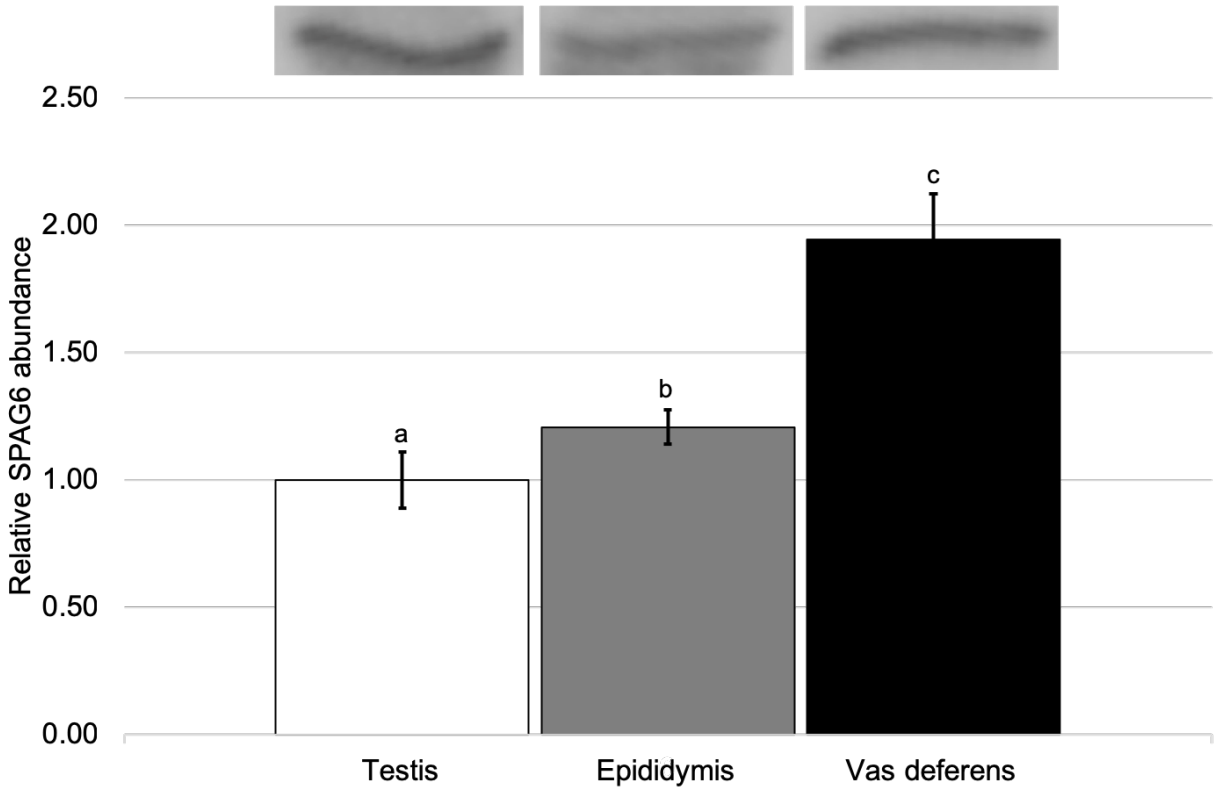

Figure 5.1. The relative abundance of SPAG6 found in the testis, epididymis and vas deferens tissues. Values represent mean ± SEM relative to the mean testis tissue SPAG6 abundance. SPAG6 abundances are normalized relative to total loaded protein per lane as measured by stain-free technology. Each biological replicate represents a pool of samples from 10 individuals. [a-c]Indicates significant difference in means, $p < 0.05$ ($n = 2$).

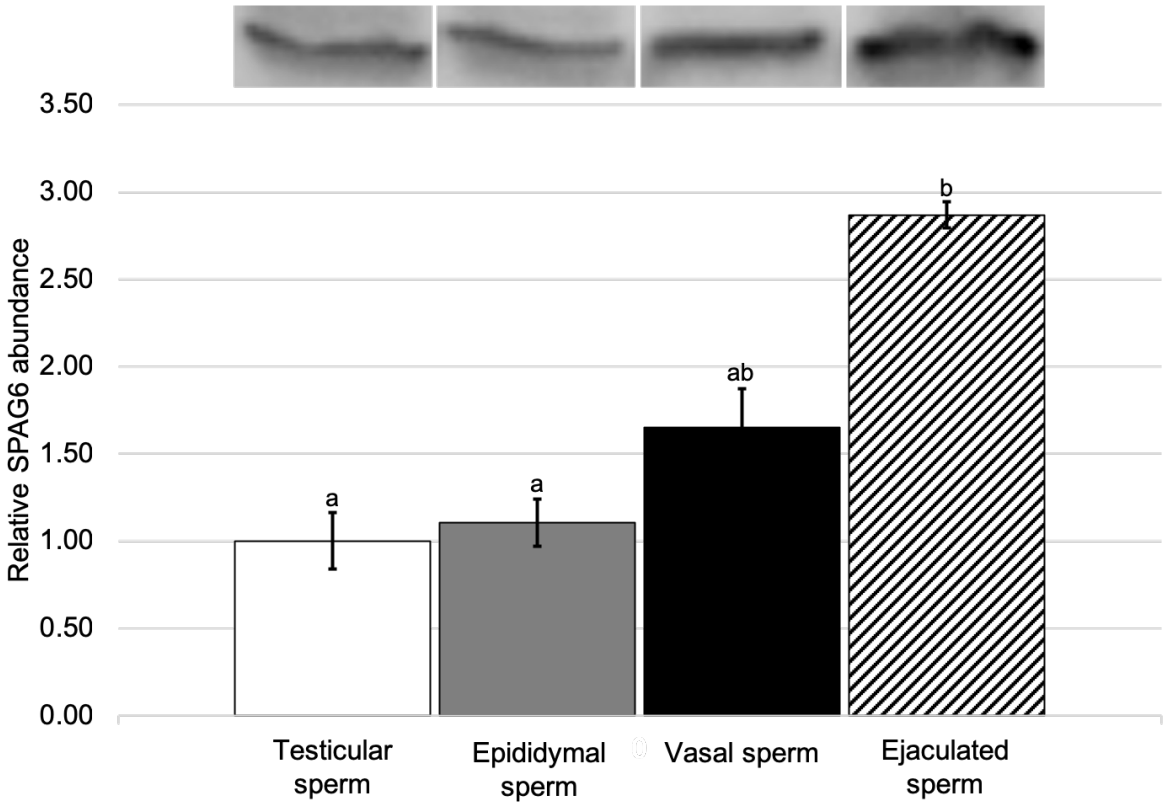

Figure 5.2. The relative abundance of SPAG6 found in sperm collected from the testis, epididymis, vas deferens and in ejaculate. Values represent mean ± SEM relative to the mean testicular sperm SPAG6 abundance. SPAG6 abundances are normalized relative to total loaded protein per lane as measured by stain-free technology. Each biological replicate represents a pool of samples from 10 individuals. [a-b]Indicates significant difference in means, $p < 0.05$ ($n = 2$).

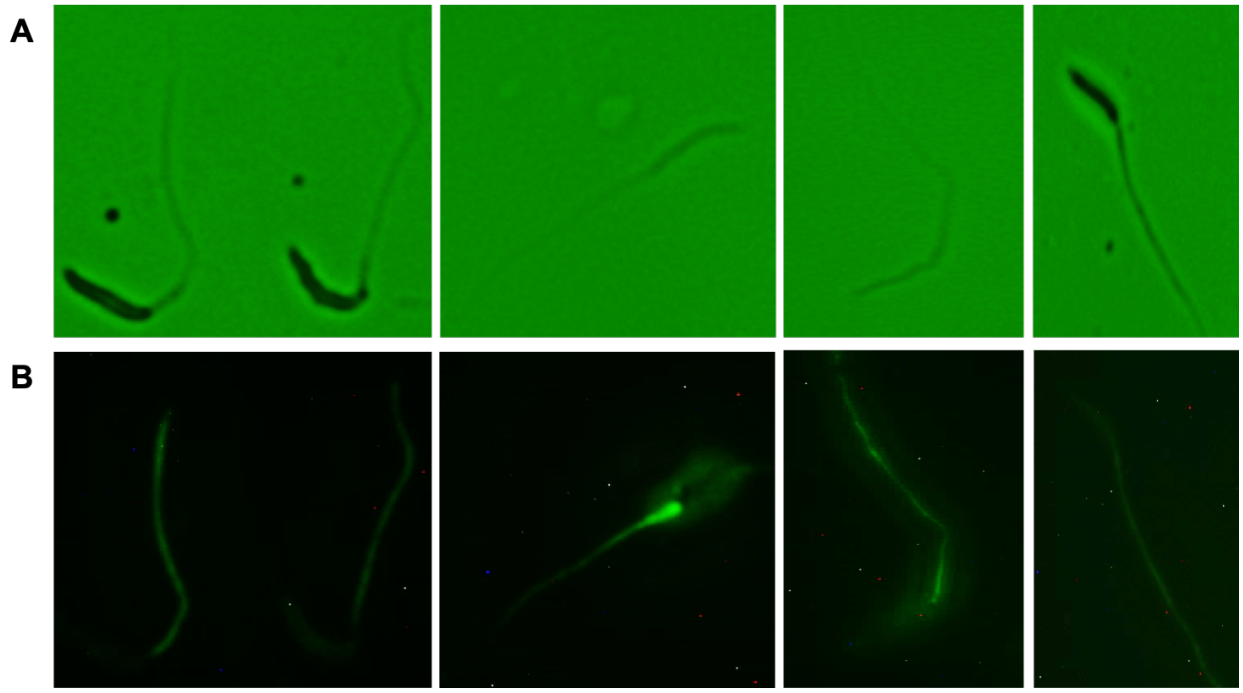

Figure 5.3. Location of sperm SPAG6 by fluorescence microscopy. A) Phase contrast microscopic images of sperm treated for fluorescence microscopy. B) Fluorescence imagery corresponding to each phase contrast image, displaying clear localization of SPAG6 in the flagellum, as well as strong fluorescence in the sperm midpiece and mild fluorescence in the head region.

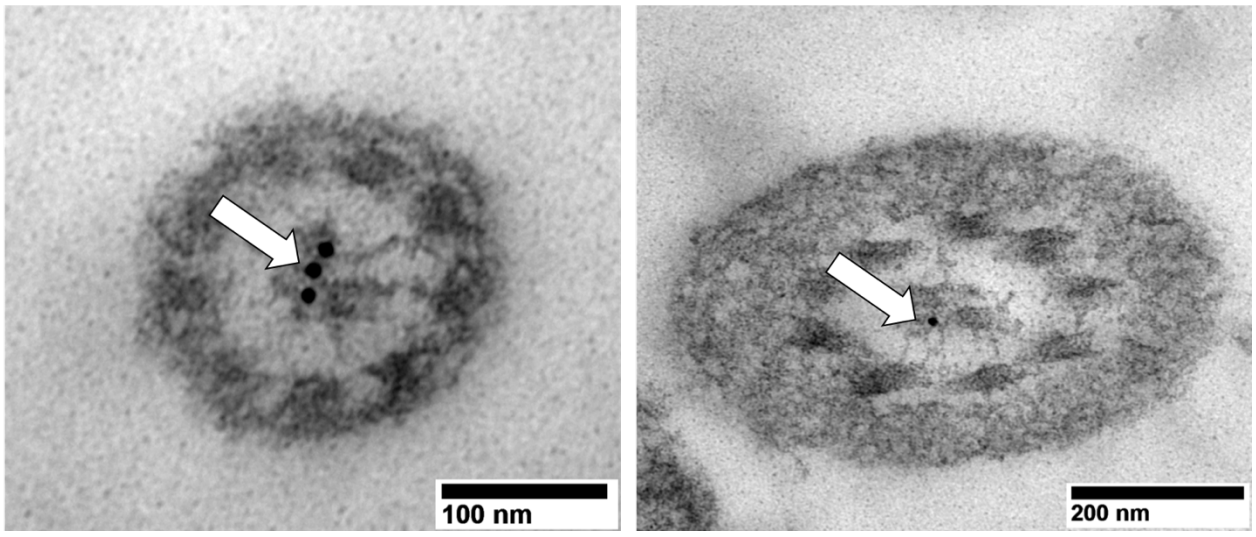

Figure 5.4. Immunogold transmission electron microscopy of the localization of SPAG6 in the rooster sperm axoneme. White arrows emphasize presence of gold nanoparticle, indicating localization of SPAG6 between the central pair of microtubules.

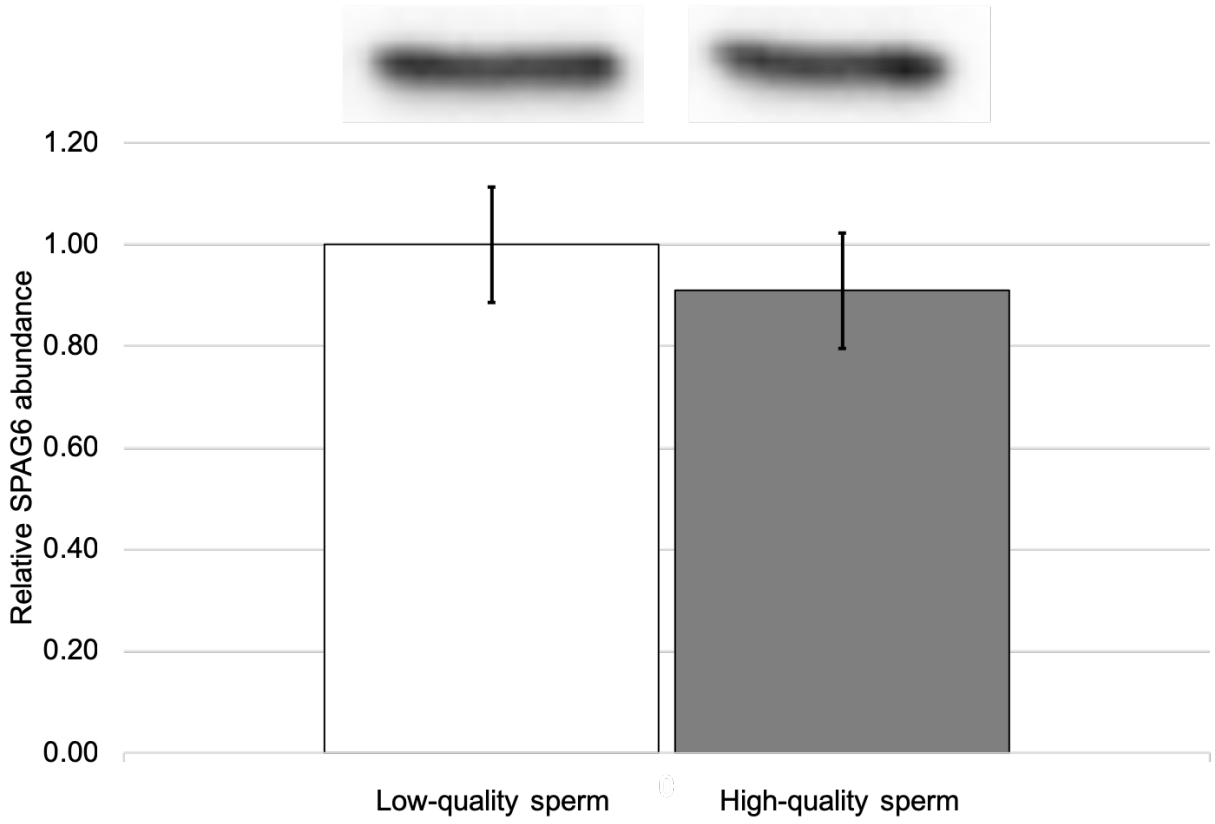

Figure 5.5. The relative abundance of SPAG6 found in low- and high-quality sperm separated by Percoll density gradient centrifugation. SPAG6 abundances are normalized relative to total loaded protein per lane as measured by stain-free technology. Values represent mean ± SEM, $p > 0.05$ ($n = 3$).

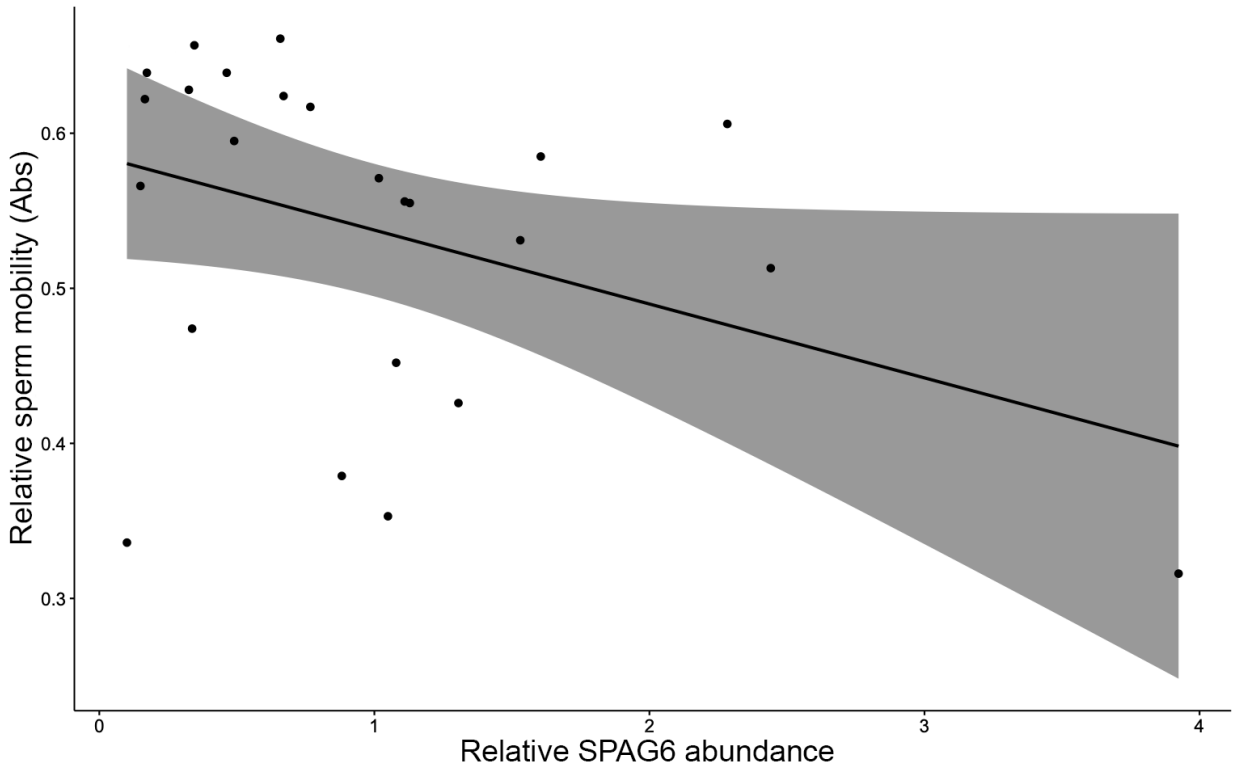

Figure 5.6. The correlation between SPAG6 abundance and sperm mobility. The shaded area represents the confidence interval of the regression line shown in black. SPAG6 abundances are normalized relative to total loaded protein per lane as measured by stain-free technology. $p > 0.05$, $R = -0.39$ ($n = 24$).

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CHAPTER 6

HEPARIN-STABILIZED GOLD NANOPARTICLE-BASED QUANTIFICATION OF ROOSTER (*GALLUS DOMESTICUS*) SPERM PROTAMINATION AND SPERM PROTAMINATION AS AN INDICATOR OF ROOSTER SPERM QUALITY[1]

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Abstract

Replacement of sperm histones with protamine is an important mechanism of spermiogenesis, and adequate protamination is necessary for the fertilizing ability of individual sperm cells. Sperm protamination is inconvenient and/or expensive to directly assess in large volumes of samples in most species. The advent of heparin-stabilized gold nanoparticles (Hep-AuNP) and the interaction which occurs between heparin and protamine provide a route for simple assessment of sperm protamination when appropriately paired with sperm protamine extraction techniques. In this study, a joint method of sperm protamine extraction and Hep-AuNP-based quantification of sperm protamination was developed and applied for characterization of sperm protamination as an indicator of rooster (*Gallus domesticus*) sperm quality and fertility. After successful development of methodology to use Hep-AuNP-based quantification with freshly extracted sperm protamine, sperm protamination was found to be a predictor of rooster sperm chromatin condensation but not of sperm mobility nor viability, nor did it correlate with rooster fertility. Chromatin condensation was found to be decreased in high-quality sperm relative to low-quality sperm. Hep-AuNP-based quantification of sperm protamination is a promising and economical method for measuring sperm protamination; however, its application in the domestic chicken is limited.

Keywords: sperm protamination, heparin, gold nanoparticle, broiler breeder, fertility

Introduction

During spermiogenesis, the conversion of a round spermatid to an elongated spermatid, several cellular rearrangements occur. Notable of these is the near-total epigenetic reprogramming of the sperm genome and the intense condensation of sperm chromatin, which contributes to the ability of the sperm head to take on a hydrodynamic shape (Aire, 2014; Balhorn, 2007). This is accomplished by the replacement of most sperm histones by protamines, proteins much smaller and more densely cationic than histones. Among vertebrates, protamines contain anywhere from 30-70% arginine residues, making them highly positively charged at physiologic pH and capable of very tightly binding the negatively charged DNA backbone (Balhorn, 2007). The chromatin condensation which protamination accomplishes protects the sperm genome against damage and fragmentation, and inadequate chromatin condensation typically results in abnormal morphology of the sperm head (Boe-Hansen et al., 2018; Menkveld et al., 2011). Degree of protamination of sperm has also been suggested to influence the rate of decondensation of the sperm pronucleus during fertilization (Águila et al., 2017; Perreault et al., 1988). Degree of sperm protamination has been implicated to influence male fertility in several mammalian models, and, while protamination has not been investigated in the domestic chicken (*Gallus domesticus*), chromatin condensation has been of recent interest in study of broiler breeder rooster fertility (Banaszewska et al., 2015; Dogan et al., 2015). Protamination is a sperm characteristic which may serve as a parameter of interest in selecting sires for breeding programs employed for agriculturally important animals.

Several methods for quantifying sperm protamination have been previously applied. These include immunoassays, chromatographic methods and indirect measurement of chromatin condensation. Each of these techniques possess limitations for use in assessing protamination to

inform selection of breeding stock. Immunoassays require anti-protamine antibodies, and due to the high degree of variation in protamine sequences between closely related species, these techniques usually require production of species-specific antibodies (Dogan et al., 2015; Rhim et al., 1995). Chromatographic methods employed are useful for analysis of sperm of any species but relatively expensive to perform for a large volume of samples to inform breeding programs (Hamilton et al., 2019; Schindler et al., 1991). Assessment of chromatin condensation is relatively simple and inexpensive; however, it is not a true measurement of sperm protamination (Dogan et al., 2015).

The high density of positive charges exhibited by protamines of any vertebrate species results in the ability of protamine to interact with heparin in solution, as heparin is a highly anionic macromolecule (Sommers et al., 2017). Heparin and protamine aggregate when in solution together, and this interaction between protamine and heparin has been utilized for a variety of medical and diagnostic applications in the last 50 years, with protamine frequently being used to remove the effects of a previous heparin injection (Hemker, 2016; Sommers et al., 2017). Recently, heparin-stabilized gold nanoparticles (Hep-AuNP) have been shown to be useful for quantifying the protamine concentration of a solution. Hep-AuNP, which are normally red in color, shift to a blue color with an absorbance peak at 750 nm when treatment with protamine causes the nanoparticles to aggregate (Bener et al., 2018; Vidya & Saji, 2018). Hep-AuNPs have not yet been applied for directly measuring protamination of a sperm sample but have been used to accurately measure known protamine concentrations in protamine-spiked seminal plasma (Vidya & Saji, 2018). It still remains to be seen whether Hep-AuNPs may be applied for measuring protamine which has been directly extracted from sperm sample.

The ability of Hep-AuNP to quantify protamine concentration based only on the cationicity of protamine, which is extraordinarily high in all studied vertebrates, has potential to be used as a low-cost diagnostic tool for measuring sperm protamination in any vertebrate. The objectives of this study were 1) to develop a method of Hep-AuNP-based quantification of protamine freshly extracted from sperm and 2) to apply Hep-AuNP-based quantification of sperm protamination in the characterization of sperm protamination as an indicator of sperm quality in broiler breeder roosters.

Materials and Methods

Synthesis and preparation of heparin-gold nanoparticle (Hep-AuNP)

All glassware was washed in 10% HCl and rinsed in deionized (DI) water. Hep-AuNPs were prepared as described (Guo & Han, 2008) with the following modifications. After 20 mL of DI water was brought to a rapid boil under reflux and constant stirring under a fume hood, 20 mL 0.025% $HAuCl_4$ (Sigma-Aldrich, St. Louis, MO, USA) was added into the reaction vessel. After 5 min of reflux, 10 mL 5% porcine heparin sodium salt (MilliporeSigma, Burlington, MA, USA) was added to the vessel. The reaction continued under reflux and constant stirring for 90 min in low light. At the end of the 90 min period, the solution formed a brilliant pink color, indicating the formation of Hep-AuNPs.

The solution was dispensed in 1.5 mL aliquots into microcentrifuge tubes and washed twice by centrifugation at 6000 x *g* for 30 min followed by removal of supernatant and reconstitution in DI water. Following washing, Hep-AuNPs were centrifuged at 6000 x *g* for 30 min and supernatant removed. Hep-AuNPs were reconstituted at 50x concentration of the original solution, and the concentration of Hep-AuNPs in the solution was quantified by dehydration via a SpeedVac vacuum concentrator (Thermo Fisher Scientific, Waltham, MA,

USA) followed by weighing. Hep-AuNPs were kept in a stock solution at 1.25 mg/mL in DI water at 4 ºC.

Animals

Twenty roosters were sampled from the University of Georgia's Athens Canadian Random Bred (ACRB) breeder flock for comparison between sperm protamination and traditional parameters of rooster sperm quality. Three Cobb 500 broiler breeder roosters were sampled for comparison between sperm protamination and fertility, in order to test the efficacy of Hep-AuNP as an indicator of broiler breeder fertility. Fifteen Hy-Line layer hens were used for artificial insemination. The birds were grown to target weight and moved to individual cages for photostimulation. They were allowed water and a commercial layer ration (Southern States, Richmond, VA, USA) *ad libitum*. ACRB semen samples were taken via the massage method described by Burrows & Quinn (1937) at 38 and 45 weeks of age and transported in glass tubes, with 38 week semen samples being used for comparison of individual sperm protamination against traditional parameters and 45 week semen samples being used for comparison of chromatin condensation and sperm protamination between low- and high-quality sperm. Cobb 500 semen samples were taken when the roosters were 70 weeks of age. The roosters were reared and maintained according to the rules set forth by the University of Georgia's Institutional Animal Care and Use Committee.

Optimization of Hep-AuNP aqueous environment

Hep-AuNP was tested for sensitivity to KH_2PO_4 buffer at various buffer concentrations. Stock Hep-AuNP was diluted to a final concentration of 50 µg/mL in 0.01, 0.05, 0.10 & 0.20 M KH_2PO_4, all previously adjusted to a pH of 7.4 using 10 M KOH. After 10 min for reaction in low light, absorbance was measured via spectrophotometer at 750 nm. Buffer materials were

chosen in order to minimize exposure of Na^+ to Hep-AuNP, as minor sensitivity of Hep-AuNP to Na^+ has been documented (Vidya & Saji, 2018). Hep-AuNP was similarly tested for sensitivity to the pH of loaded samples by the addition of HCl solutions at a range of pHs of 1-13. Sensitivity was tested by measuring for shifts in absorbance. Stock Hep-AuNP was diluted in 0.10 M KH_2PO_4 buffer to a final concentration of 50 µg/mL. HCl solutions were added 1:44 into diluted, buffered Hep-AuNP in a cuvette.

Extraction of sperm protamine

Sperm protamine was extracted by a modification of the methods described by Gill et al. (2006). Fresh semen was added to a microcentrifuge tube in 200 µL aliquots, centrifuged at 1000 x *g* for 10 minutes and seminal plasma discarded. The sperm pellet was reconstituted in 200 µL 0.325 M HCl and incubated at RT for 1 hour. Acid digested sperm samples were centrifuged at 6000 x *g* for 20 min, and 30 µL supernatant was added to a microcentrifuge tube containing 1.230 mL 0.1 M KH_2PO_4 buffer. The buffered sample was centrifuged at 6000 x *g* for 30 min. Supernatant was stored in at 4 ºC in a clean microcentrifuge until measurement with Hep-AuNP.

Hep-AuNP-based quantification of sperm protamination

After optimization of the Hep-AuNP environment for protamine extraction methods, the following standard protocol was used for Hep-AuNP-based quantification of sperm protamination. A stock standard salmon protamine sulfate (Sigma-Aldrich) was prepared at 10 µg/mL in 0.10 M KH_2PO_4. A standard curve was generated adding 27 µL of protamine sulfate standards at 6, 4, 2, 1 and 0.5 µg/mL to 1107 µL 0.1M KH_2PO_4 in a square polystyrene cuvette. Extracted protamine samples were diluted in 0.10 M KH_2PO_4 by a factor suitable for comparison against the standard curve, typically 100-1200 times, and 1134 µL diluted sample were added to a square polystyrene cuvette. A polystyrene cuvette containing 1134µL 0.10 M KH_2PO_4 was

used as a blank. For the blank, each standard and each sample, 45 µL stock Hep-AuNP was mixed into the cuvette by pipetting and incubated at RT in low light for 10 min. Absorbance was measured via spectrophotometer at 750 nm within 15 min after the end of incubation. Standard measures were performed in triplicate, and sample measures were performed in duplicate. Protamine quantification was calculated against the generated standard curve and total sample dilution.

Sperm mobility

Semen was dispensed in 0.5 mL aliquots into microcentrifuge tubes. Semen was washed twice by centrifugation at 1000 x *g* followed by removal of supernatant. The sperm pellet was reconstituted in 1.0 mL phosphate-buffered saline (PBS; pH 7.4) after the first centrifugation and to a total volume of 0.5 mL in motility buffer (MB: 111 mM NaCl, 25.0 mM glucose, 4.00 mM $CaCl_2$, 50.2 mM TES; pH 7.4) after the second centrifugation. Prior to analysis for sperm mobility, sperm count was assessed by hemacytometer counting as previously described (Freund & Carol, 1964).

The Accudenz assay, validated for objective quantification of chicken sperm mobility prior (Froman & McLean, 1996), was performed with the following modifications. A 30% (w/v) Accudenz (Accurate Chemical & Scientific Corp., Westbury, NY, US) stock solution (3 mM KCl, 5 mM TES; pH 7.4) was diluted 1:4 with MB. The 6% Accudenz solution was preheated to 41ºC and 1.00 mL of Accudenz solution was added to polystyrene cuvettes. Prepared cuvettes were overlaid with 100 µL sperm sample diluted in MB to a concentration of 5.0 x 10^8 cells/mL and incubated at 41ºC for 10 minutes. After incubation, absorbance at 550 nm was read using a DU530 spectrophotometer (Beckman Coulter, Inc., Brea, CA, USA). Measures of sperm mobility by the Accudenz assay were performed in triplicate.

Sperm viability

Eosin-nigrosin vital staining was performed as previously described (Chalah & Brillard, 1998) with the following modifications. In a microcentrifuge tube, 50 µL of washed sperm sample was prepared at a concentration of 1 x 10^8 cells/mL by dilution in MB. Eosin-nigrosin stain (2.5% (w/v) eosin, 5% (w/v) nigrosin) was added to the sample 1:1 and mixed by vortexing. The mixture was incubated at RT for 5 min. A 20 µL drop of the mixture was added to a glass slide, smeared, allowed to air-dry, and cover-slipped. Slides were observed under a TH4 100 microscope (Olympus Corp., Tokyo, Japan) and dead sperm and total sperm counted. Percentage viability reported reflects the percentage of live sperm out of a minimum of 100 cells counted.

Sperm chromatin condensation

Aniline blue staining for chromatin condensation as previously described (Banaszewska et al., 2015) was performed with the following modifications. Fresh semen was diluted 1:15 in PBS and smeared on a clean glass slide. After air-drying, smears were overlaid with glutaraldehyde (Sigma-Aldrich) diluted to 3% in PBS at RT. After 30 min incubation, fixed smears were rinsed in DI water and air-dried. Dried smears were stained with 2.5% aniline blue stain in 0.70 M acetic acid (pH 3.5) for 7 min at RT. Stained slides were rinsed in DI water and air-dried. Slides were observed under a TH4 100 microscope and blue stained sperm and total sperm counted. Percentage normal head condensation was calculated based on a minimum of 100 total cells counted.

Rooster fertility

For each of the Cobb 500 broiler breeder roosters sampled, 5 layer hens were artificially inseminated (AI) intravaginally with 0.05 mL fresh semen. Starting at two days after AI and

continuing for a total of 6 days, eggs were collected from each hen and incubated. After 10 days of incubation, eggs were candled, and percent fertile eggs of the total eggs incubated from each hen was calculated. Fertilized eggs were euthanized by CO_2 gas asphyxiation.

Separation of sperm by quality

Percoll density gradient centrifugation (PDGC), previously described and validated for separation of avian sperm by quality (Ahammad et al., 2018), was performed with the following modifications to produce sperm samples of differing quality. Gradients consisting of 3 mL each of 1.08 and 1.07 g/mL Percoll solutions were overlaid with a total of 3 mL fresh semen diluted 1:2 in PBS. Each semen sample was a pooled sample, representing at least 5 individual ACRB roosters. PDGC was performed in 3 mechanical replicates for each of 3 biological replicates (n = 3). Gradients were centrifuged at 1500 x g for 20 min. The low-quality sample layer remaining above the 1.07 g/mL Percoll solution and the high-quality sample pellet collected below the 1.08 g/mL Percoll solution were collected for analysis by aniline blue staining and Hep-AuNP-based quantification of sperm protamination. Separated samples washed by dilution in PBS and centrifugation at 1000 x g, followed by exchange of supernatant with fresh PBS. Due to high variability in cell concentration of low- and high-quality sperm resulting from separation by PDGC, low- and high-quality sperm samples were counted via hemacytometer and adjusted to 1.0 x 10^9 cells/mL in PBS.

Statistical analysis

Statistical analyses were computed with R 3.5.1 software (The R Foundation for Statistical Computing, Vienna, Austria). The relationships of sperm protamination against sperm mobility, chromatin condensation and rooster fertility were tested by the Pearson rank correlation coefficient test. The relationship between sperm protamination and sperm viability was tested by

the Spearman rank correlation coefficient test, as sperm viabilities were not normally distributed as tested by the Shapiro-Wilk normality test. Low- and high-quality sperm were tested for differences in chromatin condensation by Student's t-test. Low- and high-quality sperm were tested for differences in sperm protamination by Welch's t-test, as sperm protamination failed Bartlett's test of homogeneity of variances. Analysis results were considered statistically significant at $p \leq 0.05$.

Results

Optimization of Hep-AuNP-based quantification of sperm protamination

In order to pair Hep-AuNP-based quantification of sperm protamination with a method for sperm protamine extraction, it was necessary that the assay be modified to tolerate the low pH required to extract protamine. KH_2PO_4 buffered to pH 7.4 with KOH did not disrupt the function of Hep-AuNPs at a concentration of 0.10 M or less. Higher concentrations resulted in a shift of the solution from red to blue (Figure 6.1.A). A 0.10 M KH_2PO_4 buffer used as a diluent of stock Hep-AuNP served to buffer Hep-AuNP analyses. Buffered Hep-AuNPs at 50 µg/mL were found to exhibit no sensitivity to any sample volume with a pH within the range of 3-13. Addition of a sample volume of pH 1 resulted in a complete shift in the color of Hep-AuNP from red to blue (Figure 6.1.B).

Commercial protamine sulfate served as a standard for Hep-AuNP-based quantification of sperm protamination. Concentrations of protamine sulfate from 0.5-6.0 µg/mL consistently generated a linear standard curve of Hep-AuNP absorbance (Figure 6.2).

Sperm protamination against traditional indicators of rooster sperm quality

Sperm protamination was compared against traditional indicators of rooster sperm quality in order to test its efficacy as a practical determinant of sperm quality. Sperm protamination did

not serve as a predictor of sperm mobility (Figure 6.3) and viability (Figure 6.4), but it did, however, serve as a strong predictor of sperm chromatin condensation ($p < 0.01$). Sperm protamination accounted for 37% of variation in sperm chromatin condensation among individual roosters assessed ($R = 0.6089$) (Figure 6.5). As with sperm mobility and viability, sperm protamination did not serve as a predictor of rooster fertility as determined by intravaginal AI (Figure 6.6).

Sperm populations were phenotyped into low- and high-quality groups by PDGC in order to determine the differences between the two groups in chromatin condensation and sperm protamination. High-quality sperm, as determined by PDGC, exhibited significantly lower chromatin condensation when compared with low-quality sperm ($p < 0.05$) (Figure 6.7.A). A matching but nonsignificant decrease in sperm protamination in high-quality sperm was also observed (Figure 6.7.B).

Discussion

The objectives of this work were to develop Hep-AuNP-based quantification of protamine for application with protamine freshly extracted from sperm and to use the method to characterize sperm protamination as a measure of sperm quality in the domestic rooster. This research indicates that Hep-AuNP-based quantification of protamine can be paired with a simple method of sperm protamine extraction and that Hep-AuNP-based quantification of rooster sperm protamination serves as a predictor of rooster sperm chromatin condensation but not of sperm quality in general.

Hep-AuNP-based quantification of protamine functions with freshly extracted sperm protamine when the aqueous interaction environment is adequately buffered. All common methods of sperm protamine extraction involve acid digestion followed by purification in some

type of precipitant and filtration system (Gill et al., 2006). In order to conserve quantitative differences between samples, precipitant and filtration steps must be avoided, meaning that the pH of the extract serves as an obstacle to Hep-AuNP-based quantification. The negative charges on heparin are necessary for Hep-AuNP to remain dispersed in solution when not in the presence of protamine (Vidya & Saji, 2018). Usage of 0.10 M KH_2PO_4 at physiologic pH stabilizes the Hep-AuNP against any effects of a loaded sample volume exhibiting a pH of 3 or higher. After digestion in 0.325 M HCl, protamine extracts are moderately acidic and require a minimum of 100x dilution in buffer, bringing samples well within the acceptable range of pHs for Hep-AuNP usage.

Hep-AuNP-based quantification of sperm protamination accounts for much of the variation seen in sperm chromatin condensation among individual roosters. Sperm protamination should be expected to serve as a predictor of chromatin condensation, as its presence is the direct cause of chromatin condensation in avian sperm (Balhorn, 2007). Sperm protamination is not a predictor of sperm mobility nor viability, two of the most important determinants of rooster sperm quality, nor does it show any correlation with rooster fertility (Bilgili & Renden, 1984; Froman et al., 1999). This is despite a previous claim that broiler breeder roosters from genetic lines with lower fertility exhibited lower progressive motility of sperm and lower chromatin condensation. That previous claim was, however, based on comparisons of two genetic lines which were not shown to be significantly different in fertility (Banaszewska et al., 2015). This lack of relationship between sperm protamination and traditional parameters of sperm quality is further supported the finding that high-quality sperm as separated by PDGC are actually lower in chromatin condensation and protamine content than low-quality sperm. PDGC has been

validated to separate sperm by quality according to more traditional sperm parameters like mobility and viability (Ahammad et al., 2018).

The lack of correlation with rooster fertility is contrary to all studies of sperm protamination in mammals, where adequate sperm protamination has been found to be necessary for male fertility and protamine has shown promise as a biomarker of sperm quality (Dogan et al., 2015; Francis et al., 2014; Steger & Balhorn, 2018). The lack of evidence for sperm protamination as an influencer of rooster fertility may be due to the fact that Aves undergo polyspermic fertilization (Snook et al., 2011). In cases where only one sperm is allowed inside the ovum, as is the case in mammals, the likelihood that each sperm contains an adequate amount of protamine and intact DNA is important. In the case of the chicken, where anywhere from 20 to as much as 60 spermatozoa gain access to the ova, it may be less imperative that each and every sperm cell is adequately protaminated (Nakanishi et al., 1990).

Hep-AuNP-based quantification of sperm protamination would not function well as an indicator of rooster fertility or sperm quality in broiler breeder lines, but it does show promise as a measure of sperm protamination to be applied in other species. The fault of the method in terms of application with broiler breeder roosters is that what it measures does not appear to carry much significance in domestic chickens. The method may show promise if applied for use in species where sperm protamination is already a demonstrated indicator of sperm quality and male fertility, such as in cattle (Boe-Hansen et al., 2018; Dogan et al., 2015). Hep-AuNP-based quantification of sperm protamination should be investigated and optimized for application in agriculturally important mammals, where it may serve a simple, inexpensive, low-training-required diagnostic of male fertility or infertility.

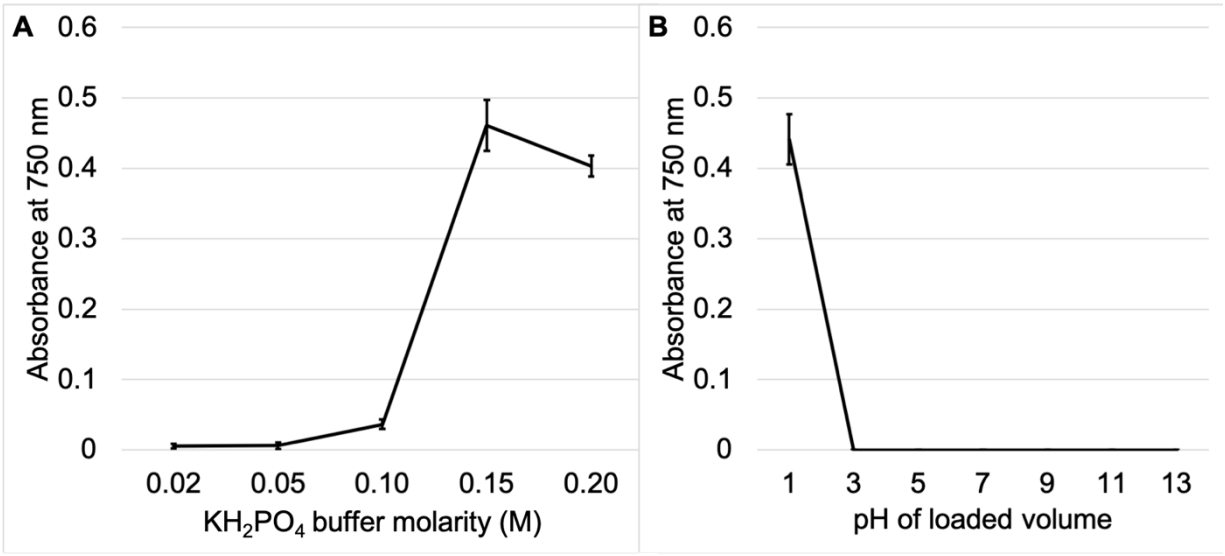

Figure 6.1. The sensitivity of Hep-AuNP to its aqueous environment. A) The absorbance of Hep-AuNP at 750 nm when diluted from stock concentration of 1.25 mg/mL to 50 µg/mL in KH_2PO_4 buffers (pH 7.4) of varying concentrations. B) The absorbance of 50 µg/mL Hep-AuNP in 0.10M KH_2PO_4 buffer at 750 nm after addition of HCl solutions of known pH at 1:44 HCl solution to HepAuNP solution. Values represent mean ± SEM (n = 3).

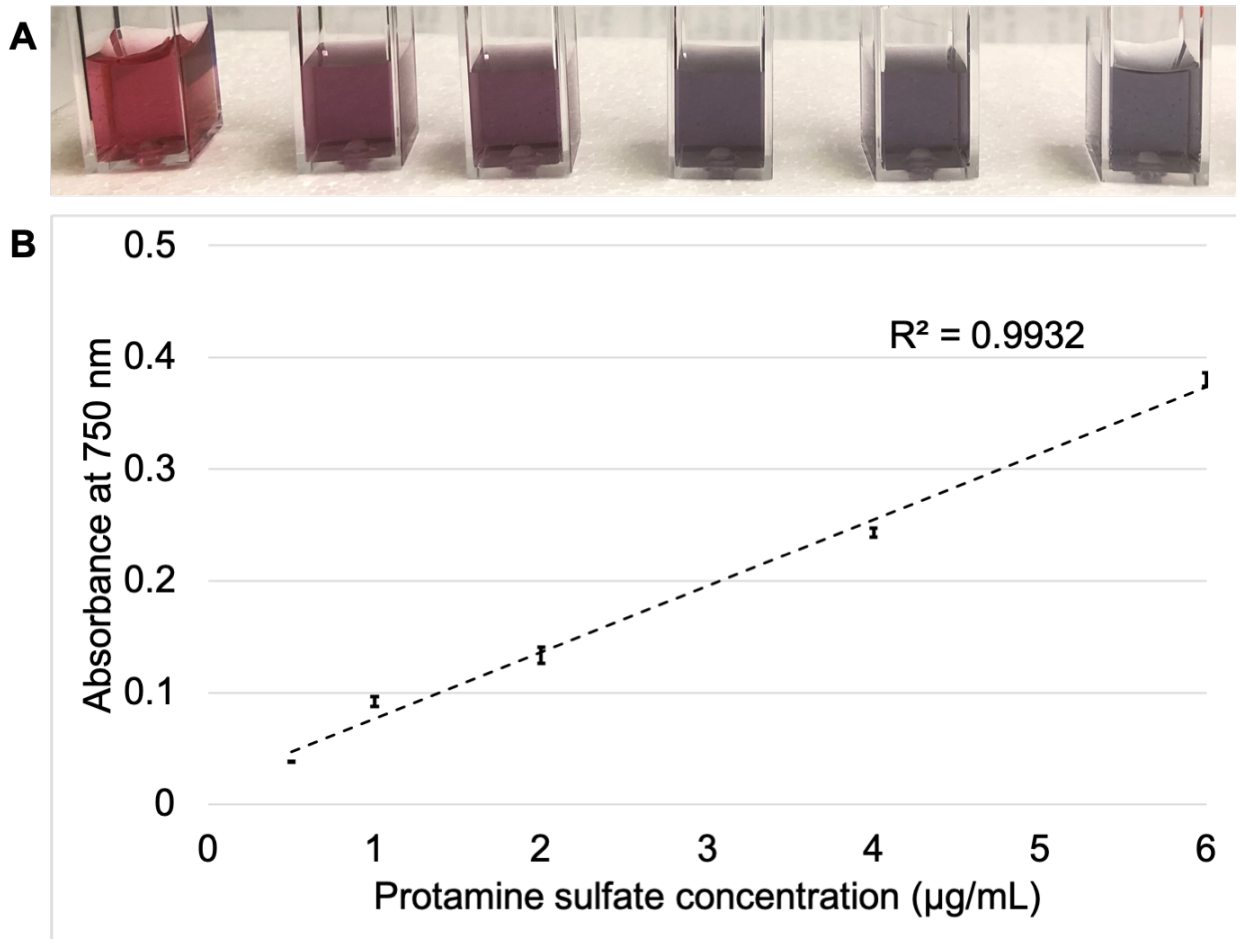

Figure 6.2. The standard curve of Hep-AuNP absorbance at 750 nm generated by addition of known protamine sulfate concentrations. A) The shift in visual tint of 50 µg/mL Hep-AuNP in 0.1 M KH_2PO_4 (pH 7.4) when dosed 1:44 with 0.0, 0.5, 1.0, 2.0, 4.0 and 6.0 µg/mL protamine sulfate (L-R). B) The absorbance at 750 nm of 50 µg/mL Hep-AuNP in 0.1 M KH_2PO_4 (pH 7.4) when dosed 1:44 with known protamine sulfate concentrations. Values represent mean ± SEM (n = 3).

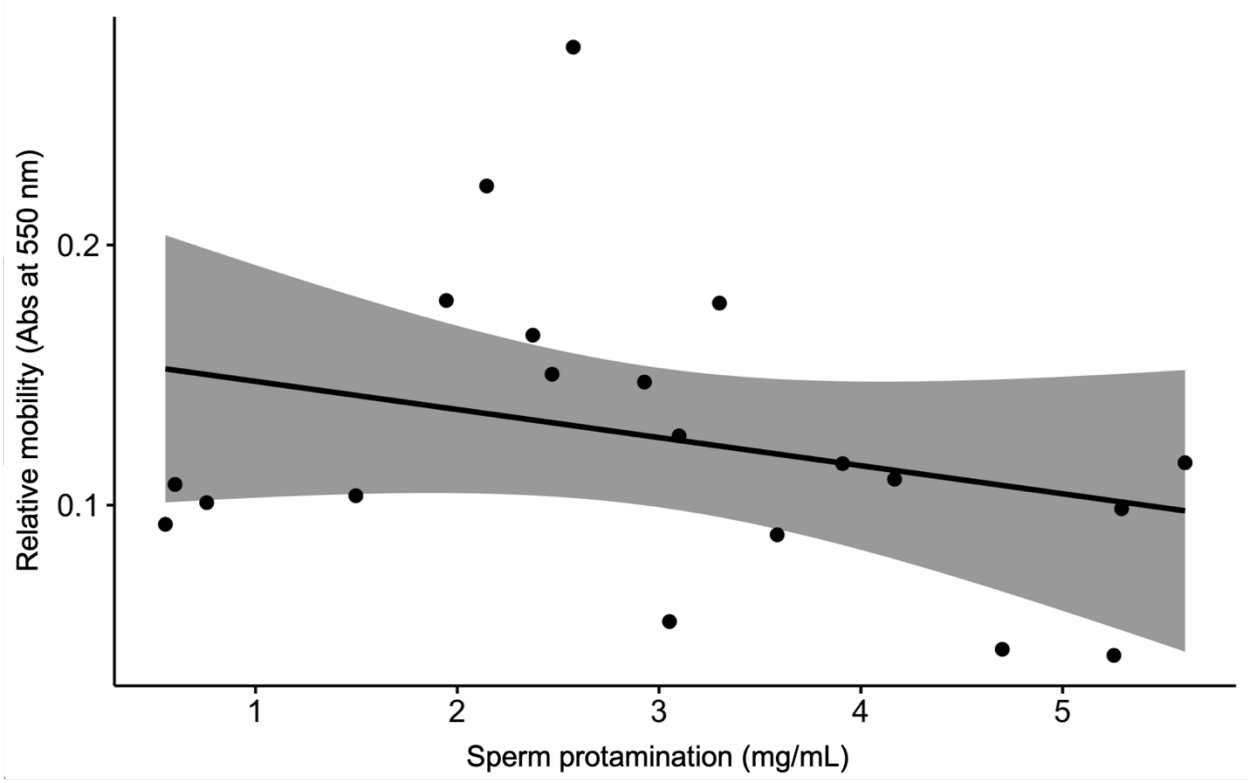

Figure 6.3. Sperm protamination as it relates to sperm mobility. The linear correlation between sperm mobility and sperm protamination. The shaded area represents the confidence interval of the regression line shown in black. $p > 0.05$ ($n = 20$).

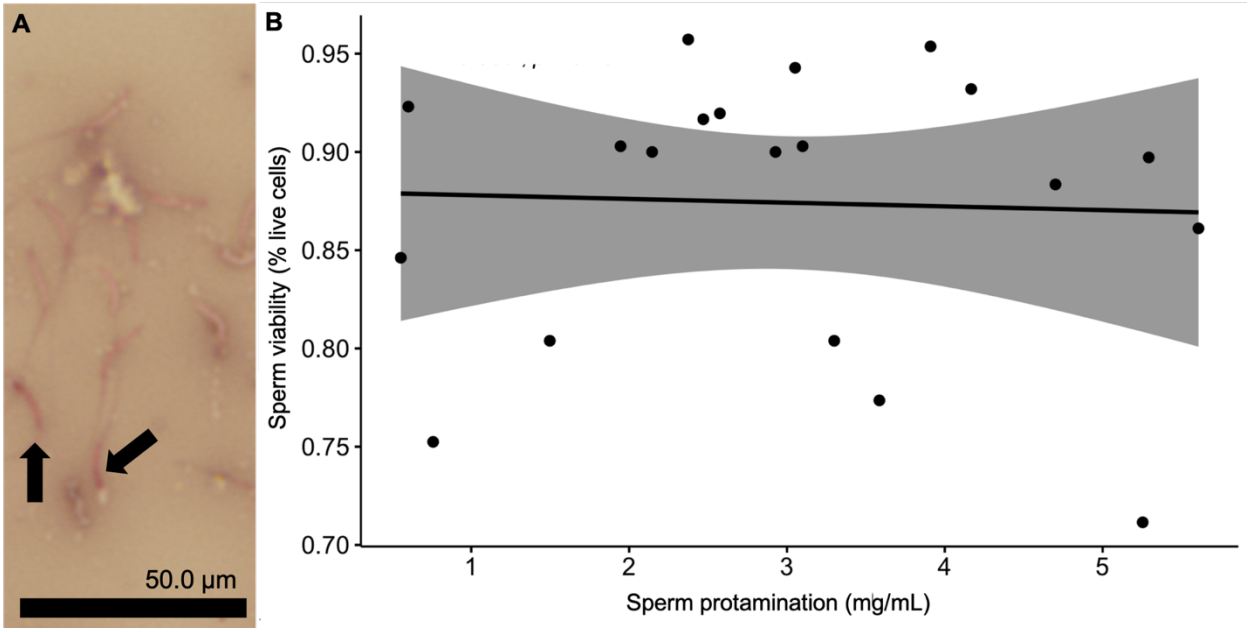

Figure 6.4. Sperm protamination as it relates to sperm viability. A) Eosin-nigrosin staining for cell viability. Black arrows indicate stained, dead sperm. B) The linear correlation between sperm protamination and sperm mobility. The shaded area represents the confidence interval of the regression line shown in black. $p > 0.05$ ($n = 20$).

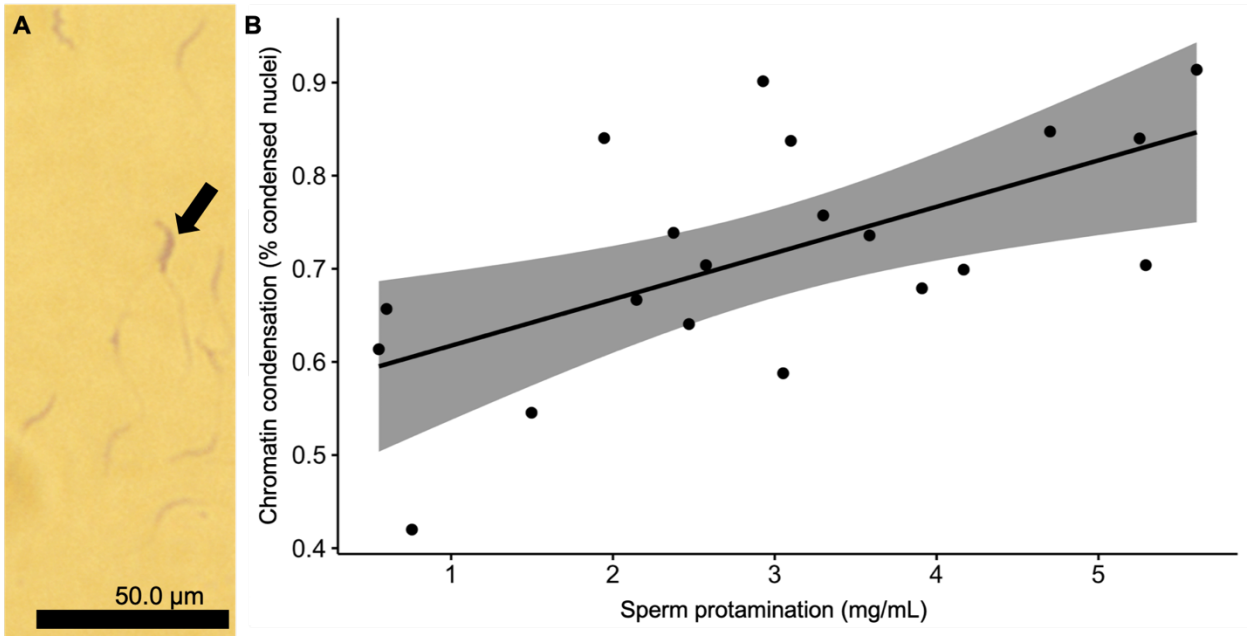

Figure 6.5. Sperm protamination as it relates to sperm chromatin condensation. A) Aniline blue staining for cell chromatin condensation. The black arrow indicates sperm stained for low chromatin condensation. B) The linear correlation between sperm protamination and sperm chromatin condensation. The shaded area represents the confidence interval of the regression line shown in black. $p < 0.01$, $R = 0.6089$ ($n = 20$).

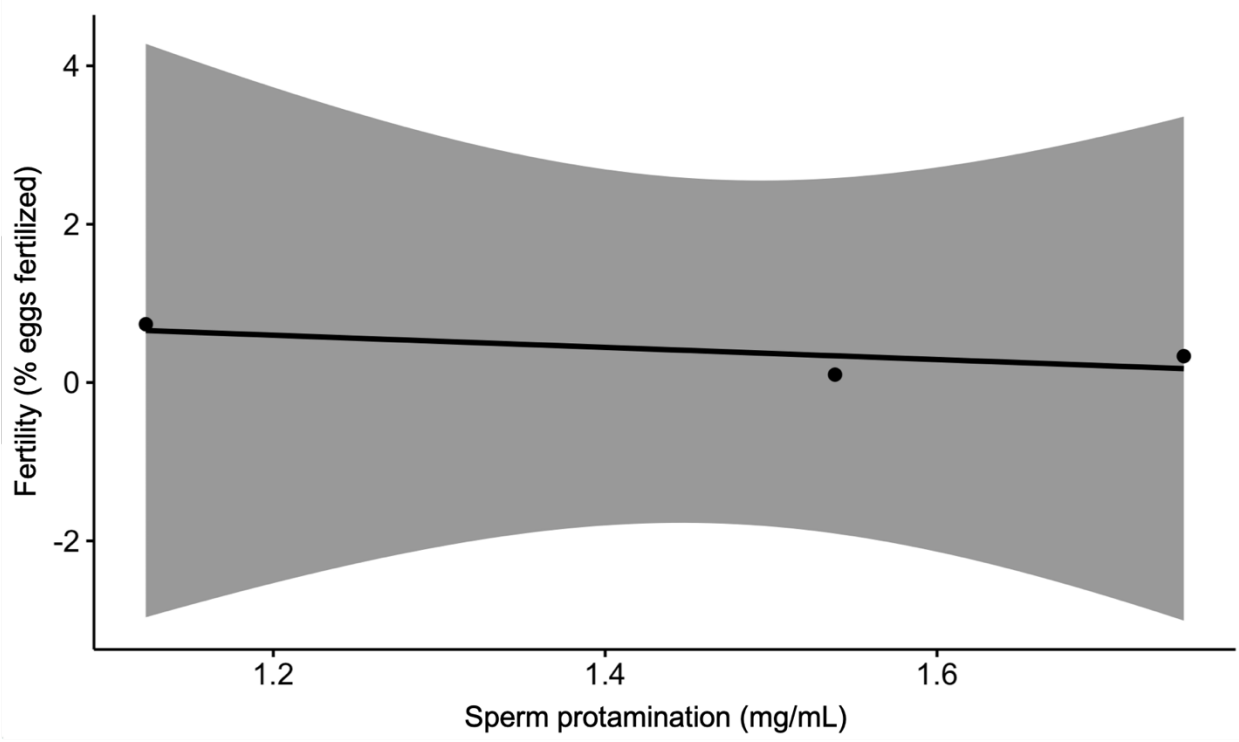

Figure 6.6. Sperm protamination as it relates to rooster fertility. The linear correlation between rooster fertility and sperm protamination. The shaded area represents the confidence interval of the regression line shown in black. $p > 0.05$ ($n = 3$).

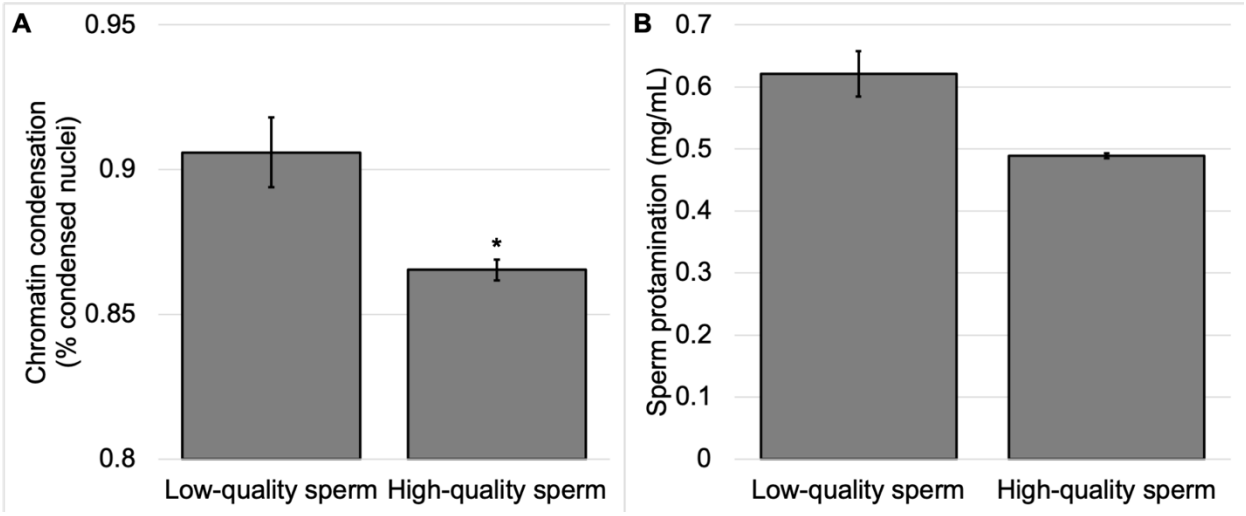

Figure 6.7. Chromatin characteristics of phenotyped low- and high-quality sperm. A) Chromatin condensation of low- and high-quality sperm separated by Percoll density gradient centrifugation (PDGC). B) Sperm protamination of low- and high-quality sperm separated by PDGC. Values represent mean ± SEM (n = 3). *Indicates significant difference in means ($p < 0.05$).

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CHAPTER 7

ROUNDUP®-TREATED FEED AS A SOURCE OF REPRODUCTIVE TOXICANTS IN BROILER BREEDER ROOSTERS AND THE COUNTERACTION OF GLYPHOSATE IN FEEDS WITH HUMIC ACIDS[1]

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Abstract

Glyphosate-based herbicides (GBHs) such as RoundUp® are a staple of modern crop production, and as a result, residues of their ingredients are typically found in animal feeds. GBH ingredients have repeatedly been shown to impact the male reproductive health of various animals, but at present, the impact of GBH exposures on reproductive health have not been investigated in broiler breeder roosters. This study sought to determine the effect of these exposures on roosters as well as the ability of humic acids (HA) to counteract the effect of GBH exposure. Through 18 weeks of treatment with RoundUp®-added or HA-added feeds compared against a common broiler breeder mash, negative effects of RoundUp® exposure were seen on testis morphology as well as sperm mobility, and HA supplementation served as a promising neutralizing additive. RoundUp® exposure was shown to be a negative influence on broiler breeder rooster reproductive health while HA improved reproductive health.

Keywords: broiler breeder, glyphosate, RoundUp, humic acids, sperm mobility

Introduction

Crops genetically modified for tolerance to glyphosate (*N*-(phosphonomethyl)glycine), a common herbicide, have become increasingly popular since their introduction by the Monsanto Company in 1996 (Benbrook, 2012). In present day production, herbicide-tolerant corn and soy varieties comprise 90 and 94%, respectively, of the acres of these crops planted in the United States, and glyphosate-tolerant (GT) varieties account for roughly 50% of corn and soy grown in the United States (Kniss, 2018; USDA, 2013, 2018) These GT cultivars are typically exposed to glyphosate-based herbicides (GBHs) throughout their production period, and due to their tolerance to glyphosate, crop production is unaffected (Arregui et al., 2004; Green, 2018). Glyphosate is still incorporated into the tissues of the GT plant, but a modification of enolpyruvylshikimate-3-phosphate synthase, the target enzyme of glyphosate, allows the GT plant to remain healthy (Arregui et al., 2004; Feng et al., 2003). This results in chemical residues of glyphosate typically being found in the tissues of these crops postharvest in addition to the presence of common GBH inert ingredients on the surface of the produce (Arregui et al., 2004; Cuhra, 2015; Duke et al., 2003).

Glyphosate is generally considered a moderate toxicity chemical, however its low dose negative impacts on mammalian reproductive systems are well established (Cuhra, 2015; Henderson et al., 2010). Glyphosate is especially damaging when exposures occur in combination with its co-formulant polyoxyethylene tallow amine, as is the case when RoundUp (RU) is used (Defarge et al., 2016, 2018). The modes of toxic action for glyphosate and its metabolite, aminomethylphosphonic acid (AMPA), in an animal model are unknown, but they are reported to produce oxidative damage (Abarikwu et al., 2015; Myers et al., 2016).

Humic acids (HA), a class of organic acids derived from humic substances, have been shown to be strong neutralizers of glyphosate in solution in *in vitro* studies (Piccolo et al., 1996; Shehata et al., 2014; Van Oosten et al., 2017). Though typically used as soil additives to act as biostimulants, HAs are theorized to neutralize glyphosate in media through adsorption (Arroyave et al., 2016; Piccolo et al., 1996; Van Oosten et al., 2017). This ability to sequester glyphosate from solution is due to the large molecular surface area and flexibility of humic acids, allowing for a high capacity for hydrogen bonds (Piccolo et al., 1996). *In vitro*, HAs have been shown to adsorb as much as 4.5 mg glyphosate per gram of HA (Piccolo et al., 1996).

Specific to male reproductive physiology, exposure to the ingredients of RU has been reported to result in endocrine disruption, abnormal morphologies of reproductive tissues and dysfunctional sperm production at RU or glyphosate exposure levels no higher than 5 mg/kg bodyweight in murine studies, although, the endocrine effects of RU exposure are widely disputed (Abarikwu et al., 2015; Dallegrave et al., 2007; EFSA, 2017; Owagboriaye et al., 2017). An investigation of RU toxicity in drakes reported endocrine disruption and abnormal testis and epididymal morphologies with treatment with RU at 5 mg/kg bodyweight, indicating similar reproductive toxicity in avian models (Oliveira et al., 2007). This is despite glyphosate being listed as having very low toxicity in avian models, both in overall toxicity and reproductive toxicity (Cuhra, 2015; Henderson et al., 2010).

In the same years that GT crops and GBHs have tandemly grown in popularity and use, broiler breeder populations have displayed a steady decline in fertility, due to a number of issues. The fault of much of this issue is attributed to the broiler breeder paradox, the reality of losses in fertility resulting from intense selective pressure for rapid growth (Decuypere et al., 2010; Hocking, 2014). While the reproductive consequences of intense selection are without contest,

there is little to no evidence to suggest that it is the only contributor to the observed, gradual loss in fertility of broiler breeders. Given what is known about the toxic nature of RU ingredients towards male reproductive tissues and the likelihood of exposure to these toxicants through GT feed crops, the effect of RU exposure on the reproductive health of broiler breeder roosters should be addressed. The objectives of this study were 1) to explore the effects of RU exposure through feed on the reproductive health of broiler breeder roosters and 2) to test the efficacy of HAs as a remedy for RU residues found in GT feed crops.

Materials and methods

Chemicals

The Roundup PRO® Concentrate Herbicide formulation (Monsanto, St. Louis, MO, USA) was used for treatment of feed with glyphosate-based herbicide. This RU formulation consists of 50.2% isopropylamine salt of glyphosate, 13.0% surfactant and 36.8% inert ingredients. The HA was purchased as a water-soluble powder at 90% HA and 10% fulvic acid and inert soil contents (Earthworks Health, Norfolk, NE, USA).

Animals

144 Cobb 500 broiler breeder roosters were grown to target weight and photostimulated (14L:10D) at 15 weeks of age. At 25 weeks of age, roosters were randomly assigned to individual cages. Prior to treatment, roosters were allowed water *ad libitum* and given 120 g broiler breeder developer mash (BBDM) each day at the start of their lighted period. The roosters were reared and maintained according to the rules set forth by the University of Georgia's Institutional Animal Care and Use Committee.

Experimental design

Treatments were administered through feed. Feed was mixed fresh every two weeks for one of four treatments: 1) control BBDM, 2) BBDM treated with 1.25 mL RU/kg feed, 3) BBDM treated with 2.50 mL RU/kg feed and 4) BBDM treated with 0.30% (w/w) HA. A HA treatment was used to evaluate HA as a method for counteracting glyphosate exposures. Dosages of RU were chosen based on previous study of RU exposures in drakes, and the HA dosage was chosen based on previous study of *in vitro* neutralization of glyphosate with HA (Oliveira et al., 2007; Piccolo et al., 1996).

After being allowed 2 weeks for habituation to individual cages, roosters began treatment at 27 weeks of age. Roosters were randomly assigned to 1 of 4 experimental groups; each group consisted of 4 replicates of 9 roosters (n = 4). Throughout the treatment period, roosters were allotted 120 g treatment feed and allowed water *ad libitum* at the start of the light period each day. Treatment lasted for 18 weeks. At the end of treatment, all experimental groups were returned to the control BBDM diet for a 4-week long recovery period in order to observe the permanence of any effects observed with respect to RU or HA treatment. Two roosters per replicate were weighed every 2 weeks to verify homologous bodyweights between treatments.

Feed (n = 2) and fecal (n = 4) glyphosate and AMPA levels were assessed by HPLC analysis. Sample extraction and HPLC analyses were performed by the UGA Laboratory for Environmental Analysis as described by Miles et al. (1986). Samples were twice extracted by subjecting 5.0 g sample to sonication for 20 min diluted first in 15 mL deionized water and second in 10 mL deionized water. After pooling and centrifugation of duplicate extracts, supernatants were collected and derivatized with 9-fluoromethyl chloroformate for HPLC analysis.

Histology

At the end of the treatment period as well as at the end of the 4-week recovery period, 2 roosters per experimental group were euthanized by CO_2 gas asphyxiation for sampling of reproductive tracts (n = 2). Reproductive tracts were immediately fixed in 10% buffered formalin and stored at room temperature (RT). For each sample, an approximately 4 mm thick sample was taken from the transverse equator of one testis. Samples were dehydrated, embedded in paraffin, sectioned transversely at approximately 5 µm thickness, hematoxylin and eosin stained and mounted by the UGA College of Veterinary Medicine Histology Laboratory.

Morphometry

Hematoxylin and eosin stained testes (n = 2) sections were imaged using a TH4 100 microscope (Olympus Corp., Tokyo, Japan) at 10X. For each sample, 25 images were taken, with each image centered on a single, transverse section of a seminiferous tubule. Morphometrical analysis was performed as described by Montoto et al. (2012) with the following modifications. A horizontal and vertical measure of the tubule diameter was taken of each tubule along with 2 horizontal and 2 vertical measures of the epithelial height. Mean epithelial height was calculated relative to each tubule's mean radius. Measurements were obtained using ImageJ v1.52k (National Institutes of Health, Bethesda, MD, USA). Seminiferous tubules were also assessed for number of individual vacuoles present.

Sperm quality

Roosters were habituated to weekly semen collection by the dorso-abdominal massage method as described by Burrows & Quinn (1937) for 2 weeks prior to treatment. From weeks 0 to 22 of the experiment, at least 0.50 mL semen was sampled weekly from one rooster from each replicate (n = 4) and transported in glass tubes. Semen samples in 500 µL aliquots were twice

washed by centrifugation at 1000 x *g*. After the first wash, semen was reconstituted in 1.5 mL phosphate-buffered saline (PBS; pH 7.4), and after the final wash, semen was reconstituted to its original volume of 0.50 mL with motility buffer (MB: 111 mM NaCl, 25.0 mM glucose, 4.00 mM $CaCl_2$, 50.2 mM TES; pH 7.4). Washed sperm sample was assessed for cell count by hemacytometer counting as previously described (Freund & Carol, 1964).

Sperm viability was assessed by the eosin-nigrosin vital staining assay as previously described (Chalah & Brillard, 1998) with the following modifications. Sperm samples were prepared in 50 µL aliquots at a concentration of 1.0 x 10^8 cells/mL by diluting in MB in a microcentrifuge tube. Eosin-nigrosin stain (2.5% (w/v) eosin, 5% (w/v) nigrosin) was mixed 1:1 into the sample by pipetting followed by vortexing. Samples were incubated in the stain at RT for 5 min. After incubation, 20.0 µL stained sample was added to a glass slide, smeared, air-dried and cover-slipped. Slides were photographed under an Olympus TH4 100 microscope and counted for total sperm and total dead sperm. Percentage viability was calculated by number of live sperm out of a minimum of 100 cells counted per sample.

Sperm mobility was assessed by the Accudenz assay, a method previously validated for the objective quantification of chicken sperm mobility (Froman & McLean, 1996), with the following modifications. A 30% (w/v) Accudenz (Accurate Chemical & Scientific Corp., Westbury, NY, US) stock solution was prepared in a 3 mM KCl, 5 mM TES solution (pH 7.4) and diluted 1:4 in MB to prepare a working 6% Accudenz solution. The Accudenz solution was preheated to 41 ºC, and 1.00 mL aliquots were added to polystyrene cuvettes kept at 41 ºC. Sperm samples were prepared at a concentration of 5.0 x 10^8 cells/mL, and 100 µL sperm sample overlaid onto Accudenz solution prepared in cuvettes. Samples were incubated in cuvettes at 41

ºC for 10 min. Absorbance was read at 550 nm using a DU 530 spectrophotometer (Beckman Coulter, Inc., Brea, CA, USA). The Accudenz assay was performed in duplicate.

Testosterone assessment

At weeks 0, 9 and 18 of treatment as well as at the end of the 4-week recovery period 2 roosters per experimental group were sampled for blood (n = 2). Blood samples were collected from the wing vein into a sterile syringe and injected into EDTA coated tubes. Blood samples were centrifuged at 3000 x g for 10 min at 4 ºC to collect plasma. Plasma samples were stored at -20 ºC. Plasma samples were subjected to diethyl ether extraction for separation of steroid content, and concentrations of plasma testosterone were determined by a testosterone ELISA kit according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI, USA).

SDS-PAGE and western blotting

At end of treatment and after 4 weeks of recovery, 2 roosters per experimental group were euthanized by CO_2 and their reproductive tracts harvested, placed in PBS and immediately stored at -80 ºC (n = 2). Testes tissues were later thawed and an approximately 1 cm^3 sample cut from the caudal end of the testis. Samples were suspended 1:5 in lysis buffer (50 mM NaCl, 10 mM Tris base, 1 mM EGTA, 1 mM EDTA and 1% (v/v) Triton X) and homogenized with a 10 mm X 115 mm saw-tooth generator probe (VWR International, Radnor, PA, USA) for 2 repetitions of 30 s at medium power followed by 1 min on ice. Samples were then sonicated by an Artek Model 150 sonic dismembrator (Thermo Fisher Scientific, Waltham, MA, USA) at 60% output for 5 repetitions of 15 s followed by at least 1 min on ice. Sonicated samples were then centrifuged at 12000 x g for 30 min at 4 ºC and supernatant collected. Protein was quantified using the Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with a bovine serum albumin standard. Measurements of absorbance were taken at 750 nm with a

Spectronic 200 spectrophotometer (Thermo Fisher Scientific) per the manufacturer's instructions. Lysates were stored at -80 ºC.

Testis protein lysates were thawed, and concentrations adjusted to 1.2 mg/mL. Samples were diluted 1:1 in 2X sample buffer (4% SDS, 20% glycerol, 120 mM Tris-HCl, 200 mM dithithreitol, 0.02% bromophenol blue; pH 6.8) and then denatured at 95 ºC for 5 min. In duplicate lanes, 50 µL protein samples were loaded into Mini-PROTEAN® TGX Stain-Free™ 10% precast gels (Bio-Rad) alongside Precision Plus Protein™ All Blue Standards (Bio-Rad). SDS-PAGE was performed on the gels in Tris-glycine-SDS running buffer (25 mM Tris base, 192 mM glycine and 0.1% SDS; pH 8.3) at 70 V for 10 min followed by 120 V until completion in a Mini-PROTEAN® Tetra Cell system (Bio-Rad). Gels were UV-activated using a ChemiDoc™ MP Imaging System (Bio-Rad) and then transferred to Immun-Blot® polyvinylidene difluoride membranes (Bio-Rad) in Towbin transfer buffer (25 mM Tris base, 192 mM glycine and 20% (v/v) methanol) via wet-blot transfer system (Bio-Rad) at 100 V for 1 hr. Membranes were washed in Tris-buffered saline with Tween (TBST: 20 mM Tris base, 150 mM NaCl and 0.1% (v/v) Tween 20; pH 7.4) and then blocked in 5% (w/v) skim milk in TBST for 30 min. Membranes were imaged for total protein normalization using the Chemidoc™ MP Imaging System. Blocked membranes were probed with a polyclonal human anti-androgen receptor IgG antibody produced in goat (product no. PA1-9005; Thermo Fisher Scientific) diluted 1:1000 in TBST overnight at 4 ºC under slow rocking. Membranes were washed in TBST and then probed for 1 hr at RT in horseradish peroxidase (HRP)-conjugated donkey anti-goat IgG secondary antibody (product no. A16005; Thermo Fisher Scientific) diluted 1:10000 in TBST, followed by washing in TBST. Membranes were subjected to visualization by Clarity™ Western ECL Substrate (Bio-Rad) and images developed on the Chemidoc™ MP Imaging

System. Membranes probed with only secondary antibody at RT for 1 hr served as secondary controls. Abundances of androgen receptor were quantitated by normalizing the densities of the androgen receptor protein bands to that of the total loaded protein per lane as measured by stain-free imaging technology. Calculations of relative androgen receptor abundances were performed using Image Lab™ Software (Version 5.2.1; Bio-Rad).

Statistical analysis

Statistical analyses were performed with R 3.5.1 software (The R Foundation for Statistical Computing, Vienna, Austria). Differences in bodyweight, tissue morphology, sperm parameters, androgen receptor abundance and plasma testosterone between experimental groups were analyzed by one-way ANOVA. Bodyweights and sperm parameters assessed throughout the treatment period were analyzed in 6-week groupings (weeks 1-6, 7-12 & 13-18) in order to limit the number of statistical tests performed. Significant differences were compared by Tukey's honest significant difference test, with differences considered statistically significant at $p \leq 0.05$.

Results

Treatment validation

In order to validate treatment with differing levels of glyphosate, acting as a proxy for relative levels of other RU ingredients, feed and fecal samples were assessed by HPLC for glyphosate and AMPA levels. Control, untreated BBDM was found to contain glyphosate and AMPA residues merely as a result of the production practices used to produce the feed crops (Table 7.1). BBDM treated with 1.25 mL RU/kg and 2.50 mL RU/kg exhibited glyphosate levels increased by 54 and 132%, respectively, relative to the control BBDM (Table 7.1). AMPA levels were increased in a similar but less dramatic trend. BBDM treated with HA exhibited glyphosate levels decreased by 34% relative to the control BBDM; AMPA levels, however, were increased

by 107% when compared to that of the control (Table 7.1). Glyphosate was only found in fecal samples collected from roosters treated with 1.25 mL RU/kg and 2.50 mL RU/kg (Table 7.2). AMPA levels were similar in fecal samples between all groups except BBDM treated with HA, which exhibited AMPA increased 105% relative to that of the control treatment (Table 7.2).

Rooster bodyweights were recorded in order to ensure that differences in growth rate did not confound comparisons of differences in reproductive health. Throughout the experiment, no significant differences were observed in rooster bodyweights between experimental groups (Figure 7.1).

Tissue morphology

Tissue morphology was assessed to discern mode of effects of RU and HA treatment on reproductive tissues. No significant effects were observed on epithelial height at end of the treatment period or the end of the recovery period (Figure 7.2). At the end of the treatment period, a dose dependent increase was seen on seminiferous tubule vacuolation with respect to RU exposures ($p < 0.05$), and, in a similar pattern, treatment with HA resulted in a non-significant decrease in tubule vacuolation when compared with the control (Figure 7.3). At the end of the recovery period, no significant differences in tubule vacuolation were seen.

Sperm parameters

Sperm count, viability and mobility were measured throughout the treatment and recovery periods in order to assess the effects of treatments on sperm performance. No significant differences were observed in sperm cell counts before the initiation of treatment (Figure 7.4). Roosters treated with HA-added feed displayed increased sperm counts throughout the first and second thirds of the treatment period ($p < 0.05$) (Figure 7.4). No clear trend in sperm count with increasing presence of RU ingredients was observed, with the control group

exhibiting the highest sperm counts throughout the last third of the treatment period (Figure 7.4). At the end of 4 weeks of recovery, all significant differences were removed.

No significant differences were observed in sperm cell viability before treatment began or after the end of the recovery period (Figure 7.5). Viability decreased with increased glyphosate levels and increased with HA-added feed during the middle 6 weeks of the treatment period ($p < 0.05$), however this pattern was not observed throughout the rest of the treatment period (Figure 7.5).

Sperm mobility exhibited a clear response to treatment with either increased glyphosate or added HA. Across all of the treatment period, sperm mobility decreased with a dose-dependent response to added glyphosate, while sperm mobility increased with HA-treatment of feed ($p < 0.05$) (Figure 7.6). Significant differences were not observed in sperm mobility neither before start of treatment nor after 4 weeks of recovery (Figure 7.6).

Endocrine effects

In order to determine the influence of any endocrine-related effects on reproductive health, plasma testosterone levels were assessed by ELISA at start of treatment (week 0), mid-point of treatment (week 9), end of treatment (week 18) and after 4 weeks of recovery (week 22), and androgen receptor abundance was assessed by western blotting at end of treatment and end of recovery.

Plasma testosterone levels were significantly higher in the RU-added groups prior to treatment ($p < 0.05$). Midway through treatment, testosterone levels were more than double that of the control group for those roosters treated with 0.30% HA and those treated with the lower dose of added RU ($p < 0.05$). No significant differences were observed at the end of treatment nor after recovery (Figure 7.7). Androgen receptor abundance was not significantly different

between any groups at the end of treatment. After 4 weeks of recovery, androgen receptor abundance was decreased by over 60% in roosters treated with the higher dose of added RU ($p < 0.05$) (Figure 7.8).

Discussion

The objectives of this work were to explore the effects of RU exposure through feed on the reproductive health of broiler breeder roosters and to test HAs as a remedy of RU residues found in feed. Here in this study, we show that exposure to increased levels of RU negatively impact the health of the seminiferous epithelium of the testes and sperm mobility, while treatment of feed with HA improves health of the seminiferous epithelium and sperm mobility in roosters.

While no effect is seen on epithelial height of the seminiferous tubules, a clear pattern of effect can be seen by increasing effective glyphosate levels on the degree of vacuolation in the seminiferous epithelium. Vacuolation was more dramatic in roosters exposed to higher levels of RU, and it was decreased in those fed with HA included to counteract glyphosate. Vacuolation of the seminiferous epithelium and germ cells has been previously shown with glyphosate exposure in both *in vitro* studies and *in vivo* studies of the multigenerational effects of glyphosate exposure (Dallegrave et al., 2007; Jiang, et al., 2018; Kubsad et al., 2019). This type of vacuolation of the seminiferous epithelium is typically indicative of Sertoli cell damage. The Sertoli cells are fairly resilient to toxic insults, relative to germ cells; however, their level of exposure to any such insults is highest, as these cells form the blood-testis barrier. With prolonged exposure and Sertoli cell damage, damage to germ cells typically follows (Creasy, 2001). The recovery period removed any differences in vacuolation between groups; in fact, all groups displayed increased vacuolation from that seen at the end of treatment. This can be explained by the age of the

roosters. By the end of the recovery period, the roosters were 49 weeks old. As roosters near the end of the their reproductive prime, typically around 50 weeks of age, overall quality of testis morphology tends to decrease even under normal physiologic conditions (Avital-Cohen et al., 2013).

A pattern of effects similar to that seen with seminiferous epithelium vacuolation was seen with regard to sperm mobility, with sperm mobility decreasing with increasing measured glyphosate levels. Transient effects were seen on sperm count and sperm viability, with HA-added feed improving the two parameters of sperm quality and RU-added feed decreasing sperm viability for part of the treatment period. These effects, however, were not consistent throughout the whole treatment period. Counts did generally increased in all groups throughout the experimental period. This is likely due to the roosters not reaching their reproductive prime until the last third of the treatment period. The effect on sperm mobility is consistent, and it is the sperm parameter which carries the most weight in broiler breeder populations. Sperm mobility is historically considered the most important determinant of broiler breeder rooster fertility (Froman et al., 1999). Sperm motility has formerly been shown to be negatively affected with glyphosate treatment at *in vivo* levels as low as 3.6 mg/kg bodyweight in murine studies and with *in vitro* treatment of human sperm at 360 ppb (Abarikwu et al., 2015; Anifandis et al., 2017, 2018; Owagboriaye et al., 2017). Our results show an effect with chronic *in vivo* treatment of feed with just over 11 ppm glyphosate residues. Based on the average weight of roosters throughout the experimental period and the feed ration allotted, that is a treatment level of 0.271 mg/kg bodyweight. Fortunately, the effects of exposure appear to be temporary, as the recovery period removed all differences between groups. The removal of differences in reproductive health between groups with respect to treatment indicates that the effects of exposure to RU may

be temporary, and intervention in broiler breeders already impacted by exposure to RU ingredients may improve their reproductive health.

The endocrine effects shown in this study are not strong. The significant differences observed in plasma testosterone follow no pattern, and significant differences were observed prior to treatment. The only effect seen on androgen receptor abundance was a dose-dependent decrease in the groups treated with RU-added feed, but this only occurred after recovery on control feed. The full scope of the effect on androgen receptor abundance in the testes, if any, was likely not captured with the relevant parameters measured in this experimental design.

This study displayed that exposure to GBH ingredients through animal feeds, at even legal levels, can significantly influence the reproductive health of broiler breeder roosters. Both the gross histopathology of the rooster testis and sperm mobility, the most important component of rooster sperm quality, are seriously impacted by exposure to GBH ingredients. This study was the first work to display this effect of GBH ingredient exposure at such low levels in an avian species, let alone an agriculturally important avian species. The addition of HA was shown reduce the effect of these residues in feed and provided no additional negative effects on reproductive health or growth of the rooster. Further study should be performed to ensure that the benefits seen with HA supplementation are related to its ability to neutralize glyphosate residues in the feed, rather than due to a mode of action outside of its relationship to glyphosate. Additionally, further study should investigate the effects of increasing GBH ingredient exposure on both male and female broiler breeders and their overall reproductive efficiency in order to determine the impact that GBH exposure imposes on broiler chick production.

Table 7.1. Glyphosate and aminomethylphosphonic acid (AMPA) residues in treatment feeds as measured by HPLC.[1]

Treatment	Glyphosate (ppm)	AMPA (ppm)
Control	11.329 ± 0.461	1.300 ± 1.300
1.25 mL RU/kg	17.441 ± 1.452	1.533 ± 0.185
2.50 mL RU/kg	26.338 ± 12.063	1.642 ± 0.954
0.30% HA	7.258 ± 7.258	2.685 ± 0.783

[1]Values are means ± SEM; n = 2; RU = RoundUp®; HA = humic acid

Table 7.2. Glyphosate and aminomethylphosphonic acid (AMPA) residues as measured in fecal samples by HPLC.[1]

Treatment	Glyphosate (ppm)	AMPA (ppm)
Control	0.000 ± 0.000	0.139 ± 0.139
1.25 mL RU/kg	3.166 ± 0.184	0.128 ± 0.128
2.50 mL RU/kg	3.321 ± 0.264	0.138 ± 0.138
0.30% HA	0.000 ± 0.000	0.285 ± 0.165

[1]Values are mean ± SEM; *n* = 4; RU = RoundUp®; HA = humic acid

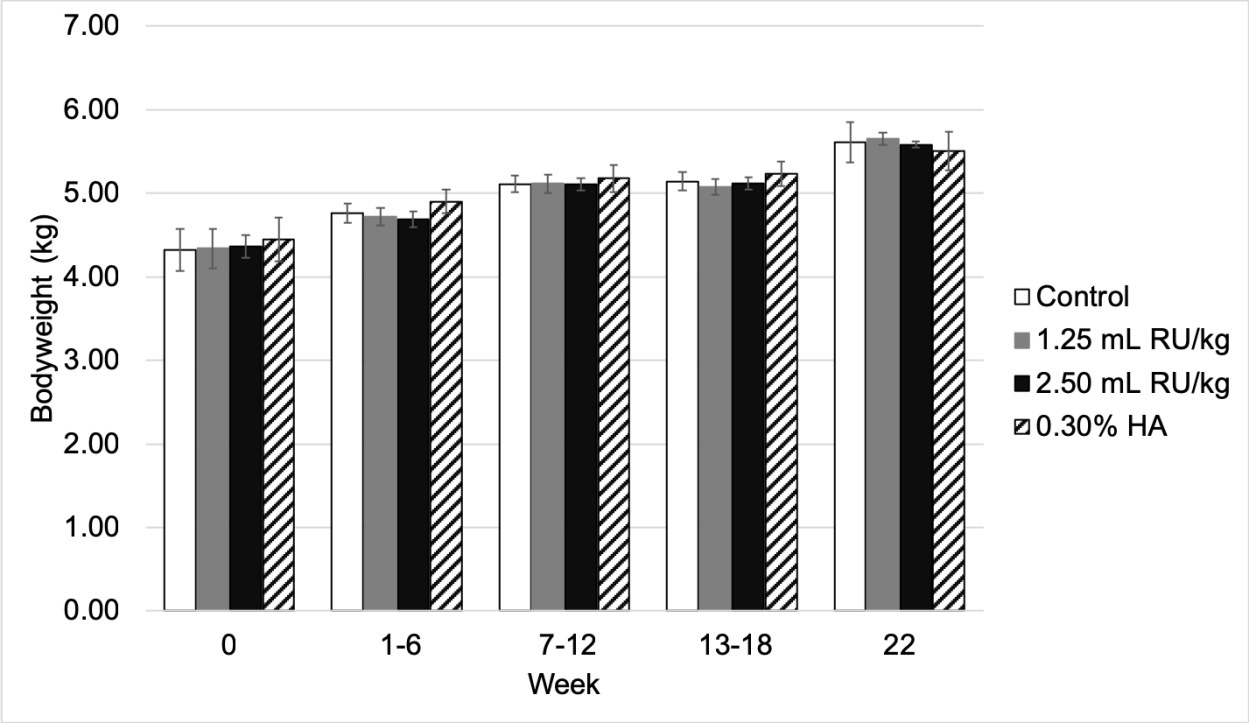

Figure 7.1. Effect of RoundUp® (RU) and humic acid (HA) treatments on rooster bodyweights. No significant differences were observed at start of treatment (n = 4), first 6 weeks of treatment (n = 12), second 6 weeks of treatment (n = 12), final 6 weeks of treatment (n = 12) nor end of the recovery period (n = 4). Values represent mean ± SEM of bodyweights taken on every even-numbered week.

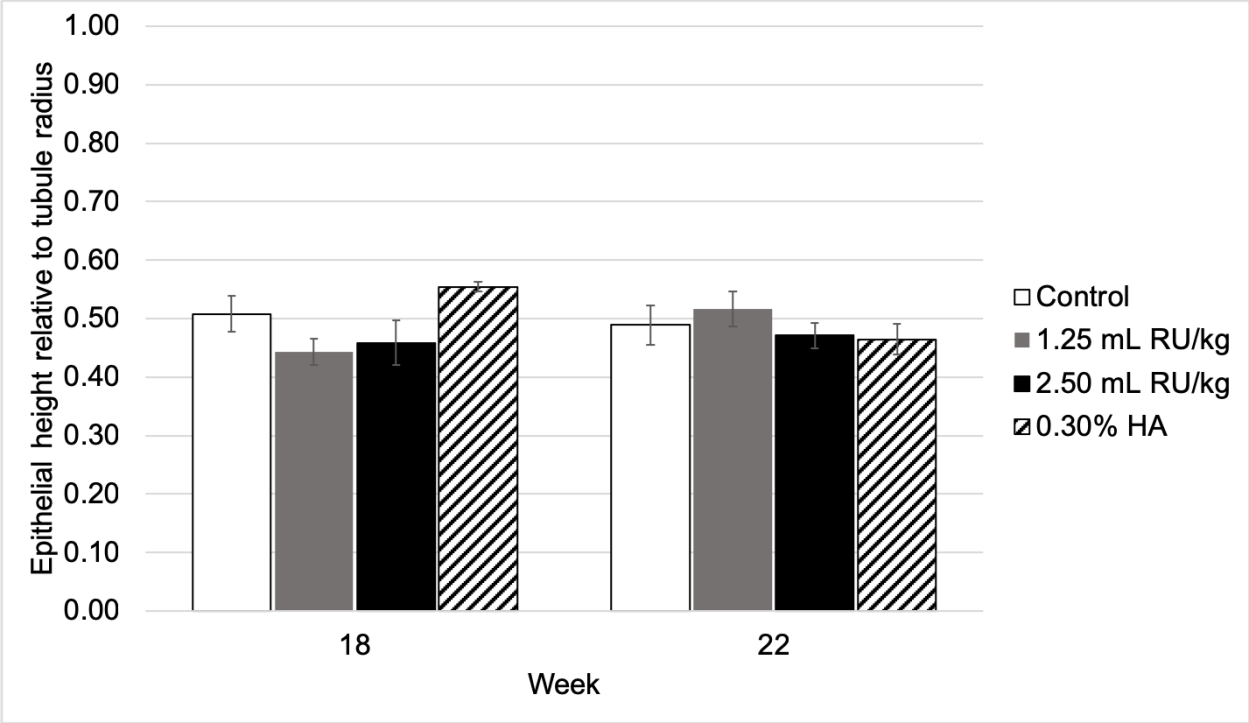

Figure 7.2. Effect of RoundUp® (RU) and humic acid (HA) treatments on epithelial height relative to overall seminiferous tubule radius. No significant differences were observed at end of treatment (week 18) and end of the recovery period (week 22). Values represent mean ± SEM of relative epithelial height (n = 2).

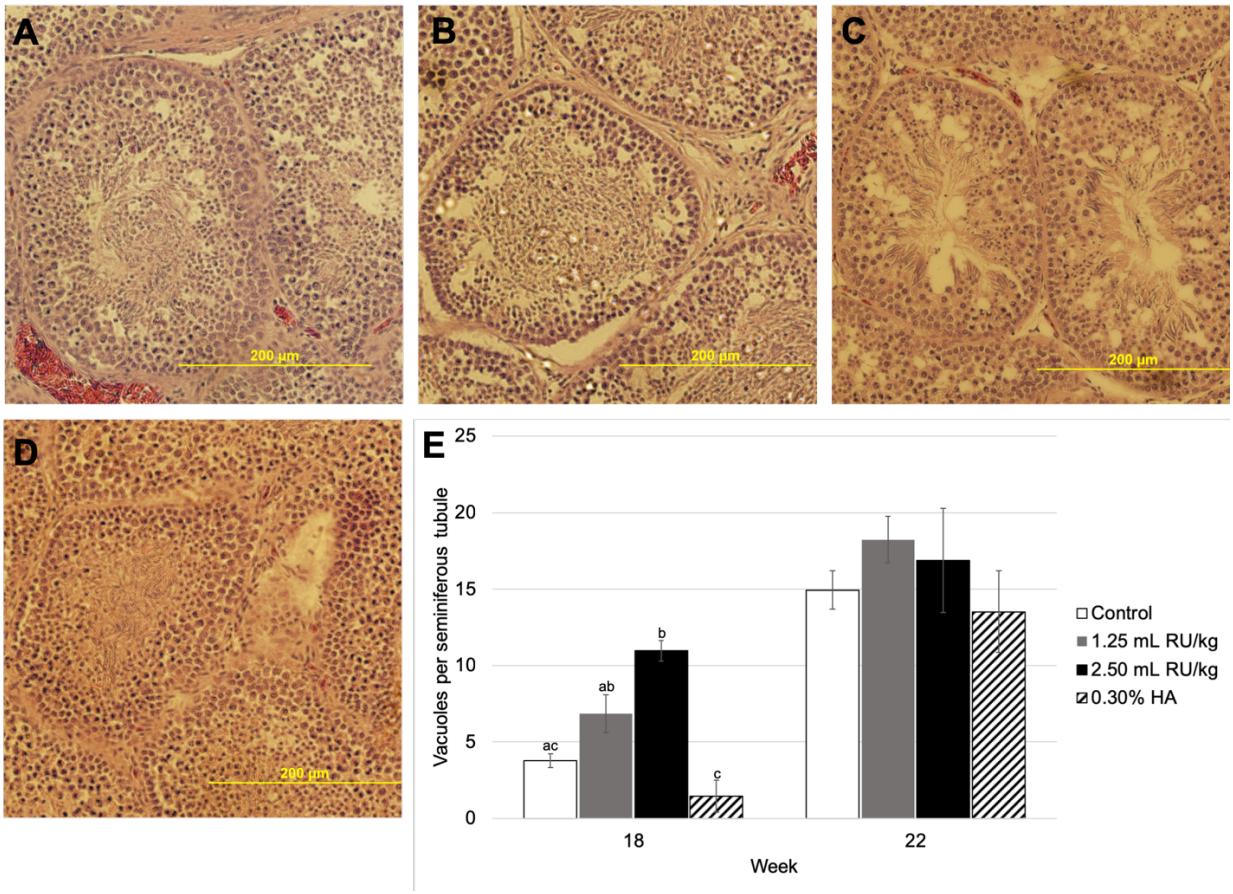

Figure 7.3. Effect of RoundUp® (RU) and humic acid (HA) treatments on tubule vacuolization. Cross sections of a seminiferous tubule of roosters treated with A) a control ration, B) 1.25 mL RU added/kg feed, C) 2.50 mL RU added/kg feed and D) 0.30% HA added. E) Degrees of seminiferous tubule vacuolation of treatment groups at end of treatment (week 18) and end of 4 weeks of recovery (week 22) are shown. Values represent mean ± SEM of relative epithelial height ($n = 2$). $^{a\text{-}c}$Indicates significant difference in means, $p < 0.05$.

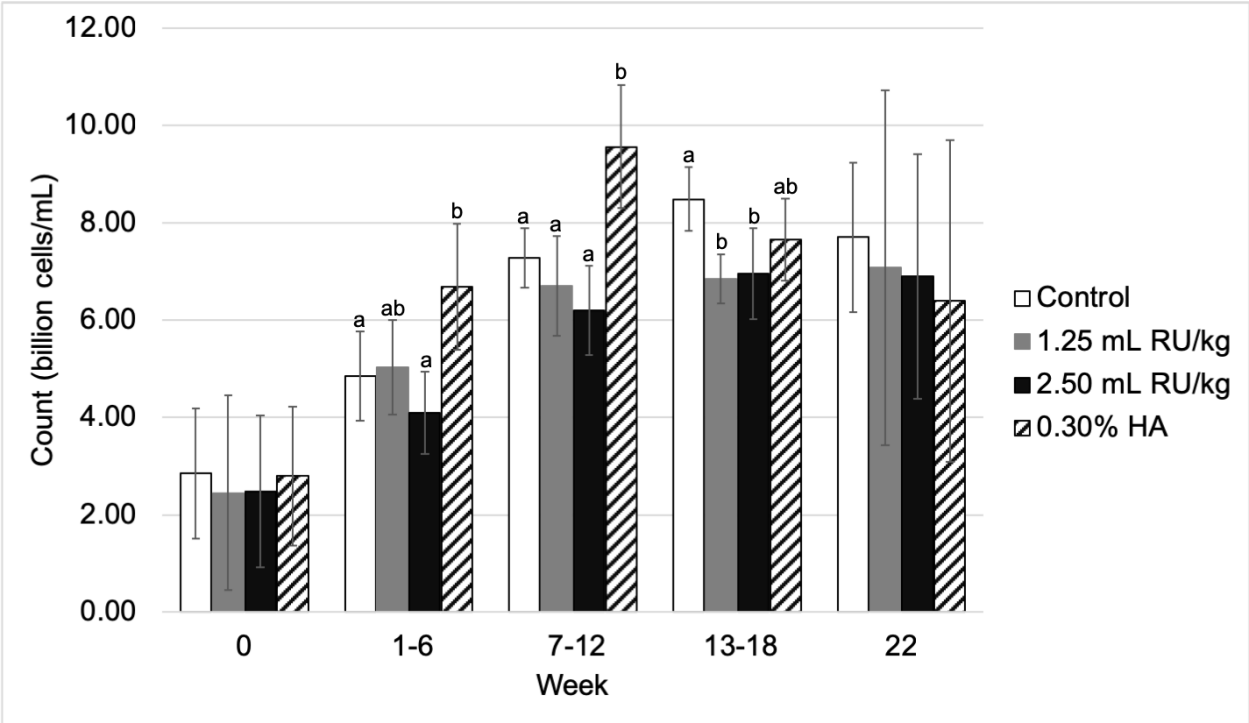

Figure 7.4. Effect of RoundUp® (RU) and humic acid (HA) treatments on sperm cell count. Sperm cell counts at start of treatment (n = 4), first 6 weeks of treatment (n = 24), second 6 weeks of treatment (n = 24), final 6 weeks of treatment (n = 24) and end of the recovery period (n = 4) are shown. Values represent mean ± SEM of weekly cell counts. [a-b]Indicates significant difference in means, $p < 0.05$.

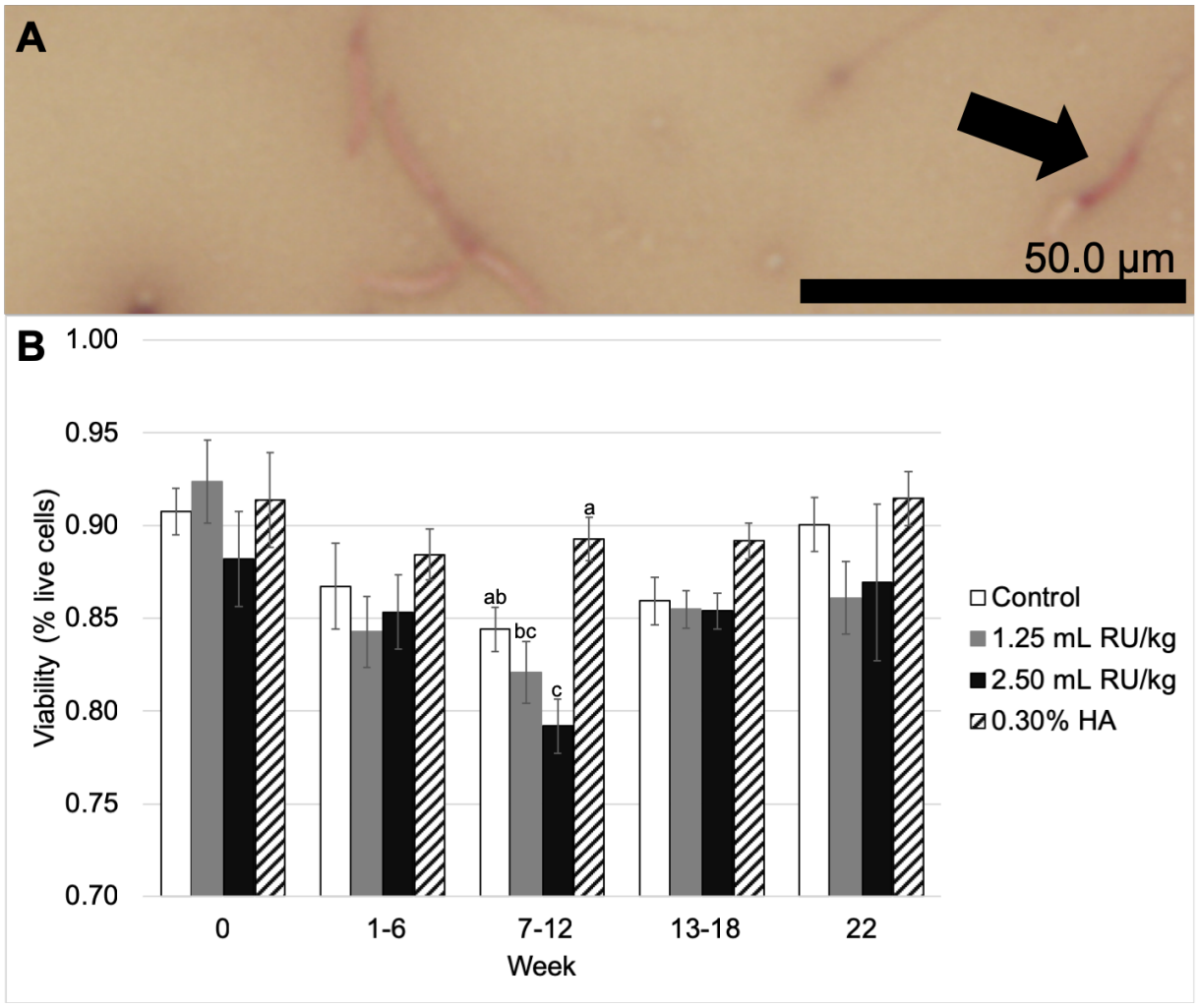

Figure 7.5. Effect of RoundUp® (RU) and humic acid (HA) treatments on sperm cell viability. A) Eosin-nigrosin staining for sperm cell viability. The black arrow indicates stained, dead sperm. B) Sperm cell viabilities at start of treatment (n = 4), first 6 weeks of treatment (n = 24), second 6 weeks of treatment (n = 24), final 6 weeks of treatment (n = 24) and end of the recovery period (n = 4) are shown. Values represent mean ± SEM of weekly cell viability assessments. [a-c]Indicates significant difference in means, $p < 0.05$.

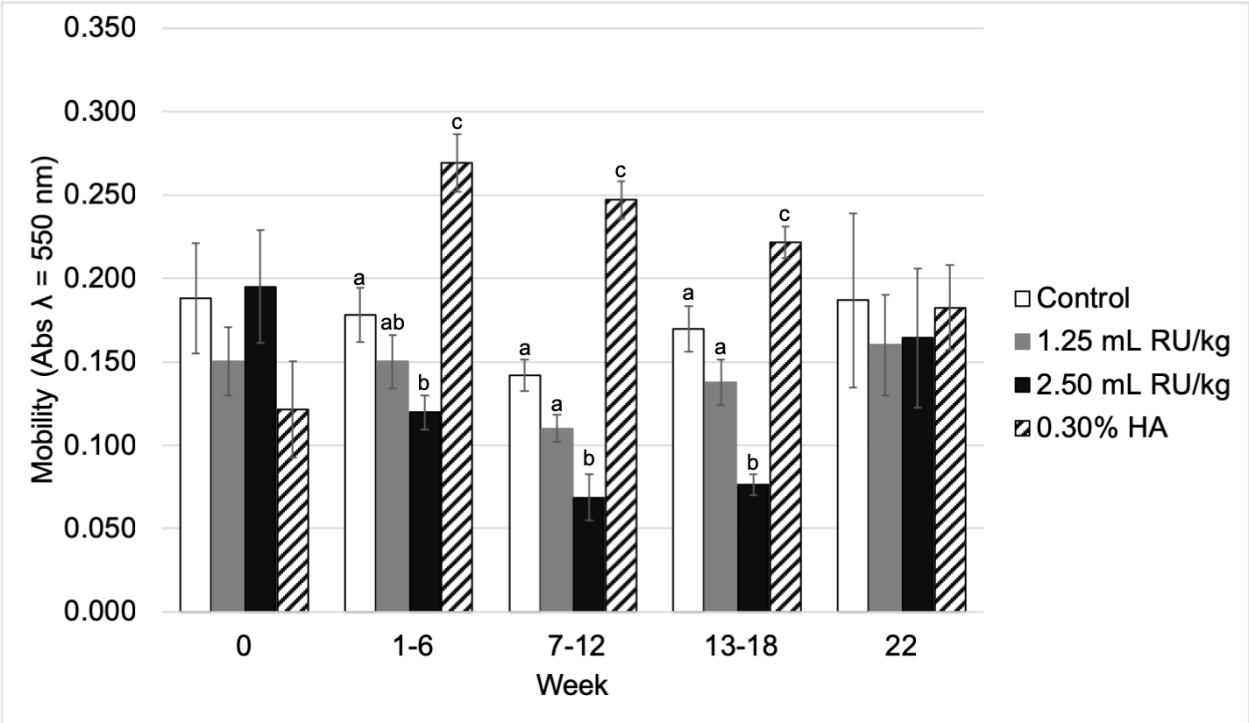

Figure 7.6. Effect of RoundUp® (RU) and humic acid (HA) treatments on sperm mobility. Sperm mobilities at start of treatment (n = 4), first 6 weeks of treatment (n = 24), second 6 weeks of treatment (n = 24), final 6 weeks of treatment (n = 24) and end of the recovery period (n = 4) are shown. Values represent mean ± SEM of weekly sperm mobility assessments. $^{a\text{-}c}$Indicates significant difference in means, $p < 0.05$.

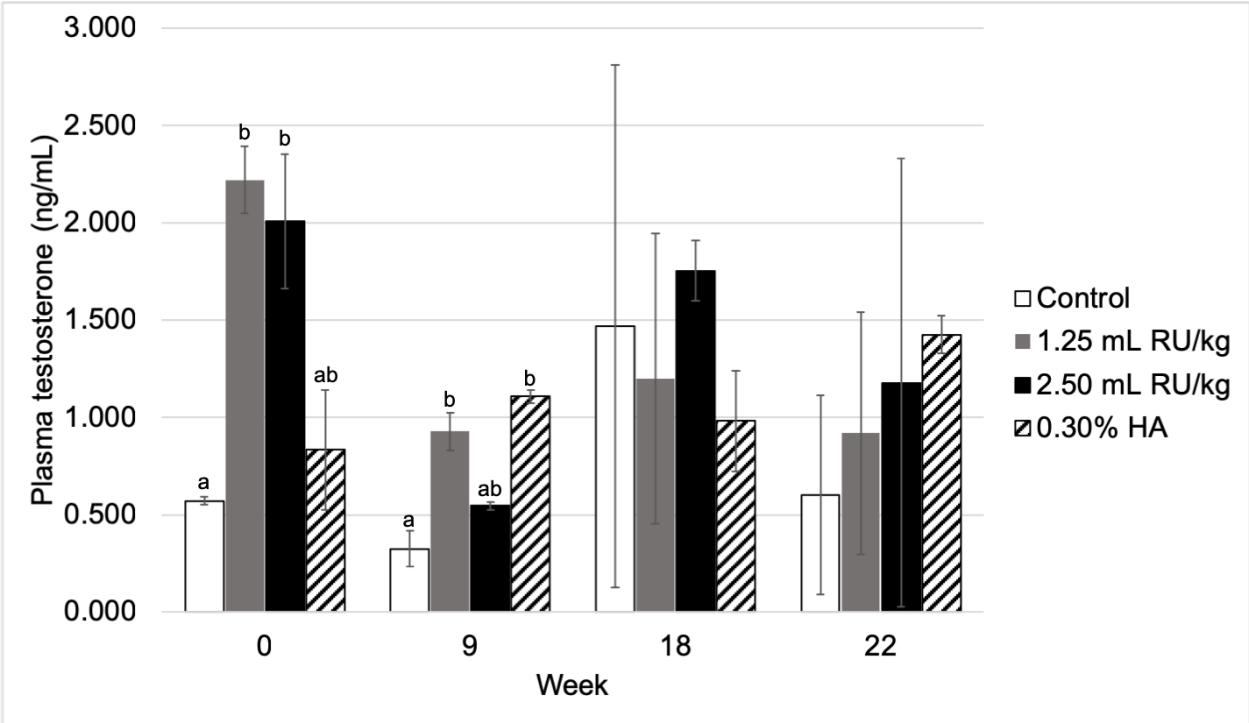

Figure 7.7. Effect of RoundUp® (RU) and humic acid (HA) treatments on plasma testosterone levels. Plasma testosterone levels at start of treatment (week 0), mid-point of treatment (week 9), end of treatment (week 18) and end of the recovery period (week 22) are shown. Values represent mean ± SEM of plasma testosterone levels ($n = 2$). [a-b]Indicates significant difference in means, $p < 0.05$.

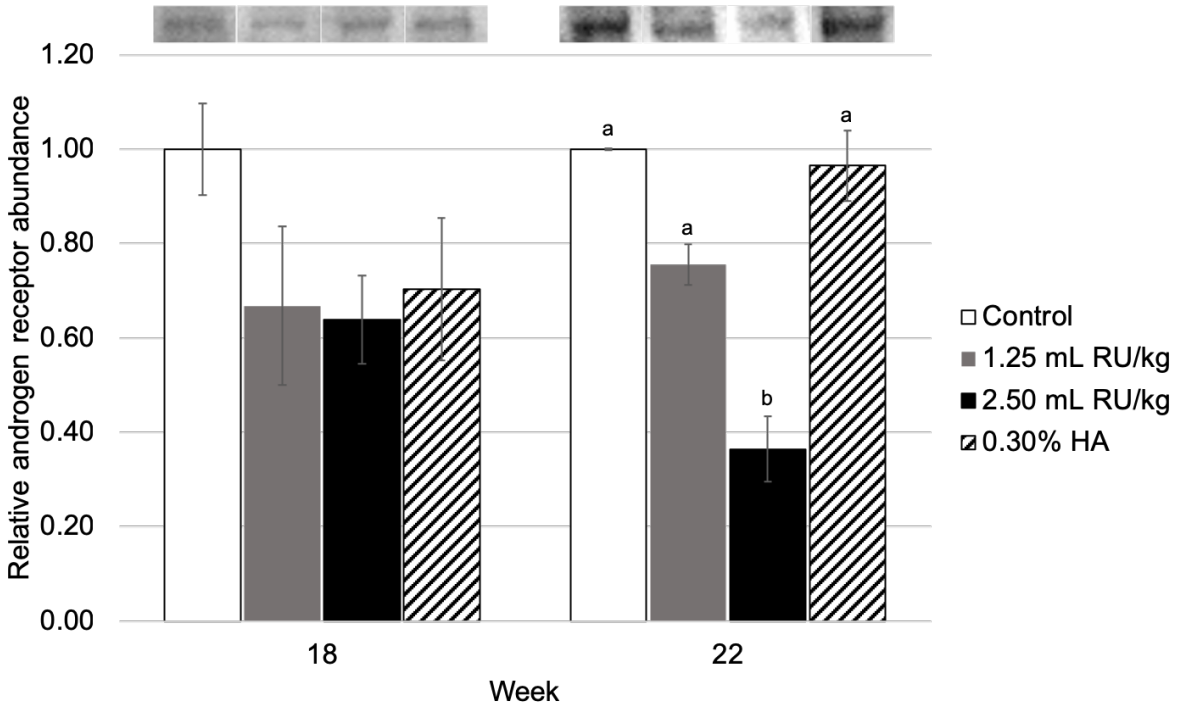

Figure 7.8. Effect of RoundUp® (RU) and humic acid (HA) treatments on relative abundance of androgen receptor protein. Relative abundance of androgen receptor protein at end of treatment (week 18) and end of the recovery period (week 22) are shown. Values represent mean ± SEM of androgen receptor abundance relative to that control tissues ($n = 2$). Androgen receptor abundances are normalized relative to total loaded protein per lane as measured by stain-free technology. [a-b]Indicates significant difference in means, $p < 0.05$.

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CHAPTER 8

CONCLUSIONS

The intense selection of broiler breeders for bodyweight gain has resulted in gradual losses of fertility in broiler breeder populations. The decline in fertility observed in broiler breeder populations stems from multiple influencers and will require a multifaced intervention to correct. This work sought to investigate three separate influencers of broiler breeder rooster fertility and the utility of each factor in improving fertility of broiler breeder roosters. These influencers were 1) sperm-associated antigen 6 (SPAG6) as a potential biomarker of rooster sperm mobility, 2) sperm protamination as an indicator of rooster sperm quality, and 3) glyphosate-based herbicide (GBH) ingredients as reproductive toxicants in broiler breeder roosters.

SPAG6 was not found to be a functional, stand-alone biomarker of sperm mobility. SPAG6 is expressed throughout the reproductive tract of the rooster and increases in abundance in sperm as they mature. It mainly localizes in the flagellar axoneme of rooster sperm but is also found in the midpiece and outside the acrosome of the head region. High abundance of SPAG6 has previously been found to be associated with sub-fertility in roosters, but its impact not exclusively through sperm mobility. The nature of the relationship between SPAG6 abundance and overall rooster fertility outside of sperm mobility has yet to be investigated and should remain a topic of study.

In effort to determine the impact of sperm protamination on rooster sperm quality, an inexpensive, non-species-specific method for quantifying protamine in sperm extracts was developed based on acid digestion of sperm samples coupled with heparin-stabilized gold nanoparticle (Hep-AuNP)-based quantification of sperm protamine. Rooster sperm protamination was not found to be a particularly useful indicator of rooster sperm quality or rooster fertility; however, the method developed for measuring protamination has potential to be used in animal models where sperm protamination is a strong indicator of sperm quality and male fertility.

Investigation of GBH ingredients as potential reproductive toxicants in broiler breeder roosters revealed a significant impact of GBH ingredients introduced through feeds on rooster reproductive health. GBH ingredients increase vacuolation of the testis' seminiferous epithelium and decrease sperm mobility in roosters with chronic exposure. Furthermore, inclusion of humic acids as a feed additive were found to reduce the effect of exposure to GBH ingredients. Humic acid inclusion in feed improves rooster testis morphology and sperm mobility by counteracting glyphosate residues found in conventionally produced feed.

The findings of this work 1) characterize the expression of a protein associated with sub-fertility in the rooster reproductive tract and detail its influence on sperm mobility as a mode of influence on fertility, 2) demonstrate an inexpensive method of quantifying sperm protamination and display a lack of importance of sperm protamination in avian sperm quality relative to that seen in mammals and 3) identify and detail the effect of and counteraction of a male reproductive toxicant to which broiler breeder populations are regularly exposed. These findings investigated three new routes through which broiler breeder rooster fertility may be improved. Application of these findings in the direction of breeding programs or in broiler breeder management will

contribute to the overall improvement of broiler breeder rooster fertility, thereby improving the productive efficiency of the broiler industry, increasing profitability of the industry and decreasing its environmental impact.