THE PROTEIN EXPRESSION OF ZONA PELLUCIDA B1, B2, AND C IN TURKEY AND BROILER BREEDER HENS

by

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(Under the Direction of Adam J. Davis)

ABSTRACT

The ovulated ovum in avian species is surrounded by a protein layer called the inner perivitelline layer (IPVL). The IPVL contains the zona pellucida (ZP) family of proteins, and in mammalian species one or more of the ZP proteins serve as the key component(s) of the sperm receptor. Sperm binding to sperm receptors located at the germinal disc (GD) region of the ovulated ovum initiates the biological steps necessary for optimum fertilization in avian species, and the amount of sperm binding at the GD reflects female fertility potential. In both chicken and turkey hens, six ZP proteins genes have been identified and hepatic produced ZPB1 and granulosa cell produced ZPB2 and ZPC appear to play key roles in sperm binding based on mRNA expression results. The goal of the current research was to determine if previously reported differences in mRNA expression in genetic lines of turkey hens and broiler breeder hens are translated to the protein level. Although ZPB2 protein expression significantly decreases as broiler breeder follicles mature from prehierarchical to hierarchical follicles, the expression of ZPB2 in the IPVL is greater in the GD region compared to nongerminal disc (NGD) region in both turkey and broiler breeder hens as mRNA expression indicated. However, protein expression of ZPB1 and ZPC in the IPVL of two genetic lines (E, high fertility and F, low

fertility) of turkey hens was opposite of mRNA expression indications. ZPC protein expression was higher in E line IPVL, and ZPC expression was not different between GD and NGD IPVL regions which also disagreed with mRNA expression indications. The results indicate that mRNA expression of ZP proteins at their site of synthesis in different genetic lines of turkey and broiler breeder hens often do not directly correlate with IPVL protein expression of these proteins. In addition, the greater protein concentration of ZPB2 in the GD region compared to the NGD region suggests that this protein may be a critical component of the sperm receptor and could serve as a genetic selection marker for female fertility.

INDEX WORDS: Inner Perivitelline layer, Broiler Breeder, Turkey, Western Blotting, Zona Pellucida

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DEDICATION

I would like to dedicate this dissertation to Melissa, Jeff, and Lindsey Steed whose guidance and presence in life penned this manuscript.

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CHAPTER 1

AVIAN FEMALE REPRODUCTIVE PHYSIOLOGY

The Avian Ovary

The mature avian ovary contains a distinct hierarchy of follicles arranged according to size and time to ovulation. In the reproductively active hen, four to six large yolk-filled follicles of 12-40 mm in diameter are referred to as the hierarchical follicles. The large, yellow hierarchical follicles are named according to size with the largest one, the F_1 follicle, ovulating within 24 hours; the next largest follicle is the F_2 follicle and it will ovulate in about 48 hours, and so on for the other follicles. The development of these hierarchical follicles is tightly regulated with an interval of 24-26 hours between each consecutive ovulation. After the F_1 follicle ovulates, successive follicles advance one place in the hierarchy and an additional follicle is then recruited into the hierarchy from the prehierarchical follicles. The prehierarchical follicles are also categorized by size and there are several follicles within each category. Small yellow follicles (SYF) are 5-12 mm in diameter, the large white follicles (LWF) are 2- 5 mm in diameter, and the small white follicles (SWF) are less than 2 mm in diameter. As hierarchical follicles are ovulated, the hierarchy must be replenished from the set of pre-hierarchical follicles, specifically the small yellow follicles. Additionally, large white follicles will begin collecting yellow yolk thereby transforming into small yellow follicles. Most small yellow and large white follicles will fail to enter the hierarchy, instead undergoing atresia by apoptosis (Gilbert et al., 1983, Johnson et al., 1996b).

Avian Follicular Tissues: Granulosa and Theca Cell Layers

Distinct tissue layers surround each yolk-filled oocyte of the avian ovary. In each hierarchical follicle, the yolk-filled oocyte is surrounded by its plasma membrane, followed in order by the ZP or inner perivitelline layer (IPVL), the granulosa cell layer, a basement membrane, and lastly the theca cell layer. The theca layer can be further subdivided into two tissue layers, the theca interna and externa. The theca layer, unlike the avascular granulosa tissue layer, is vascularized permitting the transfer of yolk precursor components from plasma to the developing follicles in the ovary (Etches and Cheng 1981).

Follicular maturation is demarcated by the accumulation of yolk and the development of endocrine functionality in follicular tissues (Huang and Nalbandov 1979). Follicular maturation is regulated by two pituitary glycoprotein hormones, luteinizing hormone (LH) and follicle stimulating hormone (FSH), and is controlled by expression of LH and FSH receptors within granulosa tissue. FSH receptor expression in the hierarchy is highest in the granulosa cells of the small yellow follicles and expression levels decrease with follicular maturation (Calvo and Bahr 1983, Ritzhaupt and Bahr 1987, You et al., 1996, Woods and Johnson 2005). Theca cell expression of FSH receptors is less than granulosa cells and the level of FSH receptor expression in the theca cells does not vary significantly with follicular maturation (Etches and Cheng 1981, Gilbert et al., 1985, You et al., 1996). In prehierarchical follicles, FSH promotes granulosa cell proliferation and maturation (Davis et al., 2000, 2001). FSH also helps maintain the follicular hierarchy through prevention of atresia (Palmer and Bahr 1992, Johnson et al., 1996, 1999), induces LH receptor, steroidogenic acute regulatory protein (StAR) and P_{450} cholesterol side chain cleavage enzyme expression in granulosa cells for the subsequent steroid production pathway (Li and Johnson 1993, Johnson and Bridgham 2001, Johnson et al., 2004), and

stimulates progesterone (P4) production (Calvo and Bahr 1983, Robinson et al., 1988, Davis et al., 1999, 2001, Johnson et al., 2004). Cumulatively, these results suggest that the prehierarchical follicle that is more receptive to FSH will escape apoptotic cell death, leading to recruitment into the avian follicular hierarchy. Inside the pool of small yellow follicles, it is postulated that only one follicle has an upregulated level of FSH receptors, specifically in the granulosa layer, and it is likely the one that advances into the hierarchy (Woods and Johnson 2005). The selective increase is an effect, and not the cause of follicular selection. Presently, a clear understanding is lacking about the nature of the mechanism responsible for up-regulating FSHR expression (as reviewed by Johnson and Woods 2009).

As the selected FSH sensitive follicle is recruited into the hierarchy, the follicle transitions from being predominantly FSH dependent to becoming primarily LH dependent (Calvo and Bahr 1983). LH receptor expression is highest in the granulosa cells of large follicles, especially the F1-F3 follicles (Calvo et al., 1981, Calvo and Bahr 1983, Gilbert et al., 1983, 1985, Johnson et al., 1996). LH receptor mRNA expression in the theca tissue varies little with follicular development (Johnson et al., 1996) and LH promotes steroidogenesis by the theca cells of prehierarchical and hierarchical follicles (Robinson et al., 1988, Kowalski et al., 1991). The mRNA expression for the LH receptor in the granulosa cells of the prehierarchical follicles is low, but failure for LH mRNA expression up-regulation in granulosa cells in small yellow follicles is associated with atresia (Johnson et al., 1996). LH promotes granulosa cell growth (Davis et al., 2000) and progesterone production (Davis et al., 1999, Johnson et al., 2004).

Steroidogenesis in the Hen Ovary

The vascularized theca cells surrounding the small yellow and white follicles are steroidically competent and secrete androgens and estrogens through the Δ^5 steroidogenic

pathway (Lee et al., 1998) and these follicles are the primary source of plasma estrogens (Senior and Furr 1975, Lee and Bahr 1989, 1993). In contrast, the avascular granulosa cell layers of these small yellow and white follicles are steroidically incompetent due to a lack of P_{450} side chain cleavage enzyme activity (Li and Johnson 1993). The first step of steroidogenesis, the conversion of cholesterol to pregnenolone is catalyzed by P_{450} side chain cleavage enzyme (Lee and Bahr 1990, Tilly et al., 1991). Plasma concentrations of estradiol peak 4-6 hours before ovulation, but estradiol does not seem to be directly involved in ovulation. Estradiol stimulates the hypothalamus and pituitary to express progesterone receptors (Wilson and Sharp 1976). Estradiol also stimulates the liver to produce lipid and vitellogenin for yolk formation (Deeley et al., 1975) and aids in calcium metabolism for shell formation and medullary bone deposition (Etches 1987). Estradiol induces the expression of its own receptor in the oviduct which increases its effects on oviductal functions (Takahashi et al., 2004). Estradiol is responsible for increasing expression of the progesterone receptor within the ovary and oviduct, promoting the formation of tubular secretary glands for albumen, shell secretion, and the contractile activity of the myometrium (Yoshimura and Bahr 1991).

In the hierarchical follicle, both the theca and granulosa cells are steroidically competent. The granulosa cells express P_{450} side chain cleavage enzyme activity (Li and Johnson 1993, Kato et al., 1995), but possess, very low levels of 17 α-hydroxylase which shunts cholesterol based intermediates to the Δ^4 steroidogenesis pathway, producing progesterone (Etches and Duke 1984, Robinson et al., 1988, Lee and Bahr 1989, 1993, Johnson et al., 1996b, Lee et al., 1998). The theca cells of the hierarchical follicles also use the Δ^4 pathway to produce progesterone, which they further process to androgens. The theca cells of the hierarchical follicles, with the exception of those of the F_1 follicle, also metabolize the progesterone produced by the granulosa cells into

androgens (Etches 1990). In the F_1 follicle, the theca cells do not process the progesterone generated by the granulosa cells (Marrone and Hertelendy 1985). This distinctive feature allows for the F_1 follicle to be the primary source of plasma progesterone (Huang and Nalbandov 1979).

Plasma progesterone produced by the F_1 follicle binds to progesterone receptors in the hypothalamus to increase production and release of LHRH I, which stimulates the release of LH from the anterior pituitary as reviewed by Advis and Contijoch (1993). The released LH travels through the circulatory system to the ovary and binds to its receptors on the granulosa cells of the F_1 follicle to stimulate more progesterone production generating a positive feedback loop resulting in a surge in LH and progesterone production that will induce ovulation (Wilson and Sharp 1976, Robinson and Etches 1986). Plasma concentrations of progesterone peak 4-6 hours before ovulation. Progesterone, along with LH, bind to their receptors in the cells along the stigma of the F_1 follicle, which activates them to produce enzymes such as collagenase that degrade the tissue along the stigma and allow the rupture of the F_1 follicle for ovulation (Isola et al., 1987, Yoshimura and Bahr 1991).

Germinal Disc Region

The germinal disc (GD) appears as a 3-4 mm diameter white spot on the surface of the white yolk core of the preovulatory oocyte or yolk of the oviposited egg and represents less than 1% of the mature follicle volume. Prior to ovulation, the avian GD resembles the mammalian oocyte both functionally and structurally with the GD containing the arrested metaphase II meiotic nucleus, first polar body, mitochondria, and granules of glycogen (Bakst and Howarth 1977a). The germinal disc floats on a column of white yolk that extends to the nucleus of Pander (Perry et al., 1978). The composition of white yolk more closely resembles normal physiological fluids as compared to the high lipid content of yellow yolk (Perry et al., 1978) and it provides a

more suited environment for early zygote development. The germinal disc region is considered the growth center of the avian preovulatory follicle because granulosa cells proximal to the GD region are more differentiated and produce greater amounts of progesterone in response to LH (Tischkau et al., 1997). Additionally, DNA synthesis is 2-fold higher in granulosa cells within the GD region compared to granulosa cells from non-germinal disc (NGD) regions (Tilly et al., 1992). There is also noted variances in quantities of mRNA of granulosa-derived proteins that exist between GD and NGD granulosa cells (Yao and Bahr 2001). The plasma membrane of the oocyte at the GD is continuous while the plasma membrane in NGD areas is discontinuous allowing yolk material to breach the plasma membrane in these areas (Bakst and Howarth 1977a).

Ovulation and the Avian IPVL

When ovulation of the F1 follicle occurs, the theca cell layer, basal lamina and granulosa cell layer are all retained on the ovary to constitute the postovulatory follicle. Post-ovulation, the oocyte is comprised of the outermost layer, the IPVL, followed by the plasma membrane, or oolemma, which surrounds the yolk material (Bellairs et al., 1963). As reviewed by Etches (1996), the fimbriated region of the infundibulum is particularly active at the time of ovulation and surrounds the F_1 follicle and captures the ovulated oocyte.

Through electron microscopy, Okamura and Nishiyama (1978) confirmed that the infundibulum is the site of fertilization. At the time of fertilization, the plasma membrane of the oocyte is only surrounded by the IPVL and therefore, avian spermatozoa must bind and penetrate through the IPVL to reach the plasma membrane of the oocyte. Initially it was believed that sperm were able to pass through the loose fibrous meshwork of the IPVL due the spacing between the fibrous network being about 2 µm in diameter and sperm are about 0.5 µm in

diameter (Bellairs et al., 1963). Bakst and Howarth (1977a) later reported that the apparent holes in the meshwork of the ZP layer actually contained granular material, preventing a simple passage of the sperm through the IPVL.

The Acrosome Reaction

Subsequent research established that sperm penetration of the IPVL was dependent on an acrosome reaction which dissolves the IPVL, creating a hole about 9 µm in diameter through which sperm may pass to gain access to the plasma membrane of the ovum (Okamura and Nishiyama 1978). Through electron microscopy, sperm were also documented within the sperm holes following hydrolysis of the IPVL (Okamura and Nishiyama 1978). *In vitro* studies using chicken IPVL indicated that the binding of the sperm to the IPVL initiated the acrosome reaction (Koyanagi et al., 1988). Sperm penetration of the IPVL is hindered by trypsin protease inhibitors that inhibit the activity of acrosin released by the sperm to digest the IPVL (Kuroki and Mori 1997, Glogowski et al., 2001) and zinc, which prevents acrosin release by the sperm (Glogowski et al., 2001). Sperm are able to bind and penetrate the IPVL whether it is isolated from preovulatory follicles (Steele et al., 1994), ovulated oocytes (Koyanagi et al., 1988), or laid eggs (Robertson et al., 1997). These studies indicate that the biochemical and physical structure of the IPVL does not significantly change after ovulation and during egg formation (Kido and Doi 1988).

Because the formation of sperm holes in the IPVL requires sperm to first bind to the IPVL and subsequently undergo the acrosome reaction, sperm holes in the IPVL are indicative of a successful sperm-IPVL interaction. The number of spermatozoa penetrating the IPVL can be determined by the number of holes or points of hydrolysis in the IPVL (Bramwell and Howarth 1992). Formation of sperm holes occur within 2.5 minutes of *in vitro* co-incubation with sperm.

These hydrolyzed holes will then over time increase in size as enzymes continue to digest the IPVL (Robertson et al., 1997).

Relationship between Sperm Hole Formation and Fertility

Although only one sperm is required for fertilization, in avian species the probability of syngamy increases if multiple sperm penetrate the egg. In chickens and turkeys, the probability of fertilization is 100% when six or more sperm penetrate the IPVL over the GD region, yet about 50% when only 3 sperm penetrate the IPVL over the GD region (Bramwell and Howarth 1997, Wishart 1997). Wishart and Staines (1999) proposed that there may be a minimum number of sperm necessary to enter an oocyte in order for syngamy success in birds. The fate of the "extra" sperm not joining with the pronucleus of the oocyte is fragmentation and disintegration, which would imply that they do not have a function related to embryonic development (Wishart and Staines 1999). Wishart (1997) even demonstrated that embryonic development proceeds normally in eggs that contain only one sperm hole in the IPVL over the GD region. The prevailing hypothesis is that supernumerary sperm increase the probability of syngamy and early zygote development by activating the egg cell (Etches 1996) to finish meiosis and increase metabolic activity. There is most likely a threshold number of sperm supernumerary that, if surpassed, might interfere with normal embryonic development and potential induce embryonic mortality. In chickens, this threshold has been proposed to be 200 sperm penetration holes with greater numbers resulting in early embryonic death (Bekhtina 1968, Bramwell et al., 1996, Hazary et al., 2000). However, one study suggested that the threshold is much higher after finding that more than 200 sperm holes did not have a significant effect in fertile chicken eggs compared to eggs with fewer than 200 sperm holes (Bramwell et al., 1995). Despite the disagreement in the exact number of maximum sperm penetration holes an ovum

may endure before embryonic death ensues, it does seem logical that there would be a threshold of sperm holes allowed in the plasma membrane before integrity was lost or of additional genetic material allowed in the ovum before abnormal development occurred.

Sperm Holes vs. Sperm Disks

Using electron microscopy, Sultana et al., (2004) identified two distinct configurations in the IPVL of fertile quail eggs: a disk in which meshwork of fibers of the membrane are dissociated but not completely dissolved, and a hole in which the components of the IPVL are absent. The first configuration of partially dissolved IPVL was defined as a sperm disk, occuring when sperm only partially dissolve the IPVL. In a later study with quail, the same laboratory reported that most of the sperm holes classified by light-microscopy were actually only disks and not complete holes when viewed under an electron microscope and, surprisingly, only 5% of the ring shaped configurations in the IPVL from fertile quail eggs were true sperm holes (Rabbani et al., 2006). A subsequent study with chickens yielded similar sperm hole to disk ratios (Rabbani et al., 2007). Although these sperm "disks" do not represent the holes through which sperm have passed through the IPVL, they can still be used as a measure of acrosome reactions on the IPVL since the meshwork of fibers in these "disks" is partially dissociated. These findings may indicate that previously reported sperm penetrations of the oocyte plasma membrane based on sperm hole penetrations of the IPVL by light microscopy may have to be reevaluated.

Preferential Binding of Sperm at GD Region

In chickens, turkeys, and quail, spermatozoa preferentially attach and digest the IPVL in a circular fashion preferentially over and around the GD area (Howarth and Digby 1973, Ho and Meizel 1975, Bramwell and Howarth 1992, Kuroki and Mori 1997, Wishart 1997). It should be

noted, however that one study performed in chickens by Steele et al., (1994) found no sperm binding preference towards the GD area compared to NGD areas. In this study, Steele and colleagues conducted an assay in which fragments from different regions of the IPVL of freshly ovulated eggs were incubated *in vitro* with sperm. A similar frequency of sperm hole formations were found in the GD and NGD regions, and the reason for the conflicting results is unclear, but these unusual results of Steele et al., (1994) have not been duplicated to date. One study performed on 27 various captive avian species, across many orders such as Galliformes, Anseriformes, Passeriformes, Gruiformes, Ciconiiformes, Charadriiformes, and Struthioniformes (Birkhead et al., 1994) and another performed in ostrich and emu (Malecki and Martin 2003) also indicate that sperm holes are preferentially formed over the GD region.

The Window of Fertilization

Fertilization occurs in the infundibulum prior to the oocyte arriving at the magnum of the oviduct (Kaupp 1918, Olsen and Neher 1948). The ovulated oocyte remains in the infundibulum of the oviduct for about 15 minutes (Warren and Scott 1935), and this period is referred to as the "15 minute window of fertilization". This narrow window exists because a new fibrous protein (Bakst and Howarth 1977b) is deposited around the IPVL in the distal infundibulum (Bellairs et al., 1963). This layer is collectively named the outer perivitelline layer (OPVL), but is comprised of two distinct layers. The initial layer of the OPVL, termed the "middle continuous layer", is secreted and deposited around the IPVL in the distal infundibulum and is followed by a subsequent proteinaceous layer as the ovum enters the upper magnum (Bellairs et al., 1963, Bain and Hall 1969). The OPVL varies in thickness from about 8.5 µm to as little as 3 µm in the hen (Bellairs et al., 1963) and consists mainly of the following proteins; ovomucin, lysozyme,

vitelline membrane outer protein I (VMOI) and vitelline membrane outer protein II (VMOII) (Back et al., 1982, Kido et al., 1992).

The short fertilization duration window in birds is allowable because of the female bird's ability to store and release sperm. Sperm storage tubules (SST) are located in the uterovaginal junction (UVJ) and the distal infundibulum of the oviduct with the UVJ being the primary site of sperm storage while the infundibulum acts as the secondary sperm storage site (as reviewed by Bakst 1998). Synchronization of ovulation with the presence of sperm at the upper infundibulum is of singular importance to assure fertilization. The mechanisms by which sperm are released from SST have yet to be elucidated (Bakst 2009).

The OPVL and Polyspermy Prevention

As discussed previously, polyspermy is normal during avian fertilization and appears to even enhance the probability of syngamy. Because polyspermy is possible in birds, there is not a mechanism analogous to the mammalian cortical reaction in avian species that prevents multiple sperm from digesting holes in the IPVL. But, the probability of pathological polyspermy and subsequent loss of zygote viability in avian species is minimized by the deposition of the OPVL which covers the sperm binding sites on the IPVL and thus prevents initiation of the acrosome reaction (Bakst and Howarth 1977b).

During the deposition of the OPVL, the proteinaceous secretions from the epithelial cells of the oviduct trap free sperm residing in the lumen of the infundibular/magnum interface. The OPVL, along with the entrapped sperm, are deposited on top of the IPVL of the oocyte. In the ovipositioned egg, these sperm are found evenly distributed through all regions of the OPVL of both chicken (Wishart 1987) and turkey (Wishart 1997) eggs. In oviposited chicken eggs, Wishart (1997) found 10 times more sperm trapped in the OPVL than the number of sperm that

had hydrolyzed the IPVL. Birkhead et al., (1994) demonstrated that the number of sperm found in the OPVL and that had penetrated the IPVL were similar across several avian orders. Sperm recovered from the OPVL fibers have not undergone the acrosome reaction (Bakst and Howarth 1977b) and are therefore distinct from the sperm that have penetrated the IPVL. OPVL preparations inhibit the sperm acrosome reaction *in vitro* (Wishart and Fairweather 1999) because the OPVL contains protease inhibitors, such as ovomucin and ovoinhibitor (Matsushima 1958), which prevent the protease activity required during the acrosome reaction. Furthermore, it is assumed that avian species are similar to mammalian species where the actual binding of the sperm to the sperm receptor of the ZP or IPVL initiates the acrosome reaction (Koyanagi et al., 1988).

Summary

The freshly ovulated, yolk-filled oocyte is surrounded by its plasma membrane and IPVL. During the 15 minute fertilization window, sperm bind with the IPVL which activates the acrosome reaction resulting in a digested hole in the IPVL and oocyte plasma membrane through which sperm can traverse through to gain access to the pronucleus of the oocyte. In avian species, sperm preferentially bind to the IPVL in the area of the GD, where the female pronucleus is located. The opportunity for sperm to bind to the IPVL ends 15 minutes after ovulation when the OPVL is deposited around the IPVL.

CHAPTER 2

THE ZONA PELLUCIDA LAYER

General Introduction to Fertilization

Fertilization is the process by which two differentiated haploid germ cells congeal together to generate progeny in a highly regulated fashion. The haploid germ cells, the egg and sperm, combine to produce a single celled, diploid, totipotent zygote (reviewed by Talbot et al., 2003). In both avian and mammalian species, the process of fertilization will ensue once the sperm provided by the male traverse the female oviduct to recognize and bind to the freshly ovulated oocyte's outer glycoprotein layer, better known as the ZP or IPVL. Sperm binding to the ZP layer and the subsequent penetration of it occur in a species-specific manner. Unsurprisingly, the ZP's sperm binding capacity makes it an integral component of the overall sperm-egg interaction.

Introduction to the ZP and ZP Protein Nomenclature

The ZP surrounds the vertebrate egg functioning in sperm binding as well as structural support for the yolk filled oocyte as it passes through the reproductive tract (Greve and Wassarman 1985, Wassarman 1999). The ZP layer has been implicated in the process of sperm binding and has been shown to exhibit species-specific specificity in this regard. These findings across species, support the role of ZP proteins as a sperm receptor for fertilization. Naturally, due to its importance in sperm binding, abundant research has ensued into elucidating the role of the ZP in fertilization. However, with research conducted in multiple species and across numerous laboratories, certain variances in nomenclature regarding the ZP in the vertebrate

lineage have materialized. For instance, the ZP is known as the chorion in fish, the vitelline envelope in amphibians, the perivitelline envelope in reptiles, the IPVL in birds and the ZP in mammals (Spargo and Hope 2003). Contrary to the varied terminology used to describe this protein layer, its overall functionality in fertilization is ubiqitous.

Across species, the ZP layer consists mainly of a family of multiple proteins that all share a common protein domain known as the ZP domain, lending to their identification as ZP proteins (Bork and Sander 1992, Spargo and Hope 2003). Initially in 1980, Bleil and Wassarman described ZP proteins in mice using molecular weight as the naming system basis whereby ZP1 had the highest molecular weight, then ZP2, and ZP3 with the lowest molecular weight. Subsequently, Harris et al., (1994) using research that spanned multiple species (cat, dog, pig, mouse, human, and rabbit) devised a nomenclature system based on gene transcript length from largest to shortest. This new method resulted in a reordering to reflect ZP2, ZP1, and ZP3 in order of gene transcript length. In addition, to address the change in ZP order, Harris et al., (1994) devised a letter based system where ZP2 became ZPA, ZP1 became ZPB, and ZP3 became ZPC. Ironically, this new nomenclature was not universally accepted by the scientific community resulting in groups choosing between lettering and numerical demarcations. ZP nomenclature ordering was forced to be readdressed in 1999, when Hughes and Barrat discovered a new genomic sequence, designated as ZP1, which was orthologous to mouse ZPB gene and paralogous to human ZPB gene. In 2003, Spargo and Hope proposed a new, unified nomenclature system. In this system, ZP genes would be divided into subfamilies based on evolutionary mapping within the ZP gene family and then named alphabetically (ZPA, ZPB, ZPC, etc.) in order of protein sequence length. Within those alphabetical subfamilies, group level paralogues were numbered in order of coding sequence length. Paralogues below group

level of phylogeny were differentiated using an additional lower case letter. For the remainder of this literature review, the nomenclature derived from Spargo and Hope (2003) will be used to refer to the ZP genes and proteins in all species.

Identification of the Avian ZP Glycoproteins

The rapid evolution of ZP proteins has led to at least six different subfamilies of ZP genes identified in various vertebrates. At this point, the chicken is the only species to exhibit all six gene subfamilies (ZPA, ZPB1, ZPB2, ZPC, ZPX1, and ZPX2) in its genome, while most species express only three or four of the subfamilies (Smith et al., 2005). Chicken ZPX2 was originally named ZPD based on its isolation and characterization from the IPVL (Okumura et al., 2004), but Smith et al., (2005) renamed it ZPX2 to conform to the new naming protocol. However, our laboratory had already published research using the ZPD designation (Benson et al., 2005), so this designation will be used throughout this dissertation for consistency sake. Each chicken ZP gene has been found on a different chromosome (Smith et al., 2005), so the avian ZP genes are not clustered as is sometimes seen with members of the ZP gene families in other species (Mold et al., 2001). The mapping of the chicken ZP genes places ZPB1 on chromosome 5, ZPA on chromosome 14, ZPC on chromosome 10, ZPB2 on chromosome 6, ZPD on chromosome 11, and ZPX1 on chromosome 3 (Smith et al., 2005). Previous proteomic analyses of the chicken IPVL revealed two more potential ZP glycoproteins, a second ZPC protein named ZP3A and a second ZPAX protein named ZPAX2, giving potentially eight distinct ZP glycoproteins in the chicken IPVL (Hughes 2007, Mann 2008).

ZPA and ZPX1

Smith et al., (2005) identified and subsequently mapped ZPA and ZPX1 in the chicken genome. Although chicken ZPA and ZPX1 were classified as part of the chicken genome,

expression analysis of ZPA and ZPX1 proteins in the chicken has yet to be characterized. In turkeys, ZPA and ZPX1 were both detected by real time RT-PCR in granulosa cells isolated from each of the hierarchical follicles (Benson 2006, Benson et al., 2017). In broiler breeder hens the mRNA expression of ZPA is greatest in granulosa cells from small white follicles less than 2 mm in diameter and expression decreases drastically as follicles mature to the SY and hierarchical stages (Benson et al., 2017). Similarly, in quail, ZPA protein expression has been found to decrease dramatically during follicular development, with highest expression observed in the large white follicles (Kinoshita et al., 2010). In the white leghorn hen, Nishio et al., (2014) demonstrated that expression of ZPA mRNA and protein was coordinately highest in the large white pre-hierarchical follicles, and decreased with follicular maturation.

ZPB2

In 1999, a cDNA sequence for ZPB was deposited in GenBank (Accession # AB025428) and subsequently renamed ZPB2 under the Spargo and Hope naming system (2003). Based on this sequence, Bausek et al., (2000) made a cDNA probe of ZPB2 for Northern analysis and detected expression of this message only in small stroma embedded follicles in the chicken ovary. Subsequently, using real time RT-PCR, Benson (2006) reported that ZPB2 mRNA expression was present in granulosa cells isolated from large preovulatory follicles of turkey hens and that the expression of ZPB2 was higher in granulosa cells isolated from the GD region than NGD regions of the F_1 and F_2 follicles. Likewise, Malloy (2011) reported expression of broiler breeder hen ZPB2 mRNA to also be higher in the granulosa cells overlaying the GD of F_1 follicles, compared to the NGD region. The mRNA expression profile for ZPB2 during follicular maturation was also analyzed, and ZPB2 expression was greatest in the granulosa cells of LWF followed by a strong significant reduction in expression as the follicle began the uptake

of yellow yolk components (Malloy 2011). Nishio et al., (2014) also noted similar results in white leghorns.

ZPD

The mRNA for ZPD has been detected in preovulatory follicular granulosa cells of the chicken and quail (Okumura et al., 2004, Sato et al., 2009). In the laying hen ZPD, like chicken ZPC, is a 42 kDa protein (Okumura et al., 2004), and the two proteins co-migrate under reducing conditions. However, the two ZP proteins separate under non-reducing conditions due to a drastic and unexplained increase in ZPC mobility (Okumura et al., 2004, Nishio et al., 2014). In quail, ZPD protein expression was investigated in the hierarchical follicles (F_1-F_4) and expression was lowest in the F_4 follicle and as the follicle matured, expression increased with the greatest expression found in the F_1 follicle (Sato et al., 2009). In agreement with prior studies, ZPD mRNA and protein expression in white leghorns was found to be initially expressed in the smaller preovulatory follicles and expression increased incrementally until reaching its highest level in the F_1 follicle (Nishio et al., 2014). In turkey hens, Benson (2006) reported that expression of ZPD mRNA did not differ between F_1 GD and NGD granulosa cells.

ZPC

The majority of the chicken's IPVL appears to be composed ZPC, ZPD and the 95 kDa glycoprotein ZPB1 (Waclawek et al., 1998, Bausek et al., 2000, Okumura et al., 2004, Hughes 2007, Mann 2008). The mRNA transcript for chicken ZPC was detected by Northern Analysis in granulosa cells of the developing preovulatory follicles (Waclawek et al., 1998, Takeuchi et al., 1999). Western blot analysis with anti-chicken ZPC detected a 42 kDa protein in the IPVL isolated from large preovulatory follicles (Waclawek et al., 1998). In quail, both ZPC protein and mRNA expression increases as the follicle matures towards ovulation (Pan et al., 2001). The

developmental mRNA and protein expression profile reported by Pan et al., (2001) was also found in white Leghorn hens (Nishio et al., 2014). Malloy (2011) reported ZPC mRNA expression in the NGD region of F_1 granulosa cells of broiler breeders was higher than the GD region in 3 of 4 genetic lines examined. In the 4th genetic line the expression of ZPD was equal in NGD and GD granulosa cells. In turkey hens, ZPC mRNA expression in F_1 granulosa cells was greater in NGD granulosa cells compared to GD granulosa cells in a low female fertility genetic line while the mRNA expression was equal between the 2 regions in a high female fertility genetic line (Benson 2006).

Interestingly, Western blot analysis of the IPVL isolated from freshly laid eggs revealed that chicken ZPC undergoes postovulatory modifications because anti-chicken ZPC antibodies only detected a single 34 kDa protein in these samples (Waclawek et al., 1998). This size reduction is independent of IPVL-sperm interaction because this smaller protein was detected in the IPVL of laid eggs from virgin hens (Waclawek et al., 1998). Pan et al., (2000) reported a similar size difference between quail ZPC detected in preovulatory follicles versus laid eggs. Both Pan et al., (2000) and Waclawek et al., (1998) suggested that the size modification is likely due to the presence of a protease secreted by the infundibulum. Pan et al., (2000), further suggested that in addition to proteolytic processing involving the removal of the N-terminal amino acids, the molecular weight reduction of ZPC also resulted from N-linked and O-linked oligosaccharide chain modifications. However, Waclawek et al., (1998) reported that the size reduction was independent of oligosaccharide side chain modifications in the chicken.

ZPB1

A 95 kDa protein component of the IPVL was identified, isolated and then subsequently cloned from a chicken liver cDNA library (Bausek et al., 2000). Based on the significant

homology of the coding sequence of their isolated clone and mammalian ZPB1 sequences, the clone was classified as chicken ZPB1 (Bausek et al., 2000). Antisera to chicken ZPB1 bound a 95 kDa protein in immunoblot samples from the IPVL and liver but not from granulosa cell samples separated on SDS-PAGE gels under reducing conditions, indicating that chicken ZPB1 is synthesized in the liver and then transported to the ovary (Bausek et al., 2000). When IPVL and liver samples are separated on SDS-PAGE gels under non-reducing conditions and transferred to immunoblots, an additional 180 kDa band is detected (Bausek et al., 2000, Takeuchi et al., 2001). The detected 180 kDa peptide, which is approximately twice the mass of the 97 kDa peptide, was determined to be a dimer of the 95 kDa peptide formed through intermolecular disulfide bonds (Bausek et al., 2000, Takeuchi et al., 2001, Okumura et al., 2004). Similarly, ZPB1 in Japanese quail is transported to the ovary from the liver and has a high level of homology (87.8%) with chicken ZPB1 (Sasanami et al., 2003a). In fact, both ZPB1 and ZPC have been found to be sufficiently conserved across avian species to allow antibodies formed against the chicken proteins to detect the orthologous proteins in other avian species including Galliformes, Anseriformes, Passeriformes and Columbiformes (Stewart et al., 2004). Using Leghorn hens, Nishio et al., (2014), reported ZPB1 expression at the IPVL increases as the oocyte developed with the greatest expression found in the F_1-F_4 hierarchical follicles.

Site of Synthesis and Regulation of ZP Protein Synthesis

The tissue and cell location of ZP protein synthesis varies across species. In mammals, the origin of the ZP glycoproteins is still unresolved. Three theories have been proposed hypothesizing that the proteins are synthesized by (1) the oocyte alone, (2) the follicle cells alone, or (3) both the oocyte and the follicle cells. In the mouse, strong evidence indicates that mZPA, mZPB, and mZPC are exclusively synthesized by the growing oocyte and not the

granulosa cells of the developing follicle (Epifano 1995, Wassarman 2008). Both the oocyte and the surrounding follicular cells synthesize ZP proteins in human, monkey, rabbit, dog, pig and cow at different stages of follicular development (as reviewed by Sinowatz et al., 2001). Some species of teleosts such as the rainbow trout (Oppen-Burntsen et al., 1992), white flounder (Lyons et al., 1993) and medaka (Sugiyama et al., 1998) produce ZP proteins in the liver, but in Gilthead Seabream, *Sparus aurata*, the ZP proteins are produced in both the liver and ovary (Modig et al., 2006). The chicken, turkey and quail, synthesize ZPB1 in the liver and then subsequently transport this glycoprotein to the developing follicles in the ovary, while the other ZP proteins are produced by the granulosa cells and possibly for some ZP proteins, the theca cells of the developing preovulatory follicle (Waclawek et al., 1998, Bausek et al., 2000, Sasanami et al., 2003a, Okumura et al., 2004, Benson 2006, Malloy 2011, Benson et al., 2017). The production of ZPB1 in the liver in avian species aligns with the fact that the vast majority of the yolk components that are deposited in the developing follicles are also produced by the liver (Lazier 1978, Nadin-Davis et al., 1980).

The mechanisms regulating the transcription of ZP genes are largely unknown for many species whose ZP genes have been cloned. Three ovary-specific DNA-binding proteins have been identified in the mouse; zona pellucida gene activating protein-1 (ZAP-1) which binds to the conserved sequence 5'–CAC(G/C)TG-3' that is within 250 base pairs upstream of the mZPA and mZPC promotor TATAA box (Millar et al., 1993), the oocyte-specific protein 1 (OSP-1) which binds to the sequence 5'–GATAA-3' within the first 100 base pairs of the mZPC promoter (Schickler et al., 1992, Wassarman 2008), and FIGL-ALPHA, which is a transcription factor regulating expression of zona pellucida proteins A, B1, and C through an E-Box motif at the promoter region (Liang et al., 1997). Genetic knockouts of FIGL-ALPHA result in a failure to

develop primordial follicles and follicular development is impaired (Liang et al., 1997, Soyal et al., 2000, Hu et al., 2012). Similar regulatory mechanisms of ZP gene expression are conserved across other investigated mammals (Liang and Dean 1993, Millar et al., 1993, Buhi et al., 2000) and also in fish (Kanamori 2003, Liu et al., 2006, Mold et al., 2009).

Expression of mRNA for all three ZP proteins in the mouse is developmentally regulated in coordinate as ZPA, ZPB1, and ZPC mRNA expression increases in concert during the early stages of oogenesis and subsequently decline together (Epifano 1995). In medaka (*Oryzias latipes*), ZPA, ZPB1, and ZPC are concomitantly expressed in primary oocytes and subsequently halt expression just prior to rapid deposition of yolk protein and lipid components (Kanamori 2000). In avian species mRNA expression patterns of the ZP proteins are distinctly different than mammals, whereby ZPA and ZPB2 are expressed early in development, and ZPB1, ZPC, and ZPD are expressed later in maturation (Pan et al., 2001, Benson 2006, Sato et al., 2009, Malloy 2011, Nishio et., al., 2014, Benson et al., 2017).

Hormonal Regulation of ZP Protein Synthesis

There is a paucity of research on the hormonal regulation of ZP protein synthesis. Exogenous estradiol was found to increase hepatic expression of the ZP proteins in female fish as well as young male fish (Hyllner et al., 1991, Oppen-Berntsen et al., 1992, Larsson et al., 1994). Because estrogens and estrogenic compounds affect hepatic expression in fish species, ZP protein expression can be used as an indicator of environmental water contamination of estrogen-like substances (Oppen-Berntsen et al., 1999, Arukwe and Rhoe 2008, Rhee et al., 2009). In domestic dogs, Kempisty et al., (2012) reported based on cell culture experiments that ZPA mRNA and protein expression was down regulated by estrogen and progesterone

individually, or in combination, while ZPC and ZPB2 expression was upregulated by these hormones.

The liver of reproductively active female chickens (Bausek et al., 2000) and quail (Sasanami et al., 2003a) synthesize ZPB1, but it is not produced by sexually active males. However, estradiol treatment of roosters results in a dramatic induction of ZPB1 production by the liver such that serum levels are elevated in these males to levels similar to those observed for an egg-producing laying hen (Bausek et al., 2000). Similar results were found in male quail after diethylstilbestrol treatment (Sasanami et al., 2003a). The induction of ZPB1 was so pronounced that Sasanami et al., (2003a) suggested that it could be used in males or immature females as a biological marker to detect environmental estrogen exposure. In contrast, estradiol and progesterone do not have an effect on ZPC expression in quail, (Pan et al., 2001). In cultured quail granulosa cells, ZPC synthesis is stimulated by testosterone (Pan et al., 2001) and FSH (Pan et al., 2003). Benson (2006) found that ZPC and ZPD mRNA expression was up-regulated in chicken granulosa cells isolated from SYF when cultured in the presence of LH, FSH, or testosterone. In cultured F_3 granulosa cells, FSH stimulates ZPC and ZPD mRNA expression while testosterone and estradiol stimulate ZPD mRNA expression (Benson 2006). Similarly in quail, ZPD protein expression is significantly increased in F_3 granulosa cells cultured in the presence of FSH, but FSH did not have a significant effect in F_1 or F_2 granulosa cells. (Sato et al., 2009). In F_1 granulosa cells, LH lowered the mRNA expression of ZPC but not ZPD (Benson 2006).

Structure of ZP Glycoproteins

Across all species, egg coat proteins have retained some common essential features in their primary structure that enable them to perform important functions during oogenesis,

fertilization, and early development within vertebrate lineages (Wassarman 1988, Wassarman et al., 2001, Monne et al., 2008). All ZP genes have a common domain known as the ZP domain (Bork and Sander 1992). The mammalian ZP domain, located near the C terminus, consists of ~260 amino acids and has either 8 (type I domain found in ZPC) or 10 (type II domain found in ZPB1/2) conserved cysteine residues that participate in intramolecular disulfide bonds (Bork and Sanders 1992, Jovine et al., 2005, Han et al., 2010). These bonds are responsible for the polymerization of these proteins into filaments that collectively form the zona pellucida protein matrix (Jovine et al., 2002, 2005, Han et al., 2010). Deletion of the ZP domain in oocytic ZP proteins also prevents their incorporation into the ZP (Jovine et al., 2002). This loss of function suggests a larger function for the ZP domain.

While sequence analyses shed light on the existence of ZP domain within the zona proteins (Bork and Sanders 1992) it did not provide much insight into structure and function of the ZP proteins. Years later, Monne and colleagues (2008), elucidated the high resolution crystal structure of the ZP domain of mouse ZPC. This development provided key advancements in our understanding of the zona layer, however, a pitfall to the partial crystal structure was its inability to offer information on functional features of ZP proteins. Shortly thereafter, the full crystal structure of chicken ZPC was published (Han et al., 2010), this structure in conjunction with the previous partial structure (Monne et al., 2008) of ZPC provided insight into ZPC functionality. For instance, the full length crystal structure provides clarity into the ZP domain identified by Bork and Sanders (1992) which is actually made up of two sub domains, ZP-N and ZP-C. Both ZP-N and ZP-C form an IG-like protein structural fold made up of two beta sheets. The two sheets contain specifically, A-B-E and C-F-G strands. Additionally, the beta sheet folds also accommodate the cysteine residues involved in the notable conserved disulfide bonds of the ZP

proteins. Each ZP domain, shares a similar topology and structural similarity with one another which is remarkable given they do not share the same primary structure and sequence (Han et al., 2010).

The sub-domain ZP-N is a distinct type of IG super family protein as it contains a unique E' strand in the E-F-G strand of the beta sheet as well as two disulfide bonds that lock the beta sheet together (Monne et al., 2008, Han et al., 2010). Those disulfide bonds are C1-C4 and C2-C3 and connect the 4 conserved cysteine residues found in all ZP proteins identified to date (Bork and Sanders 1992, Jovine et al., 2005, Kanai et al., 2008). Deletion and mutational studies on ZP-N have demonstrated ZP-N's involvement in the secretion of ZP proteins (Han et al., 2010), Additionally, Each ZP glycoprotein contains a hydrophobic N-terminal signal sequence that varies in length and targets nascent ZP glycoproteins to the secretory pathway (Wassarman et al., 2005). The signal peptide is cleaved off of the mature ZP glycoprotein, however, it is not known at which stage of ZP biosynthesis and assembly into the zona matrix, the signal peptide is removed.

Secretion of the ZP Glycoproteins

ZPC must be secreted as a homodimer according to mutational work by Han et al., (2010). If the electrostatic interactions between the homodimers are impaired, secretion will cease to occur. This necessitation for ZPC to be secreted as a homodimer suggests it may be assembled as a homodimer at the IPVL. Prior research by Jovine and colleagues (2004) suggested for all ZP proteins, successful secretion and subsequently polymerization of the ZP filaments, likely depends in part on the external hydrophobic patch (EHP) located on the C terminal end of the ZP-C domain. In quail ZPC, proteolytic processing of the C-terminal end is required for secretion and if this cleavage event is blocked, pro-ZPC will accumulate in the

endoplasmic reticulum (Sasanami et al., 2003b). The EHP is located between a consensus furin cleavage site (CFCS) and the transmembrane domain (TMD) which are both required for successful secretion and polymerization. Jovine et al., (2002) demonstrated that mutant forms of ZPA and ZPC, with the TMD region removed, were successfully secreted, but not incorporated into the ZP matrix. In addition, avian granulosa cells of the hierarchical follicles are arranged on the surface of the oocyte as a single layer enabling several studies to investigate the intracellular trafficking and highly polarized secretion of avian ZPC. Waclawek et al., (1998) performed immunohistochemical staining on chicken granulosa cells and demonstrated the exclusive presence of chicken ZPC on the apical side of the granulosa cells and proposed that a hydrophobic domain near the carboxy-terminus of ZPC may serve as a glycosylphosphidylinositol (GPI) anchor which directs the newly synthesized ZPC to the apical side of the granulosa cells. These results are supported by prior work postulating that the TMD is used to anchor the glycoproteins in secretory vesicles and the plasma membrane of the endoplasmic reticulum (Wassarman et al., 2005).

In their proposed mechanism for ZP protein assembly, Jovine et al., (2004) predict that the EHP functions as a control switch that prevents the premature polymerization of ZP precursors. When ZP glycoproteins are synthesized in the trans-golgi in the cytoplasm of the oocyte, premature assembly must be avoided while the proteins are still in the cytoplasm. This delay was suggested to be achieved by the interaction of the EHP and IHP. Subsequent cleavage of ZP proteins by a furin-like enzyme would lead to the loss of the EHP. After this cleavage, the glycoproteins can assemble into crosslinked filaments using the IHP within the ZP domain (Jovine et al., 2004). Mutations to the EHP and the IHP resulted in impaired prevention of premature polymerization of ZP proteins (Jovine et al., 2004).

Assembly of the ZP Matrix

The ZP is a pronounced extracellular matrix and varies in thickness from less than 1 μm to more than 27 μm in studied mammalian species (Wassarman 1988, Dunbar et al., 1994, Mugnier et al.,2009). The 6 μm-thick mouse ZP has a sponge-like, porous structure, allowing relatively large molecules such as antibodies and small viruses to permeate through. Assembly of the ZP matrix is best understood in the mouse where ZPC associates with ZPA and is crosslinked by ZPB1 to form filaments (Greve and Wassarman 1985, Wassarman 1988). Regretfully the molecular mechanisms of mouse ZP assembly cannot be directly applied to other species that differ in the number of expressed ZP proteins. For instance the human ZP consists of five ZP glycoproteins (Smith et. al., 2005) while the Xenopus Laevis vitelline envelope has six ZP proteins (Smith et. al., 2005), and the chicken IPVL consists of at least six ZP glycoproteins (Hughes and Barratt 1999, Smith et al., 2005, Mann 2008).

The understanding of the IPVL assembly in avian species is limited. Rodler et al., (2012) completed research with Japanese quail, whose inner perivitelline layer is 1-3 um-thick. In Japanese quail primordial follicles localization of ZP proteins was devoid. As development ensued, synthesis and localization of ZPA and ZPB2 were observed in the granulosa cells and developing IPVL of white follicles. As the follicles matured, localization of ZPA and ZPB2 declined to nearly unobservable levels in the hierarchical F_4 follicle. ZPB1 localization was absent in primordial follicles and became detectable in the IPVL by the F_5/F_4 follicular stage. ZPC and ZPD were localized to granulosa cells, where ZPC was first located in the white follicles (1-2 mm in diameter) where it incorporated into the IPVL matrix containing ZPA and ZPB2. Localization of ZPC into the IPVL was notably increased in larger follicles, becoming a major component of the IPVL layer. However, ZPD protein was not observed until the

hierarchical F_5 stage, suggesting its incorporation into the avian IPVL was the last of all the ZP proteins.

Based on their quail ZP protein expression findings, Rodler and colleagues (2012) hypothesized that ZPA and ZPB2, may act as a pre-matrix protein complex to form a scaffold by which the two largest ZP components of the IPVL, ZPB1 and ZPC, integrate into together. Last, ZPD, a minor component of IPVL can then be secreted into the IPVL during the late stages of follicular development by the neighboring granulosa cells. Support for this assembly hypothesis is supported by similar IPVL protein expression work in other avian species (Mann 2008, Kinoshita et al., 2010, Nishio et al., 2014). Additionally, Ohtsuki et al., (2004) reported that granulosa cell cultures secrete ZPC in a soluble form, but ZPC becomes insoluble when IPVL homogenates are added to the cell culture media, implying protein interactions lead to aggregation. In addition, chicken and quail ZPB1 and ZPC have been known to spontaneously form fibrous aggregates of ZPB1-ZPC hetero-complexes *in vitro* through disulfide cross-linked bonds (Ohtsuki et al., 2004, Okumura et al., 2007b). As these ZPB1-ZPC complexes accumulate on the surface of the oocyte, they may act as scaffolding for subsequent matrix construction including ZPD association (Okumura et al., 2007a, Rodler et al., 2012).

Sperm-ZP Adhesion

The ZP layer acts as a protective barrier for the oocyte and sperm must hydrolyze a hole through the ZP before reaching the oocyte's plasma membrane. For successful fertilization to occur, the plasma membranes of sperm and egg must bind and fuse together. This fusion is the mixing of the sperm inner acrosomal and oocyte plasma membranes, which facilitates cytoplasmic continuity between the two cells (Stein et al., 2004). The ZP glycoprotein component responsible for sperm recognition and binding and initiation of the acrosome reaction

varies from species to species. Most of the understanding on the functional aspects of ZP glycoproteins during fertilization has been developed from the mouse model. Early fertilization studies using mice indicated ZPC as the primary sperm receptor in the ZP based on its ability to induce sperm acrosomal exocytosis upon sperm binding (Bleil and Wassarman 1980a, 1983, 1986). Mouse ZPA may act as the secondary receptor for sperm acting to maintain the binding of the acrosome-reacted spermatozoa to the ZP (Bleil et al., 1988). The third protein component of the mouse oocyte ZP, ZPB, simply acts as a structural component of the ZP (Wassarman et al., 2001).

Subsequent research is leading to potential modifications of the original model of sperm binding to the ZP of the mouse ovum. Both Baibakov et al., (2012), and Arella et al., (2014) completed research where the ZP proteins of mice were replaced with human othologs which indicated that ZPA may actually play a larger role in the mediation of sperm binding process than was previously thought. Many studies with mice have focused on the oligosaccharides linked to ZPC and have found O-linked oligosaccharides located near the C-terminus may be critical for sperm-egg binding and initiating the sperm acrosomal reaction (Florman and Wassarman 1985, Chen et al., 1998, Kerr et al., 2004). However, Liu and Wassarman (1995) reported that mutated serine residues in mouse ZPC, which are residues that accomodate O-linked oligosaccharides, had no effect on fertilization when mutated.

Carbohydrates may not be the only factors involved in sperm-egg recognition. In both humans (Chapman et al., 1998, Chakravarty et al., 2005) and in mice (Ding et al., 2007) the ZP glycoprotein's polypeptide backbone also takes part in the species-specificity of the sperm-ZP interaction. The human ZP is composed of four proteins ZPA, ZPB1, ZPB2, and ZPC (Hughes and Barratt 1999, Lefievre et al., 2004). Several studies indicate that ZPC can induce an

acrosomal reaction in human sperm (Van Duin et al., 1994, Dong et al., 2001, Bray et al., 2002a, 2002b). Other studies have reported that ZPB2 also induces the acrosome reaction (Chakravarty et al., 2005, Caballero-Campo et al., 2006, Chakravarty et al., 2008). Subsequently, Chiu et al., (2008) suggested that both ZPC and ZPB2 act to induce the sperm acrosome reaction and ZPA acts as a secondary sperm receptor. In addition, recent studies conducted with human ZPB1 indicated that it, too, may have a role in inducing the acrosome reaction (Gupta et al., 2009, Ganguly et al., 2010). Therefore, in humans, ZPB1, ZPB2, and ZPC have all been found to induce the acrosome reaction, and more research is needed to determine how these glycoproteins interact specifically with sperm.

The inability to clarify the specifics of sperm binding in humans may be due, in part, to research on the functional attributes of human ZP glycoproteins being hindered by the lack of human oocytes and thus the nonavailability of sufficient amounts of native purified human ZP proteins. Researchers have overcome this obstacle by resorting to recombinant DNA technology and the use of Escherichia coli (E. coli) and baculovirus expression systems. However the glycosylation of recombinant human ZP proteins are likely not exactly the same as native human ZP proteins and thus influence sperm binding artificially. In non-human species, purified glycoproteins are comparatively more accessible and therefore the research is plentiful and has yielded results that support a great deal of variety in specific sperm-binding glycoprotein(s) across vertebrate species.

Similar to humans, studies from various other species have suggested that more than one ZP protein is involved in binding sperm and inducing the acrosome reaction. In the pig, a ZPB-ZPC heterodimer acts as the primary sperm receptor (Yurewicz et al., 1998). In addition, in contrast to mice, N-linked carbohydrates, rather than O-linked carbohydrates, are involved in

sperm binding to the ZP in pigs (Yonezawa et al., 1995). In the cow, ZPB1 exhibits the strongest sperm-binding activity among the three identified bovine ZP proteins (Yonezawa et al., 2001). In the rabbit, both ZPB1 and ZPC have been found to bind sperm (Yamasaki et al., 1995), and baculovirus-expressed ZPB1 has been shown to bind to the sperm in a dose-dependent manner (Prasad et al., 1996). In Xenopus laevis, ZPA and ZPC are both individually able to bind sperm, yet ZPC appears to be the major ligand for sperm binding (Vo and Hedrick 2000). However, combining egg glycoproteins ZPA, ZPB, and ZPC in the natural ratio found in Xenopus egg envelopes, enhances sperm binding (Vo and Hedrick 2000).

Interestingly, recent evidence suggests that the acrosome reaction should not be used as an indicator of sperm binding to ZP or for the search for the sperm receptor(s). Inoue and colleagues (2011) performed key experiments in which acrosome reacted sperm from the perivitelline space was recovered and shown to have the capacity to once more reach the ZP layer, and fertilize the egg. Jin et al., (2011) also reported the acrosome reaction occurs independently of sperm binding, as most sperm had undergone the reaction prior to ZP contact in the study. These results seem to indicate that the process of sperm-zona recognition and binding in mammals may be much more complex than previously thought.

In avian species, all three major ZP components of the egg envelope, ZPB1, ZPC, and ZPD, have been implicated in sperm-egg interaction (Bausek et al., 2004, Okumura et al., 2004). In the quail, there is evidence that ZPB1 is responsible for activating the acrosomal reaction (Sasanami et al., 2007). Mori et al., (1998) and Pan et al., (1999) reported that antiserum against ZPC inhibits sperm binding and penetration of the IPVL in quail. Bausek et al. (2004) also presented evidence which suggested that ZPC may play a role in the sperm-egg interaction because chicken ZPC binds with far more affinity than ZPB1 to two isolated and purified

chicken sperm proteins. Both chicken ZPC and ZPB1 have an ability to bind individually to the acrosomal region of rooster sperm (Bausek et al., 2004, Ichikawa et al., 2017). However, sperm binding to the IPVL leads to a proteolytic degradation of ZPB1 into discrete fragments, while chicken ZPC degradation is minimal (Bausek et al., 2004). The degradation of ZPB1 suggests that it may be degraded more during the acrosome reaction and therefore is more intimately involved in the sperm-IPVL interaction. Through these studies, Bausek et al., (2004) suggested that the binding of rooster sperm to the IPVL is mediated by both chicken ZPC and chicken ZPB1 with the initial adhesion occurring through ZPC. In addition, antiserum directed against ZPB1 significantly reduced the number of sperm holes dissolved in the IPVL (Takeuchi et al., 2001, Bausek et al., 2004). Okumura et al., (2004) reported that dimeric ZPB1 was capable of stimulating the sperm acrosome reaction during an *in vitro* sperm activation assay. These observations are in line with Ichikawa et al., (2017) where antibodies against ZPB1 generated the greatest reduction in the amount of sperm holes formed through *in vitro* analysis, followed by ZPC.

In experiments with two genetic lines of turkey hens that differ in female fertility, Benson et al., (2009) found greater levels of hepatic ZPB1 mRNA expression in the genetic line with higher fertility. These results imply that ZPB1 expression could be correlated with better fertility in turkeys. However a role for ZPC was also implicated in the work by Benson et al., (2009) because hens from the genetic line with low fertility had greater levels of ZPC mRNA expression in NGD granulosa cells than GD granulosa cells. In contrast, the hens from the high fertility line had equal levels of ZPC mRNA expression between GD and NGD granulosa cells. Benson et al., (2017) also examined ZPB2 expression in the two lines of turkey hens, and ZPB2 mRNA was greater in GD granulosa cells than NGD granulosa cells in both lines of hens. The greater

mRNA expression of ZPB2 in the granulosa cells surrounding the GD area may be important for the preferential binding of sperm to this region of the IPVL, because sperm preferentially bind at the GD region (Howarth and Digby 1973, Bramwell and Howarth 1992, Birkhead et al., 1994, Wishart 1997).

Because ZPA and ZPB2 expression is greater in the GD region in quail and chicken (Serizawa et al., 2011, Nishio et al., 2014, Benson et al., 2017) it naturally facilitates interest in them as a key component for sperm preferentially binding at this location. However, both them were found to have no significant effect in sperm hole formation *in vitro* (Ichikawa et al., 2017). This however, does not necessarily rule out ZPA and/or ZPB2 playing a role as secondary sperm receptors. These proteins may require primary receptors to lock sperm into an amiable position for attachment. Furthermore, due to the nature of plentiful antibody binding to ZPC or ZPB1 which are major components of the GD ZP matrix, it is possible that these antibodies block access to the minor, but important components, ZPB2 and ZPA and thus making it appear that they do not bind sperm.

One of the more recently discovered chicken IPVL glycoproteins, ZPD, was also reported to play a role in the sperm egg-interaction immediately following its identification based on its ability to stimulate the sperm acrosome reaction *in vitro* better than ZPC and monomeric ZPB1 (Okumura et al., 2004). Additionally, because N-linked glycans have been shown to be vital in avian sperm-egg interactions (Horrocks et al., 2000, Robertson et al., 2000), it is important to note that chicken ZPD has the most potential N-glycosylations sites of any of the known avian ZP glycoprotein. However, Benson et al., (2009), did not find a significant difference in ZPD mRNA expression between the two genetic lines of turkey hens that differ in female fertility and antibodies against ZPD did not prevent sperm binding *in vitro* (Ichikawa et al.,2017).

With research reports indicating that at least 5 of the ZP proteins found in avian species have sperm binding capacity, a great deal more research is needed to untangle which ZP protein or proteins function as the sperm receptor in avian species. In addition, as research advancements are made in identifying the avian sperm surface molecule or molecules that bind to IPVL, it will become easier to resolve the identity of the avian sperm receptor.

Oligosaccarides of the ZP

The size of the individual ZP glycoproteins across species varies due to extensive posttranslational modifications such as sulfation and glycosylation (Prasad et al., 2000). ZP glycoproteins can exhibit both N-linked and O-linked glycosylation. In N-linked glycosylation, an oligosaccharide chain is attached by oligosaccharyl transferase to asparagine's amino group when it occurs in the tripeptide sequence Asn-X-Ser or Asn-X-Thr, where X can be any amino acid except Pro. In O-linked glycosylation, N-acetyl-galactosamine is attached to a serine or threonine residue by the enzyme UDP-N-acetyl-D-galactosamine: polypeptide N-acetyl-galactosaminyl-transferase. The oligosaccharides of the ZP proteins are also sialylated and sulfated (Liu et al., 1997), which further contributes to their molecular weight heterogeneity and makes the ZP glycoproteins relatively acidic (Noguchi and Nakano 1992). To illustrate the extent of heterogeneity across species, the following comparisons are made between two closely related species: the mouse and rat. The mouse ZP consists of ZPA, ZPB1, and ZPC, which have different molecular masses of 200, 120, and 83 kDa, respectively (Bleil and Wassarman 1980b), yet in the rat, the molecular weights of ZPA, ZPB1, and ZPC are 140, 185 and 46 kDa, respectively (Akatsuka et al., 1998).

Research on the carbohydrate moieties of ZP glycoproteins has consistently supported their necessity for successful sperm binding and penetration of the IPVL. Chicken ZPD, ZPB1,

and ZPC proteins have four, three, and one potential N-glycosylation sites, respectfully (Okumura et al., 2004, 2012). In chicken, the removal of both N- and O-linked oligosaccharides from the IPVL results in a loss of sperm receptor activity (Howarth 1992). More specifically, Robertson et al., (2000) reported that the removal of N-linked, but not O-linked oligosaccharides led to an inhibition of avian sperm-IPVL interaction. Horrocks et al., (2000) also reported that the acrosomal reaction in chickens is induced by N-linked glycans which have a terminal N-acetyle-glucosamine residue. However, a study with the high resolution crystal structure of chicken ZPC indicated that the elimination of a specific O-glycan led to an 80% decrease in sperm binding to ZPC (Han et al., 2010). Interestingly, to add more complexity, Rodler (2011), noted that over the development of the avian follicle, the oligosaccaride patterns of the follicle changed. Rodler inferred that in previous studies where immature ovum were observed to fail to interact with sperm, may have been due to them not having the oligosaccharide pattern present at ovulation. Okumura et al., (2012) used 2D electrophoresis to discover that heterogeneity in lectin binding exists with chicken ZPC, because ZPC was found to reside in at least 4 different isoforms based on isoelectric points resulting from distinct oligosaccharide profiles. Taken together, identifying which ZP glycoprotein(s) carries the carbohydrate moiety(s) responsible for sperm-IPVL binding.

Practical Applications

Due to their critical role of binding sperm, ZP glycoproteins have been utilized in the development of novel approaches to control fertility. The first study to demonstrate the feasibility of using antibodies against ZP glycoproteins for immunocontraception injected heat-solubilized, mouse ZP-generated antibodies into hamsters. The mouse antibodies bound to the native hamster ZP proteins and induced partial infertility (Gwatkin and Williams 1977). Shortly

after this discovery, Gwatkin and colleagues were able to induce a complete loss of fertility in the mouse with the active immunization directed against hamster zona pellucida (Gwatkin and Williams 1977, Gwatkin et al., 1977). Since these studies, numerous investigators have published reports detailing the effectiveness of using the development of antibodies against ZP proteins as an immunocontraceptive to induce infertility (as reviewed by Kirkpatrick et al., 2009). The administration of a ZP-protein immunocontraceptive vaccine in wild populations as a humane alternative for population control has been demonstrated in several species such as feral horses (Bartholow 2007), white-tailed deer (Kirkpatrick et al., 1997), and dogs (Mahi-Brown et al., 1985). For wild animal immunocontraceptive vaccines, porcine zona pellucida (PZP) has become the antigen of choice due to the accessibility of porcine ovaries from abattoirs and the observation that antibodies developed against PZP prevents fertilization in a variety of species (Sacco 1977).

Unfortunately, the initial optimism of ZP immunocontraceptive vaccines has been marred by the appearance of complete ovarian regression in some research experiments using ZP antibody protocols. The loss of ovarian function is characterized by a disruption of folliculogenesis and ultimately by a depletion of the primordial follicle population, a situation from which the ovary cannot recover (Paterson et al., 1999). Research is currently focused on this ovarian regression because it is one of the major obstacles for the application of ZP immunocontraception as a reversible contraceptive method for humans (Gupta et al., 2004). Permanent infertility would also not be desirable for valuable exotic species such as wild horses, and elephants, where reversibility is necessary given the potential threats to population stability and the long reproductive lifespan of females from these species. Studies performed in rabbits have implicated making antibodies against glycosylated ZP proteins in the pathogenesis of

ovarian dysfunction associated with ZP antigen immunization, and the authors of these research reports encourage the use of deglycosylated zona molecules in the continued efforts to develop a safe zona-based contraceptive vaccine (Keenan et al., 1991, Jones et al., 1992). However, using bacterially expressed recombinant ZP proteins, which are devoid of carbohydrates, do not induce an effective immune response (VandeVoort et al., 1995, Kaul et al., 1997).

From a poultry production standpoint, an undesired effect from the genetic selection for rapid growth and meat yield in poultry breeders has been an associated decline in fertility (Barbato 1999, Brillard 2004). Much of the research in poultry fertility focuses on the male and in particular on the quantity and quality of sperm produced by the male (Holsberger et al., 1998, Donoghue et al., 1999, Hammerstedt et al., 1999). Female fertility should not be ignored especially with regard to the initial interaction of the sperm and egg. The preferential binding of sperm to the IPVL overlying the GD region indicates that sperm receptors may be concentrated in this area of the IPVL in order to increase the probability of fertilization (Howarth 1990; Bramwell and Howarth, 1992; Kuroki and Mori, 1997). Additionally research with 2 genetic lines of turkey hens indicate that the line selected for rapid body growth and large body size has females that have lower fertility due to decreased sperm binding at the GD region relative to the hens in their original cohort population that has been selected for egg production. In addition, Benson et al., (2009, 2017) reported that these 2 lines of turkey hens have differences in the mRNA expression of ZP1, ZPB2 and ZPC. Thus, if the sperm receptor protein or proteins could be identified in commercial avian species, it could potentially provide a genetic marker for the selection of improved female fertility.

Summary

The ZP is a glycoprotein matrix surrounding freshly ovulated oocytes. It provides structural support for the oocyte and contains proteins that play a role in species specific sperm binding during fertilization. In most species, the ZP coat consists of only 3 or 4 proteins called ZP proteins. However, the number of ZP proteins synthesized and utilized to make the ZP vary across vertebrate species. In the chicken and turkey and 6 distinct zona pellucida genes have been identified (ZPA, ZPB1, ZPB2, ZPC, ZPD and ZPX1) and all but ZPX1 have been confirmed as protein constituents of the IPVL. Once sperm are bound to ZP sperm receptors, the sperm can penetrate the ZP and make contact with the plasma membrane of the oocyte for subsequent union of the male and female pronuclei. In avian species the ZP protein or proteins that serve as the sperm receptor has not been identified.

CHAPTER 3

STATEMENT OF PURPOSE

When a follicle is ovulated in an avian species, it is surrounded by the IPVL. For successful fertilization, sperm must attach to and penetrate the IPVL. Sperm penetration of the IPVL is concentrated in the area around the GD which contains the female pronucleus. Three of the protein constituents of the IPVL are ZPC, ZPB1 and ZPB2. Both ZPC and ZPB2 are synthesized and secreted by cells from the developing follicle, while ZPB1 is produced by the liver and transported to the developing follicle. In avian species, all 3 of these ZP proteins have been implicated as the possible sperm receptor that mediates sperm binding and penetration of the IPVL at the GD region of the ovulated ovum.

Previously, our laboratory investigated the mRNA expression of ZPB1, ZPB2, and ZPC in two genetic lines of turkey hens selected for over 40 generations for either increased egg production (E-line) or increased body weight (F-line). As a result of this genetic selection, hens from the E-line have a higher rate of female fertility than hens from the F-line. Hepatic expression of the mRNA for ZPB1 was significantly greater in turkey hens from the E-line than the F-line. The total mRNA expression of ZPC in F_1 follicle granulosa cells was equal between the 2 genetic lines of turkey hens. But, hens from the F-line expressed a greater amount of ZPC in NGD regions than in the GD region, while the hens from the E-line had equal expression of ZPC in the granulosa cells between these 2 regions. Although the mRNA expression of ZPB2 was equal between the 2 lines of hens, its expression was greater in F_1 follicle GD granulosa cells than NGD granulosa cells for both genetic lines. These results suggested that the higher

rates of fertility previously observed for eggs from the E-line versus the F-line of turkeys may be related to differences in the expression of ZPB1 and ZPC. In addition, the results suggested the preferential attachment of sperm to the GD region may be related to the higher expression of ZPB2 in this area.

Subsequent research investigated the mRNA expression of ZPB1, ZPB2, and ZPC in 4 genetic lines of broiler breeder hens. As had been seen with the turkey hens, there were differences in GD versus NGD granulosa cell expression of the mRNA for ZPC in the hens of some genetic lines, but not in other lines. Differences in hepatic ZPB1 mRNA expression also existed between the lines. Similar to the previous results in turkey hens, overall expression of ZPB2 mRNA was greater in GD versus NGD granulosa cells in the 4 genetic lines of broiler hens. Previous research with broiler breeder hens also established that the mRNA expression of ZPB2 is greatest in the smallest prehierarchical follicles and decreases drastically as follicles start accumulating yellow yolk which suggests a potential role for ZPB2 in early follicular development. Finally, previous research with cultured broiler hen granulosa cells indicated that the mRNA of ZPC was responsive to gonadotropic and steroid hormone stimulation.

While this previous mRNA expression research suggests that ZP proteins could be used as genetic markers for selecting female lines of hens for greater fertility, and that ZPB2 may be important in early follicular development and the preferential attachment of sperm at the GD region of the ovum, these suggestions are only valid if the differences in mRNA expression are mirrored at the protein expression level. Therefore, the aim of the current research was 1) to determine if the protein expression of ZPB1, ZPB2, and ZPC in the perivitelline layer of eggs produced by the 2 genetic lines of turkey hens and 4 genetic lines of broiler breeder hens matches the mRNA expression of these ZP proteins at their site of synthesis in these genetic lines

of hens, 2) to determine if protein expression of ZPB2 decreases with follicular development in broiler breeder hens, 3) to determine if ZPC protein expression is hormonally regulated in granulosa cell culture.

CHAPTER 4

MATERIALS AND METHODS

Experiment 1: ZPB2, ZPC, ZPB1 Protein Expression in Two Genetic Lines of Turkey Hens

Animals

In order to obtain egg tissue samples for subsequent western blot analyses of the ZP proteins, two lines of turkey hens that differ greatly in body weight and egg production were used. The egg line, or E-line, was selected by McCartney et al., (1968) from an established random bred control line (McCartney 1964), while the growth line, or F-line, was selected from the same original random bred population for increased 16 week body weight (Nestor 1977). Selection for increased body weight in the F-line resulted in reduced egg production in this genetic line due to a decrease in the intensity of egg laying as measured by average clutch size (Nestor et al., 1996, 2000). In contrast, selection for increased egg production in the E-line has greatly reduced broodiness while vastly increasing the intensity of egg laying in the hens of this genetic line (Anthony et al., 1991, Nestor et al., 1996). Eggs produced by the F-line hens also have lower rates of sperm penetration of the IPVL and fertility than the eggs produced by the E-line hens (Nestor and Noble 1995, Christensen et al., 2005). In 2007, E-line and F-line poults were hatched at the Ohio Agriculture Research and Development Center and were soon thereafter shipped to the North Carolina State University Turkey Educational Unit. The turkeys were raised in floor pens and provided 10 hours of light per day until 25 weeks of age when the hours of light were reduced to 8. At 31 weeks of age, the turkey hens were moved to breeding pens (6 birds per pen) and photo-stimulated for reproduction by providing them 14 hours of light

per day. Each breeding pen was equipped with a nest box. The turkeys were provided with free access to appropriate commercial diets and water at all times through rearing and production. All animal procedures were approved by the North Carolina State University Animal Care and Use Committee.

Tissue Collection

To determine if there were differences in the protein expression of ZPB1, ZPB2, and ZPC, unfertilized eggs produced from the hens from each genetic line were collected over a two day period when the hens were 41, 43, and 44 weeks of age. Each egg was broken and the yolk was separated from the albumen. The yolk was placed into Krebs-Ringer bicarbonate buffer (pH 7.4). A one cm^2 section of the IPVL around the GD and a NGD area, on the opposite side of the follicle to the GD area, was collected. To obtain enough protein for subsequent Western Blot analyses, the IPVL samples from the GD and NGD regions were pooled from 3 eggs of the same genetic line for each sample. A total of 6 samples per genetic line were collected frozen and stored at -80^{o}C in 150 μL of lysis buffer containing protease inhibitors (Shirley et al., 2003).

Experiment 2: ZPB2, ZPC, ZPB1 Protein Expression in Four Genetic Lines of Broiler Breeder Hens

Animals

Female chicks from 4 genetic strains of Cobb primary broiler breeder hens designated B, O, R, and G were obtained from a commercial hatchery and reared according to the recommended guidelines of the primary breeder for each genetic line using a 4/3 skip-a-day feed restriction program. At 21 weeks of age, the pullets were separated into 2 body weight categories: control (at or below primary breeder recommended target weight) and heavy (above primary breeder recommended target weight). The pullets in the heavy category were fed to

maintain the 300 gram weight differential between the two weight categories for the rest of the experiment. One hundred pullets from each strain (50 heavy and 50 control) were placed into individual cages in random blocks of 10 cages (5 replicate blocks of cages for each weight category within each strain and 10 replicate blocks of cages per strain). Additional hens were housed in floor pens and were used to replace caged hens that died or ceased egg production during the trial that lasted until 65 weeks of age. At 21 weeks of age the pullets were photostimulated by providing 14 hours of light (14 light:10 dark) with 30 minute light increases given at 10 day intervals until 16 hours of light (16 light:8 dark) per day was reached. At 30 weeks of age all caged hens were artificially inseminated with 100 million sperm in a 50 µl volume from a pooled semen sample. Inseminations continued at 5 week intervals until 60 weeks of age. All eggs were recorded and collected daily to determine fertility by day post-insemination. All animal procedures were approved by the University of Arkansas Animal Care and Use Committee.

Tissue Collection

To determine if there were differences in the protein expression of ZPB1, ZPB2, and ZPC in four genetic lines of broiler breeder hens, eggs produced by the hens from each of the genetic lines were collected when the birds were 44 weeks of age. The samples were collected from the eggs as described in the previous turkey hen experiment, except only 2 eggs were utilized for each collected sample. A total of 6 samples per genetic line, consisting of 3 heavy weight and 3 light weight were collected.

Experiment 3: Protein Expression of ZPB2 in Granulosa and Theca cells of Hierarchy and Pre-Hierarchy during Follicular Development in Broiler Breeder Hens

Tissue Collection

ZPB2 protein expression was investigated in the theca and granulosa cells of hierarchical and prehierarchical follicles in the broiler breeder hen. Twelve Ross 708 broiler breeder hens 63 weeks of age were killed by cervical dislocation 2 to 4 hours prior to ovulation as evidenced by the presence of a hard shell egg in the shell gland. The ovary was removed from each hen. The F_1, F_2, F_3 and F_4 hierarchical follicles were removed as were the SYF and LWF. The SYF were subdivided into two groups >5 to 8 mm in diameter (SYF2) and >8 to 12mm in diameter (SYF1) and the LWF were also subdivided into size categories of <2 mm in diameter (LWF2) and >2 to 5 mm in diameter (LWF1). The granulosa cell layer was manually separated from the theca cell layers of each hierarchical follicle (Huang and Nalbandov 1979) and the theca and granulosa cells were enzymatically separated in the LWF and SYF as previously described (Davis et al., 2000). Individual theca or granulosa samples from 3 individual birds were pooled to generate 4 replicate samples of granulosa or theca tissue for each follicle size. All theca samples were homogenized for 30 seconds with a PowerGen 700 tissue disrupter (Fisher Scientific, Pittsburgh, PA). Each individual granulosa and theca sample was mixed with lysis buffer containing protease inhibitors (Shirley et al., 2003) and then frozen and stored at -80°C.

Experiment 4: ZPC and ZPB2 Protein Expression in the Presence of LH or FSH in Granulosa Cell Cultures

Animals

Cobb 500 slow feathering pullets were reared in floor pens from day 1 of age at The University of Georgia Poultry Research Center. They were provided a standard broiler breeder

pullet diet on a skip a day feed restriction program. Ten percent of the pullets were randomly selected and weighed once per week in the rearing phase to determine feed allocation. This method was used to ensure that the body weight gain of the pullets matched the recommended guidelines of the primary breeder (Cobb-Vantress 2005a). From placement on day 1 until 21 weeks of age, the pullets received 8 hours of light. The lighting program was adjusted to provide 14 hours of light per day for photostimulation at 21 weeks of age. At the time of photostimulation, 100 of the pullets were moved to individual cages and fed daily with the amount of feed provided to the hens determined using the guidelines of the primary breeder (Cobb-Vantress 2005a, Cobb-Vantress 2005b), which are based on weekly body weight measurements and egg production rates of the hens. All animal procedures were approved by the University of Georgia Animal Care and Use Committee.

Tissue Collection

At 45 weeks of age, three of the caged broiler breeder hens were selected for tissue collection based on an observed consistency in egg laying. The hens were killed by cervical dislocation 2-4 hours prior to ovulation, as evidenced by the presence of an egg in the shell gland. From each of the three hens, the ovary was removed, and the F_1, F_3, and SY follicles were collected and pooled by size. The granulosa cells from each pool of F_1, F_3, and SY follicles were isolated, dispersed, and washed, as previously described (Davis et al., 2000). Using a hemocytometer with trypan blue exclusion, cell number and viability were estimated, and cell viability was greater than 95%.

Dispersed granulosa cells from each follicle size were cultured in 6-well tissue culture plates at a density of 2.5×10^6 cells/well with 4 mL of M199 culture media, as previously described (Davis et al., 2000) except in this experiment the lipoprotein supplement was not

added to the M199 culture media. The granulosa cells were cultured for 24 hours with 0 or 50 ng/mL cell culture media of ovine LH (Lot AFP8468A) or human recombinant FSH (Lot AFP5551B). Both the LH and FSH were generously provided by Dr. A.F. Parlow of the National Hormone and Peptide Program, Torrance, CA. There were three wells per treatment for each follicle size. Cell culture media from each treatment and follicle size was saved and stored at -80 ºC for subsequent analysis of progesterone content and ZP protein expression. This experiment was repeated 5 more times to generate a total of six replicate experiments.

Experiment 5: ZPC and ZPB2 Protein Expression in the Presence of Testosterone or Estradiol in Granulosa Cell Cultures

Animals and tissue collection

The experimental procedures for this experiment were exactly the same as those utilized in experiment 4, except that the hens utilized for replicate experiments were between 50 and 54 weeks of age, and the dispersed granulosa cells were cultured in the absence or presence of 1 x 10^{-6} testosterone (Steraloids, Newport, RI) or 17-β-estradiol (Sigma, St. Louis, MO). This experiment was repeated to generate a total of 4 replicate experiments.

Progesterone RIA

Cell culture media progesterone concentrations in experiment 4 and experiment 5 were determined by RIA using the Coat-A-Count Progesterone kit (Diagnostic Products Corporation, Los Angeles, CA, catalogue #TKTPG) following the manufacturer's protocol.

Protein Isolation and Quantitation

Cell lysates from experiment 1-3 were centrifuged at 20,000 x g for 30 minutes at 4ºC. The supernatant fraction was then recovered and used for subsequent protein quantitation. Cell culture media from experiments 4 and 5 was directly used as it was for protein quantitation.

Protein was quantitated using the Lowry procedure (Lowry et al., 1951), with bovine serum albumen used as the standard.

ECL-Western Blotting

One dimensional gel electrophoresis was performed using stain free technology (BioRad, Hercules, CA). Ten well mini-PROTEAN TGX Stain Free Gels (BioRad) with a gel percentage of 10% were used. In order to accurately locate the kilodalton (kDa) size of ZPB1, ZPB2, and ZPC, Precision Plus Protein All Blue Protein Standard Ladder (BioRad) was electrophoresed in one lane of each stain free gel. The stain free gels containing 50 μg of protein per sample were transferred to PVDF membrane (Immunoblot-BioRad) as previously described (Shirley et al., 2003). Each electrophoresed and transferred gel was repeated 2 more times so that individual membranes did not have to be stripped and reused to quantify ZPB1, ZPB2 and ZPC protein expression.

After blocking each membrane for 1 hour in Tris buffered saline, pH 7.6, containing 5% Bovine Serum Albumen (Sigma-Aldrich, St. Louis, MO) the protocol and reagents for the Amersham ECL Prime Western blotting detection Kit (GE healthcare, Buckinghampshire, UK) along with goat anti-rabbit IgG-HRP conjugate (BioRad) were utilized to detect ZPB1, ZPB2, and ZPC protein using the antibodies produced against the synthetic peptides for these proteins (Malloy 2011). The antibodies were purified using the Dynabeads Protein A for Immunoprecipitation kit (Life Technologies, Carlsbad, CA) following the manufacturer's protocol. Purified primary ZPB1, ZPB2, and ZPC antibodies were diluted 1 to 1,000, while the secondary antibody dilution was 1 to 10,000 for detection.

Chemi-luminescent detection in combination with Chemidoc XRS+ software (BioRad, Hercules, CA) was used to quantify relative ZPB1, ZPB2, ZPC expression with protein

normalization and volume analysis for the stain free technology performed using the Image Lab Software (BioRad, Hercules, CA). For each replicate gel, after the quantification values of ZPB1, ZPB2 or ZPC were normalized for minimal loading differences, the sample with the highest protein expression was assigned a value of 1 and the remaining samples were given expression quantification values relative to this sample with the most expression. Therefore, all replicate data for ZPB1, ZPB2 and ZPC is expressed as the fold-difference relative to the sample with the highest expression.

Statistics

In each experiment, the data were subjected to ANOVA using the General Linear Model (GLM) procedure. Tukey's multiple-comparison procedure (Neter et al., 1990) was used to detect significant differences among genetic lines, IPVL location, follicle sizes and tissues, and cell culture treatments. Differences were considered significant when $P<0.05$. All statistical procedures were completed with the Minitab statistical software package (Release17, State College, PA).

CHAPTER 5

RESULTS

Experiment 1: ZPB2, ZPC, ZPB1 Protein Expression in Two Genetic Lines of Turkey Hens

ZPB2

Protein expression of ZPB2 was greater in the GD region compared to the NGD in perivitelline samples from eggs from turkey hens (Table 5.1) , but the expression of ZPB2 protein in the perivitelline layer of eggs did not vary between E- and F- genetic lines (Table 5.2 and Table 5.3).

Table 5.1. Expression of ZPB2 protein in the germinal disc (GD) and non-germinal disc (NGD) region of the perivitelline layer in eggs from turkey hens (Experiment 1).

Perivitelline location	Relative density[1]
GD	0.947 ± 0.048[a]
NGD	0.755 ± 0.068[b]

[1]Values are means ± SEM, n = 12 replicate samples per perivitelline location (6 E-line plus 6 F-line)

[a-b]Values with different superscripts for a given follicle size differ ($p < 0.05$).

Table 5.2. Expression of ZPB2 protein in the perivitelline layer of eggs from two genetic lines of turkey hens selected for rapid body growth (F-line) or egg production (E-line, Experiment 1).

Genetic line	Relative density[1]
E-line	0.581 ± 0.067
F-line	0.613 ± 0.076

[1]Values are means ± SEM, n = 12 replicate samples per line (6 GD plus 6 NGD samples).

Table 5.3. Expression of ZPB2 protein in the germinal disc (GD) or the non-germinal disc (NGD) region of the perivitelline layer from two genetic lines of turkeys selected for rapid body growth (F-line), or egg production (E-line, Experiment 1).

Genetic line and perivitelline location	Relative density[1]
E-line GD	0.625 ± 0.093
E-line NGD	0.536 ± 0.102
F-line GD	0.701 ± 0.095
F-line NGD	0.524 ± 0.115

[1]Values are means ± SEM, n = 6 replicate samples.

ZPC

Protein expression of ZPC in the GD and NGD regions of the perivitelline layer of eggs from turkey hens (Table 5.4) did not differ, but its expression was greater in the perivitelline layer of eggs from the E-line hens relative to F-line hens (Table 5.5). The expression of ZPC protein for the individual combinations of genetic line and perivitelline location are presented in (Table 5.6).

Table 5.4. Expression of ZPC protein in the germinal disc (GD) and non-germinal disc (NGD) region of the perivitelline layer in eggs from turkey hens (Experiment 1).

Perivitelline location	Relative density[1]
GD	0.236 ± 0.086
NGD	0.477 ± 0.109 (*p=0.09*)

[1]Values are means ± SEM, n = 12 replicate samples per perivitelline location (6 E-line plus 6 F-line)

Table 5.5. Expression of ZPC protein in the perivitelline layer of eggs from two genetic lines of turkey hens selected for rapid body growth (F-line) or egg production (E-line, Experiment 1).

Genetic line	Relative density[1]
E-line	0.500 ± 0.114[a]
F-line	0.213 ± 0.072[b]

[1]Values are means ± SEM, n = 12 replicate samples per line (6 GD plus 6 NGD samples).
[a-b]Values with different superscripts for a given follicle size differ ($p < 0.05$).

Table 5.6. Expression of ZPC protein in the germinal disc (GD) or the non-germinal disc (NGD) region of the perivitelline layer from two genetic lines of turkeys selected for rapid body growth (F-line), or egg production (E-line, Experiment 1).

Genetic line and perivitelline location	Relative density[1]
E-line GD	0.388 ± 0.149^{ab}
E-line NGD	0.612 ± 0.172^{a}
F-line GD	0.083 ± 0.032^{b}
F-line NGD	0.343 ± 0.123^{ab}

[1]Values are means ± SEM, n = 6 replicate samples.
[a-b]Values with different superscripts for a given follicle size differ ($p < 0.05$).

ZPB1

Protein expression of ZPB1 was greater in the NGD region relative to the GD region of IPVL samples of eggs from turkey hens (Table 5.7) and ZPB1 protein expression was greater in the IPVL of eggs from the F-line hens than the IPVL of eggs from the E-line (Table 5.8). The expression of ZPB1 protein for the individual combinations of genetic line and perivitelline location are presented in (Table 5.9).

Table 5.7. Expression of ZPB1 protein in the germinal disc (GD) and non-germinal disc (NGD) region of the perivitelline layer in eggs from turkey hens (Experiment 1).

Perivitelline location	Relative density[1]
GD	0.580 ± 0.086^{b}
NGD	0.897 ± 0.066^{a}

[1]Values are means ± SEM, n = 12 replicate samples per perivitelline location (6 E-line plus 6 F-line)
[a-b]Values with different superscripts for a given follicle size differ ($p < 0.05$).

Table 5.8. Expression of ZPB1 protein in the perivitelline layer of eggs from two genetic lines of turkey hens selected for rapid body growth (F-line) or egg production (E-line, Experiment 1).

Genetic line	Relative density[1]
E-line	0.293 ± 0.048^{b}
F-line	0.578 ± 0.093^{a}

[1]Values are means ± SEM, n = 12 replicate samples per line (6 GD plus 6 NGD samples).
[a-b]Values with different superscripts for a given follicle size differ ($p < 0.05$).

Table 5.9. Expression of ZPB1 protein in the germinal disc (GD) or the non-germinal disc (NGD) region of the perivitelline layer from two genetic lines of turkeys selected for rapid body growth (F-line), or egg production (E-line, Experiment 1).

Genetic line and perivitelline location	Relative density[1]
E-line GD	0.220 ± 0.048^{b}
E-line NGD	0.366 ± 0.076^{ab}
F-line GD	0.435 ± 0.123^{ab}
F-line NGD	0.721 ± 0.123^{a}

[1]Values are means ± SEM, n = 6 replicate samples.
[a-b]Values with different superscripts for a given follicle size differ ($p < 0.05$).

Experiment 2: ZPB2, ZPC, ZPB1 Protein Expression in Four Genetic Lines of Broiler Breeder Hens

ZPB2

Overall protein expression of ZPB2 did not differ in the perivitelline layer of eggs from the four genetic lines of broiler breeder hens (Table 5.10), but its overall expression was greater in the GD region of the perivitelline layer compared to the NGD region (Table 5.11).

Looking specifically within the B line, the GD region of the perivitelline layer had greater ZPB2 protein expression than the NGD region (Table 5.12). Although there was no difference in the overall expression of ZPB2 in the perivitelline layer between the eggs from hens at or below target body weight (control) versus those above the target weight (Table 5.13), individually, the expression of ZPB2 in the GD region of the perivitelline layer of the eggs from heavy hens was greater than the expression in the NGD region of the perivitelline layer of eggs from control hens (Table 5.14). The ZPB2 expression results for the O and R lines of broiler breeder hens were very similar to those of the B line hens (Tables 5.15 -5.20) with overall expression of ZPB2 being greater in the GD region compared the NGD region and with no overall expression differences between the control and heavy weight hens. In contrast to the other 3 genetic lines, the expression of ZPB2 in the GD region of the perivitelline layer did not

differ (p = 0.07) from that of the NGD region. However, like the other genetic lines there was no overall expression difference in the expression of ZPB2 in the perivitelline layer of eggs from the control weight and heavy weight hens (Tables 5.22 and 5.23).

Table 5.10. Protein expression of ZPB2 in the perivitelline layer of eggs from 4 genetic lines of broiler breeder hens (B, O, R and G, Experiment 2).

Genetic line	Relative density[1]
B	0.342 ± 0.069
O	0.384 ± 0.075
R	0.284 ± 0.070
G	0.338 ± 0.074

[1]Values are means ± SEM, n = 24 replicate samples per genetic line [12 control hen samples (6 GD, 6 NGD) plus 12 heavy hen (6 GD, 6 NGD)]

Table 5.11. Protein expression of ZPB2 in the germinal disc (GD) and non-germinal disc region (NGD) region of the perivitelline layer of eggs from 4 genetic lines of broiler breeder hens (Experiment 2).

Perivitelline location	Relative density[1]
GD	0.479 ± 0.053[a]
NGD	0.194 ± 0.038[b]

[1]Values are means ± SEM, n = 48 samples per perivitelline location [12 samples per line (6 control hens plus 6 heavy hens)].
[a-b]Values with different superscripts for a given follicle size differ ($p < 0.05$).

Table 5.12. Protein expression of ZPB2 in the germinal disc (GD) region and non-germinal disc region (NGD) region of the perivitelline layer of eggs from broiler breeders hens of genetic line B (Experiment 2).

B line	Relative density[1]
GD	0.492 ± 0.097[a]
NGD	0.191 ± 0.082[b]

[1]Values are means ± SEM, n = 12 replicate samples per perivitelline location (6 control hens plus 6 heavy hens).
[a-b]Values with different superscripts for a given follicle size differ ($p < 0.05$).

Table 5.13. Protein expression of ZPB2 in the perivitelline layer of eggs from broiler breeder hens of genetic line B that were either at or below primary breeder target weight (control) or above primary breeder target weight (heavy, Experiment 2).

B line	Relative density[1]
Control	0.209 ± 0.077
Heavy	0.474 ± 0.106

[1]Values are means ± SEM, n = 12 replicate samples per weight category (6 GD plus 6 NGD samples).

Table 5.14. Protein expression of ZPB2 in the germinal disc (GD) or the non-germinal disc (NGD) region of the perivitelline layer of eggs from hens of genetic line B that were either at or below primary breeder target weight (control) or above primary breeder target weight (heavy, Experiment 2).

B line	Relative density[1]
Control GD	0.353 ± 0.134^{ab}
Control NGD	0.065 ± 0.014^{b}
Heavy GD	0.631 ± 0.128^{a}
Heavy NGD	0.318 ± 0.152^{ab}

[1]Values are means ± SEM, n = 6 replicate samples.
[a-b]Values with different superscripts for a given follicle size differ ($p < 0.05$).

Table 5.15. Protein expression of ZPB2 in the germinal disc (GD) region and non-germinal disc region (NGD) region of the perivitelline layer of eggs from broiler breeders hens of genetic line O (Experiment 2).

O line	Relative density[1]
GD	0.431 ± 0.120^{a}
NGD	0.137 ± 0.050^{b}

[1]Values are means ± SEM, n = 12 replicate samples per perivitelline location (6 control hens plus 6 heavy hens).
[a-b]Values with different superscripts for a given follicle size differ ($p < 0.05$).

Table 5.16. Protein expression of ZPB2 in the perivitelline layer of eggs from broiler breeder hens of genetic line O that were either at or below primary breeder target weight (control) or above primary breeder target weight (heavy, Experiment 2).

O line	Relative density[1]
Control	0.346 ± 0.114
Heavy	0.223 ± 0.084

[1]Values are means ± SEM, n = 12 replicate samples per weight category (6 GD plus 6 NGD samples).

Table 5.17. Protein expression of ZPB2 in the germinal disc (GD) or the non-germinal disc (NGD) region of the perivitelline layer of eggs from hens of genetic line O that were either at or below primary breeder target weight (control) or above primary breeder target weight (heavy, Experiment 2).

O line	Relative density[1]
Control GD	0.665 ± 0.181[a]
Control NGD	0.117 ± 0.056[b]
Heavy GD	0.289 ± 0.148[ab]
Heavy NGD	0.067 ± 0.087[b]

[1]Values are means ± SEM, n = 6 replicate samples.
[a-b]Values with different superscripts for a given follicle size differ ($p < 0.05$).

Table 5.18. Protein expression of ZPB2 in the germinal disc (GD) region and non-germinal disc region (NGD) region of the perivitelline layer of eggs from broiler breeders hens of genetic line R (Experiment 2).

R line	Relative density[1]
GD	0.481 ± 0.112[a]
NGD	0.195 ± 0.084[b]

[1]Values are means ± SEM, n = 12 replicate samples per perivitelline location (6 control hens plus 6 heavy hens).
[a-b]Values with different superscripts for a given follicle size differ ($p < 0.05$).

Table 5.19. Protein expression of ZPB2 in the perivitelline layer of eggs from broiler breeder hens of genetic line R that were either at or below primary breeder target weight (control) or above primary breeder target weight (heavy, Experiment 2).

R line	Relative density[1]
Control	0.392 ± 0.108
Heavy	0.283 ± 0.104

[1]Values are means ± SEM, n = 12 replicate samples per weight category (6 GD plus 6 NGD samples).

Table 5.20. Protein expression of ZPB2 in the germinal disc (GD) or the non-germinal disc (NGD) region of the perivitelline layer of eggs from hens of genetic line R that were either at or below primary breeder target weight (control) or above primary breeder target weight (heavy, Experiment 2).

R line	Relative density[1]
Control GD	0.472 ± 0.157
Control NGD	0.312 ± 0.157
Heavy GD	0.489 ± 0.174
Heavy NGD	0.318 ± 0.026

[1]Values are means ± SEM, n = 6 replicate samples.

Table 5.21. Protein expression of ZPB2 in the germinal disc (GD) region and non-germinal disc region (NGD) region of the perivitelline layer of eggs from broiler breeders hens of genetic line G (Experiment 2).

G line	Relative density[1]
GD	0.510 ± 0.110 (*p=0.07*)
NGD	0.252 ± 0.093

[1]Values are means ± SEM, n = 12 replicate samples per perivitelline location (6 control hens plus 6 heavy hens).

Table 5.22. Protein expression of ZPB2 in the perivitelline layer of eggs from broiler breeder hens of genetic line G that were either at or below primary breeder target weight (control) or above primary breeder target weight (heavy, Experiment 2).

G line	Relative density[1]
Control	0.412 ± 0.096
Heavy	0.424 ± 0.102

[1]Values are means ± SEM, n = 12 replicate samples per weight category (6 GD plus 6 NGD samples).

Table 5.23. Protein expression of ZPB2 in the germinal disc (GD) or the non-germinal disc (NGD) region of the perivitelline layer of eggs from hens of genetic line G that were either at or below primary breeder target weight (control) or above primary breeder target weight (heavy, Experiment 2).

G line	Relative density[1]
Control GD	0.628 ± 0.159
Control NGD	0.197 ± 0.048
Heavy GD	0.464 ± 0.109
Heavy NGD	0.384 ± 0.167

[1]Values are means ± SEM, n = 6 replicate samples.

ZPC

The overall protein expression of ZPC was greater in the perivitelline layer of eggs from the O line of broiler breeder hens than in the perivitelline lay of the eggs from the G line (Table 5.24). The overall expression of ZPC did not differ between the GD and NGD regions of the perivitelline layer (Table 5.25).

Within the B line, the GD and NGD regions of the perivitelline layer did not differ in their protein expression of ZPC (Table 5.26). However, the overall expression of ZPC in the

perivitelline layer of eggs from the hens at or below target body weight (control) was greater than the expression found in eggs from hens above the primary breeder target weight (Table 5.27), and this was primarily due to differences in the expression of ZPC in the NGD regions of the perivitelline layer between the eggs from the heavy hens and control hens (Table 5.28). In contrast, in the O line, while the expression of ZPC did not differ in the GD and NGD regions of the perivitelline layer (Tables 5.29 and 5.31), it was the perivitelline layer from eggs from the hens that were above target weight that expressed a greater level of ZPC rather than the control hens (Table 5.30). For the R and G genetic lines, the protein expression of ZPC did not differ between GD and NGD regions of the perivitelline layer or between perivitelline layer from different weight hens (Tables 5.32 – 5.37).

Table 5.24. Protein expression of ZPC in the perivitelline layer of eggs from 4 genetic lines of broiler breeder hens (B, O, R and G, Experiment 2).

Genetic line	Relative density[1]
B	0.528 ± 0.054^{ab}
O	0.714 ± 0.047^{a}
R	0.664 ± 0.047^{ab}
G	0.513 ± 0.059^{b}

[1]Values are means ± SEM, n = 24 replicate samples per genetic line [12 control hen samples (6 GD, 6 NGD) plus 12 heavy hen (6 GD, 6 NGD)]
[a-b]Values with different superscripts for a given follicle size differ ($p < 0.05$).

Table 5.25. Protein expression of ZPC in the germinal disc (GD) and non-germinal disc region (NGD) region of the perivitelline layer of eggs from 4 genetic lines of broiler breeder hens (Experiment 2).

Perivitelline location	Relative density[1]
GD	0.606 ± 0.041
NGD	0.594 ± 0.036

[1]Values are means ± SEM, n = 48 samples per perivitelline location [12 samples per line (6 control hens plus 6 heavy hens)].
[a-b]Values with different superscripts for a given follicle size differ ($p < 0.05$).

Table 5.26. Protein expression of ZPC in the germinal disc (GD) region and non-germinal disc region (NGD) region of the perivitelline layer of eggs from broiler breeders hens of genetic line B (Experiment 2).

B line	Relative density[1]
GD	0.436 ± 0.074
NGD	0.620 ± 0.072

[1]Values are means ± SEM, n = 12 replicate samples per perivitelline location (6 control hens plus 6 heavy hens).

Table 5.27. Protein expression of ZPC in the perivitelline layer of eggs from broiler breeder hens of genetic line B that were either at or below primary breeder target weight (control) or above primary breeder target weight (heavy, Experiment 2).

B line	Relative density[1]
Control	0.677 ± 0.084[a]
Heavy	0.379 ± 0.032[b]

[1]Values are means ± SEM, n = 12 replicate samples per weight category (6 GD plus 6 NGD samples).
[a-b]Values with different superscripts for a given follicle size differ ($p < 0.05$).

Table 5.28. Protein expression of ZPC in the germinal disc (GD) or the non-germinal disc (NGD) region of the perivitelline layer of eggs from hens of genetic line B that were either at or below primary breeder target weight (control) or above primary breeder target weight (heavy, Experiment 2).

B line	Relative density[1]
Control GD	0.528 ± 0.133[ab]
Control NGD	0.826 ± 0.069[a]
Heavy GD	0.345 ± 0.055[b]
Heavy NGD	0.414 ± 0.033[b]

[1]Values are means ± SEM, n = 6 replicate samples.
[a-b]Values with different superscripts for a given follicle size differ ($p < 0.05$).

Table 5.29. Protein expression of ZPC in the germinal disc (GD) region and non-germinal disc region (NGD) region of the perivitelline layer of eggs from broiler breeders hens of genetic line O (Experiment 2).

O line	Relative density[1]
GD	0.769 ± 0.069
NGD	0.659 ± 0.064

[1]Values are means ± SEM, n = 12 replicate samples per perivitelline location (6 control hens plus 6 heavy hens).

Table 5.30. Protein expression of ZPC in the perivitelline layer of eggs from broiler breeder hens of genetic line O that were either at or below primary breeder target weight (control) or above primary breeder target weight (heavy, Experiment 2).

O line	Relative density[1]
Control	0.619± 0.069[b]
Heavy	0.811 ± 0.054[a]

[1]Values are means ± SEM, n = 12 replicate samples per weight category (6 GD plus 6 NGD samples).
[a-b]Values with different superscripts for a given follicle size differ ($p < 0.05$).

Table 5.31. Protein expression of ZPC in the germinal disc (GD) or the non-germinal disc (NGD) region of the perivitelline layer of eggs from hens of genetic line O that were either at or below primary breeder target weight (control) or above primary breeder target weight (heavy, Experiment 2).

O line	Relative density[1]
Control GD	0.636 ± 0.103
Control NGD	0.598 ± 0.100
Heavy GD	0.902 ± 0.135
Heavy NGD	0.721 ± 0.197

[1]Values are means ± SEM, n = 6 replicate samples.

Table 5.32. Protein expression of ZPC in the germinal disc (GD) region and non-germinal disc region (NGD) region of the perivitelline layer of eggs from broiler breeders hens of genetic line R (Experiment 2).

R line	Relative density[1]
GD	0.612 ± 0.064
NGD	0.677 ± 0.070

[1]Values are means ± SEM, n = 12 replicate samples per perivitelline location (6 control hens plus 6 heavy hens).

Table 5.33. Protein expression of ZPC in the perivitelline layer of eggs from broiler breeder hens of genetic line R that were either at or below primary breeder target weight (control) or above primary breeder target weight (heavy, Experiment 2).

R line	Relative density[1]
Control	0.582 ± 0.069
Heavy	0.707 ± 0.061

[1]Values are means ± SEM, n = 12 replicate samples per weight category (6 GD plus 6 NGD samples).

Table 5.34. Protein expression of ZPC in the germinal disc (GD) or the non-germinal disc (NGD) region of the perivitelline layer of eggs from hens of genetic line R that were either at or below primary breeder target weight (control) or above primary breeder target weight (heavy, Experiment 2).

R line	Relative density[1]
Control GD	0.587 ± 0.109
Control NGD	0.577 ± 0.095
Heavy GD	0.637 ± 0.075
Heavy NGD	0.777 ± 0.093

[1]Values are means ± SEM, n = 6 replicate samples.

Table 5.35. Protein expression of ZPC in the germinal disc (GD) region and non-germinal disc region (NGD) region of the perivitelline layer of eggs from broiler breeders hens of genetic line G (Experiment 2).

G line	Relative density[1]
GD	0.608 ± 0.094 (*p=0.07*)
NGD	0.418 ± 0.066

[1]Values are means ± SEM, n = 12 replicate samples per perivitelline location (6 control hens plus 6 heavy hens).

Table 5.36. Protein expression of ZPC in the perivitelline layer of eggs from broiler breeder hens of genetic line G that were either at or below primary breeder target weight (control) or above primary breeder target weight (heavy, Experiment 2).

G line	Relative density[1]
Control	0.479 ± 0.091
Heavy	0.547 ± 0.080

[1]Values are means ± SEM, n = 12 replicate samples per weight category (6 GD plus 6 NGD samples).

Table 5.37. Protein expression of ZPC in the germinal disc (GD) or the non-germinal disc (NGD) region of the perivitelline layer of eggs from hens of genetic line G that were either at or below primary breeder target weight (control) or above primary breeder target weight (heavy, Experiment 2).

G line	Relative density[1]
Control GD	0.575 ± 0.158
Control NGD	0.383 ± 0.087
Heavy GD	0.641 ± 0.117
Heavy NGD	0.453 ± 0.106

[1]Values are means ± SEM, n = 6 replicate samples.

ZPB1

The protein expression of ZPB1 relative to perivitelline locations and hen body weights are presented in Table 5.38 through Table 5.51. The overall protein expression of ZPB1 did not differ in the perivitelline layer of eggs from the four genetic lines of broiler breeder hens (Table 5.38). However, the overall expression of ZPB1 was greater in the NGD region of the perivitelline layer compared to the GD region (Table 5.39). This overall difference in ZPB1 expression was the result of differences in the expression of ZPB1 in the NGD and GD regions of the perivitelline layer in genetic lines R and G (Tables 5.46 and 5.49). The expression of ZPB1 in the perivitelline layer of eggs from the hens at or below target body weight (control) did not differ from the expression of ZPB1 found in eggs from hens above the primary breeder target weight for any of the genetic lines (Tables 5.41, 5.44, 5.47 and 5.50).

Table 5.38. Protein expression of ZPB1 in the perivitelline layer of eggs from 4 genetic lines of broiler breeder hens (B, O, R and G, Experiment 2).

Genetic line	Relative density[1]
B	0.400 ± 0.065
O	0.376 ± 0.062
R	0.365 ± 0.065
G	0.341 ± 0.068

[1]Values are means ± SEM, n = 24 replicate samples per genetic line [12 control hen samples (6 GD, 6 NGD) plus 12 heavy hen (6 GD, 6 NGD)]

Table 5.39. Protein expression of ZPB1 in the germinal disc (GD) and non-germinal disc region (NGD) region of the perivitelline layer of eggs from 4 genetic lines of broiler breeder hens (Experiment 2).

Perivitelline location	Relative density[1]
GD	0.241 ± 0.046[b]
NGD	0.574 ± 0.100[a]

[1]Values are means ± SEM, n = 48 samples per perivitelline location [12 samples per line (6 control hens plus 6 heavy hens)].
[a-b]Values with different superscripts for a given follicle size differ ($p < 0.05$).

Table 5.40. Protein expression of ZPB1 in the germinal disc (GD) region and non-germinal disc region (NGD) region of the perivitelline layer of eggs from broiler breeders hens of genetic line B (Experiment 2).

B line	Relative density[1]
GD	0.432 ± 0.092
NGD	0.368 ± 0.095

[1]Values are means ± SEM, n = 12 replicate samples per perivitelline location (6 control hens plus 6 heavy hens).

Table 5.41. Protein expression of ZPB1 in the perivitelline layer of eggs from broiler breeder hens of genetic line B that were either at or below primary breeder target weight (control) or above primary breeder target weight (heavy, Experiment 2).

B line	Relative density[1]
Control	0.368 ± 0.095
Heavy	0.432 ± 0.092

[1]Values are means ± SEM, n = 12 replicate samples per weight category (6 GD plus 6 NGD samples).

Table 5.42. Protein expression of ZPB1 in the germinal disc (GD) or the non-germinal disc (NGD) region of the perivitelline layer of eggs from hens of genetic line B that were either at or below primary breeder target weight (control) or above primary breeder target weight (heavy, Experiment 2).

B line	Relative density[1]
Control GD	0.183 ± 0.070
Control NGD	0.553 ± 0.146
Heavy GD	0.269 ± 0.062
Heavy NGD	0.596 ± 0.150

[1]Values are means ± SEM, n = 6 replicate samples.

Table 5.43. Protein expression of ZPB1 in the germinal disc (GD) region and non-germinal disc region (NGD) region of the perivitelline layer of eggs from broiler breeders hens of genetic line O (Experiment 2).

O line	Relative density[1]
GD	0.336 ± 0.092
NGD	0.416 ± 0.085

[1]Values are means ± SEM, n = 12 replicate samples per perivitelline location (6 control hens plus 6 heavy hens).

Table 5.44. Protein expression of ZPB1 in the perivitelline layer of eggs from broiler breeder hens of genetic line O that were either at or below primary breeder target weight (control) or above primary breeder target weight (heavy, Experiment 2).

O line	Relative density[1]
Control	0.489 ± 0.102 (p=0.08)
Heavy	0.263 ± 0.051

[1]Values are means ± SEM, n = 12 replicate samples per weight category (6 GD plus 6 NGD samples).

Table 5.45. Protein expression of ZPB1 in the germinal disc (GD) or the non-germinal disc (NGD) region of the perivitelline layer of eggs from hens of genetic line O that were either at or below primary breeder target weight (control) or above primary breeder target weight (heavy, Experiment 2).

O line	Relative density[1]
Control GD	0.467 ± 0.169
Control NGD	0.511 ± 0.130
Heavy GD	0.205 ± 0.044
Heavy NGD	0.321 ± 0.105

[1]Values are means ± SEM, n = 6 replicate samples.

Table 5.46. Protein expression of ZPB1 in the germinal disc (GD) region and non-germinal disc region (NGD) region of the perivitelline layer of eggs from broiler breeders hens of genetic line R (Experiment 2).

R line	Relative density[1]
GD	0.198 ± 0.044[b]
NGD	0.534 ± 0.102[a]

[1]Values are means ± SEM, n = 12 replicate samples per perivitelline location (6 control hens plus 6 heavy hens).
[a-b]Values with different superscripts for a given follicle size differ ($p < 0.05$).

Table 5.47. Protein expression of ZPB1 in the perivitelline layer of eggs from broiler breeder hens of genetic line R that were either at or below primary breeder target weight (control) or above primary breeder target weight (heavy, Experiment 2).

R line	Relative density[1]
Control	0.429 ± 0.100
Heavy	0.303 ± 0.081

[1]Values are means ± SEM, n = 12 replicate samples per weight category (6 GD plus 6 NGD samples).

Table 5.48. Protein expression of ZPB1 in the germinal disc (GD) or the non-germinal disc (NGD) region of the perivitelline layer of eggs from hens of genetic line R that were either at or below primary breeder target weight (control) or above primary breeder target weight (heavy, Experiment 2).

R line	Relative density[1]
Control GD	0.247 ± 0.069^{ab}
Control NGD	0.611 ± 0.162^{a}
Heavy GD	0.148 ± 0.050^{b}
Heavy NGD	0.458 ± 0.130^{ab}

[1]Values are means ± SEM, n = 6 replicate samples.
[a-b]Values with different superscripts for a given follicle size differ ($p < 0.05$).

Table 5.49. Protein expression of ZPB1 in the germinal disc (GD) region and non-germinal disc region (NGD) region of the perivitelline layer of eggs from broiler breeders hens of genetic line G (Experiment 2).

G line	Relative density[1]
GD	0.204 ± 0.064^{b}
NGD	0.478 ± 0.110^{a}

[1]Values are means ± SEM, n = 12 replicate samples per perivitelline location (6 control hens plus 6 heavy hens).
[a-b]Values with different superscripts for a given follicle size differ ($p < 0.05$).

Table 5.50. Protein expression of ZPB1 in the perivitelline layer of eggs from broiler breeder hens of genetic line G that were either at or below primary breeder target weight (control) or above primary breeder target weight (heavy, Experiment 2).

G line	Relative density[1]
Control	0.239 ± 0.065
Heavy	0.442 ± 0.116

[1]Values are means ± SEM, n = 12 replicate samples per weight category (6 GD plus 6 NGD samples).

Table 5.51. Protein expression of ZPB1 in the germinal disc (GD) or the non-germinal disc (NGD) region of the perivitelline layer of eggs from hens of genetic line G that were either at or below primary breeder target weight (control) or above primary breeder target weight (heavy, Experiment 2).

G line	Relative density[1]
Control GD	0.269 ± 0.113^{b}
Control NGD	0.209 ± 0.074^{b}
Heavy GD	0.138 ± 0.060^{b}
Heavy NGD	0.747 ± 0.137^{a}

[1]Values are means ± SEM, n = 6 replicate samples.
[a-b]Values with different superscripts for a given follicle size differ ($p < 0.05$).

Experiment 3: Protein Expression of ZPB2 in Granulosa and Theca Cells of Hierarchy and Pre-Hierarchy during Follicular Development in Broiler Breeder Hens

The granulosa cell expression of ZPB2 was greater in the LWF of 2 mm or less in diameter then in the granulosa cells from any of the hierarchical follicles (Table 5.52). Expression of ZPB2 protein was not detected in any theca cell sample.

Table 5.52. Protein expression of ZPB2 in granulosa cells from individual hierarchical follicles (F_1 through $F_{4)}$ and prehierarchical follicles pooled by size; small yellow follicles >8 to 12mm in diameter (SYF1), small yellow follicles >5 to 8 mm in diameter (SYF2), large white follicles >2 to 5 mm in diameter (LWF1) and. large white follicles <2 mm in diameter (LWF2, Experiment 3).

Follicle size	Relative density[1]
F_1	0.116 ± 0.049^{bc}
F_2	0.066 ± 0.033^{c}
F_3	0.128 ± 0.068^{bc}
F_4	0.089 ± 0.028^{bc}
SYF1	0.533 ± 0.213^{abc}
SYF2	0.589 ± 0.157^{abc}
LWF1	0.614 ± 0.197^{ab}
LWF2	0.878 ± 0.122^{a}

[a-c]Values with different superscripts differ ($p < 0.05$).
[1]Values are means ± SEM, n = 4 replicate experiments.

Experiment 4: ZPC and ZPB2 protein expression in the presence of LH or FSH in granulosa cell cultures

Progesterone concentration in the media of the granulosa cells cultured with LH was greater than the progesterone accumulation in the untreated cell cultures for the F_1 and F_3 follicles, but not for the SYF (Table 5.53). The addition of FSH to the granulosa cell cultures increased the accumulation of progesterone in the culture media for the granulosa cells from all three follicle sizes (Table 5.53). The addition of FSH or LH to cultured granulosa cells had no effect on the accumulation of ZPC protein in the culture media from the F_1, F_3 or SY follicles 5.54). The expression of ZPB2 protein could not be determined in the cell culture media as an

abundant protein component of the fetal calf serum used in the cell culture media shared a molecular weight with ZPB2 and given its abundance it also reacted with the antibodies used for detecting ZPB2.

Table 5.53. Progesterone concentration in granulosa cell culture media from F_1, F_3, or small yellow (SY) follicles cultured for 24 hours in the presence of 0 (control) or 50 ng/mL culture media of LH or FSH (Experiment 4).

Follicle size and treatment	Progesterone concentration[1] (ng/mL)
F_1 control	356 ± 35^{c}
F_1 LH	3600 ± 390^{a}
F_1 FSH	1800 ± 346^{b}
F_3 control	134 ± 9^{c}
F_3 LH	1900 ± 179^{a}
F_3 FSH	1460 ± 154^{b}
SY control	0.168 ± 0.08^{b}
SY LH	0.959 ± 0.46^{ab}
SY FSH	1.275 ± 0.54^{a}

[1]Values are means ± SEM, n = 4 replicate experiments
[a-b]Values with different superscripts for a given follicle size differ ($p < 0.05$).

Table 5.54. Concentration of ZPC in granulosa cell culture media from F_1, F_3, or small yellow (SY) follicles cultured for 24 hours in the presence of 0 (control) or 50 ng/mL culture media of LH or FSH (Experiment 4).

Follicle size and treatment	Relative density
F_1 control	0.813 ± 0.119
F_1 LH	0.682 ± 0.094
F_1 FSH	0.708 ± 0.097
F_3 control	0.599 ± 0.182
F_3 LH	0.520 ± 0.106
F_3 FSH	0.671 ± 0.150
SY control	0.463 ± 0.140
SY LH	0.689 ± 0.139
SY FSH	0.526 ± 0.193

[1]Values are means ± SEM, n = 6 replicate experiments.

Experiment 5: ZPC and ZPB2 Protein Expression in the Presence of Testosterone or Estradiol in Granulosa Cell Cultures

Progesterone accumulation in the media of granulosa cells cultured with testosterone was greater than in control granulosa cell culture media (Table 5.55). In contrast, the addition of 17-β estradiol to the cultured granulosa cells had no effect on progesterone secretion (Table 5.55). The presence of $1x10^{-6}$ M testosterone increased ZPC protein accumulation in the media of cultured granulosa cells from the SYF (Table 5.56). As with the previous experiment, the expression of ZPB2 protein could not be determined in the cell culture media as an abundant protein component of the fetal calf serum used in the cell culture media shared a molecular weight with ZPB2 and given its abundance it also reacted with the antibodies used for detecting ZPB2.

Table 5.55. Progesterone concentration in granulosa cell culture media from F_1, F_3, or small yellow (SY) follicles cultured for 24 hours in the presence of 0 (control) or 1 x 10^{-6} M 17-β estradiol or testosterone (Experiment 5).

Follicle size and treatment	Progesterone concentration[1] (ng/mL)
F_1 control	335 ± 39[b]
F_1 estradiol	297 ± 40[b]
F_1 testosterone	619 ± 68[a]
F_3 control	150 ± 16[b]
F_3 estradiol	137 ± 12[b]
F_3 testosterone	679 ± 36[a]
SY control	0.40 ± 0.1[b]
SY estradiol	0.29 ± 0.1[b]
SY testosterone	3.24 ± 0.49[a]

[1]Values are means ± SEM, n = 4 replicate experiments

[ab]Values with different superscripts for a given follicle size differ ($p < 0.05$).

Table 5.56. Concentration of ZPC in granulosa cell culture media from F_1, F_3, or small yellow (SY) follicles cultured for 24 hours in the presence of 0 (control) or 1 x 10^{-6} M 17-β estradiol or testosterone (Experiment 5).

Follicle size and treatment	Relative density[1]
F_1 control	0.698 ± 0.175
F_1 estradiol	0.778 ± 0.077
F_1 testosterone	0.698 ± 0.211
F_3 control	0.908 ± 0.036
F_3 estradiol	0.783 ± 0.078
F_3 testosterone	0.771 ± 0.191
SY control	0.449 ± 0.125[b]
SY estradiol	0.664 ± 0.177[ab]
SY testosterone	1.000 ± 0.000[a]

[1]Values are means ± SEM, n = 4 replicate experiments.
[ab]Values with different superscripts for a given follicle size differ ($p < 0.05$).

CHAPTER 6

DISCUSSION

The present research enhanced previous mRNA ZP protein research reported by our laboratory by determining the protein expression of ZP proteins in the perivitelline layer of eggs from two genetic lines of turkeys that differ in fertility and four genetic lines of broiler breeder hens. Furthermore, ZPB2 protein expression in the granulosa cells of the hierarchical and prehierarchal follicles was elucidated and the results strengthened our original hypotheses based on mRNA expression of ZPB2 in these follicles. Finally, the present research also furthered our understanding of hormonal regulation of ZPC protein expression.

Protein Expression of ZPB2

In the current research, expression of ZPB2 protein was greater in the GD region of the perivitelline layer of eggs than in NGD regions of the perivitelline layer in the two genetic lines of turkey hens. This finding was in agreement with previous mRNA research with these two genetic lines (Benson et al., 2017), as granulosa cells from the GD region of the F_1 and F_2 follicles had greater expression of ZPB2 mRNA than NGD granulosa cells from these two hierarchical follicles from both genetic lines of hens. Additionally, in the current research the protein expression of ZPB2 was greater in the GD portion of the perivitelline layer than in the NGD portion of the perivitelline layer in three genetic lines of broiler breeder hens. Even in the genetic line in which the expression of ZPB2 protein was not significantly greater in the GD versus NGD region of the perivitelline layer, there was a strong numerical trend ($p = 0.07$) for ZPB2 expression to be greater in the GD region than the NGD region of the perivitelline layer of

eggs produced by hens of this strain. Malloy (2011) had previously reported that the mRNA expression of ZPB2 was greater in GD granulosa cells than in NGD granulosa cells from F_1 follicles obtained from an equal number of hens from each of these genetic lines of broiler breeder hens.

Given avian sperm bind preferentially to the GD region of the ovulated ovum (Howarth and Digby 1973, Ho and Meizel 1975, Bramwell and Howarth 1992, Birkhead et al., 1994, Wishart 1997), the current research strongly supports the hypothesis that the greater expression of ZPB2 protein at the GD region relative to the NGD region of the IPVL plays a critical role in attracting sperm to preferentially bind at the GD region. Additional research will be necessary to determine if ZPB2 may be the avian sperm receptor by itself or whether it works in concert with another ZP protein such as ZPB1, ZPC or ZPD to form the sperm receptor. However, although ZPB1, ZPC, and ZPD have been reported to bind sperm in one or more avian species (Pan et al., 1999, Bausek et al., 2004, Okumura et al., 2004) none of these ZP proteins has a greater expression at the GD region relative to the NGD region.

The avian sperm receptor is likely a 3-D complex consisting of multiple entities, possibly in the form of other ZP proteins, which have been previously suggested to play roles in sperm binding. The current research and previous research (Rodler et al., 2012, Nishio et al., 2014) indicates that ZPB2 expression is most abundant in the prehierarchical follicles. It seems reasonable to suggest that when ZPB2 is expressed early in development and localizes at the GD region, that it may form an anchor network to which other ZP components (ZPB1, ZPC and/or ZPD) may bind with when they are subsequently added to the IPVL during late hierarchy follicular maturation. Upon binding of one more of these later produced ZP protein components to the ZPB2 network at the GD, specific 3-D ZP protein structures found almost exclusively at

the GD region may form and serve as the sperm receptors. Further research will have to be conducted that observes the structural biology of ZP proteins at the GD to determine exact conformation, as well as knockout studies that facilitate a better understanding of function or loss of function of ZP proteins such as ZPB2.

However, the current and previous research suggests that a ZPB2 knockout could be unsuccessful. As just discussed, the current and previous research (Benson 2006, Malloy 2011, Rodler et al., 2012, Nishio et al., 2014 , Benson et al., 2017), supports our continued hypothesis that ZPB2 plays a role in early follicular development across various avian species. It is interesting to note that antibodies directed against the ZP glycoproteins can cause infertility in mammalian species via a harmful effect on ovarian function, and this loss of ovarian function is characterized by a disruption of folliculogenesis and ultimately by a depletion of the primordial follicle population as reviewed by Paterson et al., (2000). This suggests that some ZP proteins play a role in early follicular maturation and in maintaining viability. The abundant production of ZPB2 protein in the smallest prehierarchical follicles supports a role for it in early follicular dynamics that may be completely unrelated to sperm binding. Future research will need to determine if ZPB2 expression acts as a survival factor for the maintenance of the smallest prehierarchical follicles which can persist for years in avian species before being activated to start maturing.

ZPB1

The mRNA expression of ZPB1 had been previously investigated in the hepatic tissue of both genetic lines of turkey and the four genetic lines of broiler breeder hens (Malloy 2011, Benson et. al., 2017). However, due to ZPB1's mRNA expressional absence in granulosa cells an indication of its potential localization in the GD or NGD tissue of the perivitelline layer was

unknown. The hepatic mRNA expression of ZPB1 was greater in the E-line turkey hens selected for egg production than in the F-line hens selected for rapid growth. Thus, the expectation in the current research was that the expression of ZPB1 protein in the perivitelline layer of the eggs produced by hens from these genetic lines would reflect the hepatic mRNA expression data. However, it was the perivitelline layer of the eggs from the F-line hens that expressed more ZPB1 protein. Within the perivitelline layer, the expression of ZPB1 protein was greater in NGD region than the GD region in turkey hens and a similar difference in NGD and GD ZPB1 expression was seen in broiler breeder hens.

The discrepancy between the hepatic mRNA expression and the perivitelline protein expression of ZPB1 between the two genetic lines of turkey hens may have an explanation based on follicular maturation dynamics. The F-line hens have an egg production rate that is less than half the rate of the E-line hens (Hoffman et. al., 2007). Furthermore, Hoffman et al., (2007) reported that the F-line hens have an extended hierarchy ($F_1 – F_{16}$) relative to the E-line hens (F_1-F_{10}). Although it is well established that ZPB1 is produced in the liver, the mechanism involved in its transport to the developing follicles is not known. However, previous research indicates that ZPB1 protein expression is only detectable in follicles containing yellow yolk (Rodler et al., 2012) and ZPB1 expression is highly responsive to estrogenic compounds (Kudzma et al., 1975, Dashti et al., 1983, Bausek et al., 2000, Sasanami et al., 2003a). Both vitellogenin and the specialized VLDL particles that make up yellow yolk are produced by the liver under the stimulation of estrogens and then transported to the ovary. It would not be surprising if ZPB1 was transported with one of these components or in a similar independent receptor mediated process to the developing follicles. But, more importantly, it is likely given the extended hierarchy and low rate of egg production that hierarchical follicle development occurs for a

longer period of time in the F-line hens than E-line hens, which would allow a longer period of ZPB1 deposition to the IPVL to occur which could account for a greater overall concentration in the IPVL of F-line eggs. Clearly, further research is needed to determine ZPB1 transport mechanisms from the liver to the ovary and their regulation.

ZPC

Overall, the expression of ZPC protein in the perivitelline layer or cell culture media did not statistically correlate well with previously obtained granulosa cell mRNA expression results. This was a bit surprising given the excellent correlations between granulosa cell mRNA expression and perivitelline layer protein expression for ZPB2. For the turkey hens, the mRNA expression for ZPC was greater in the NGD than the GD granulosa cells for the F_1 follicle of the F-line hens and the expression of ZPC did not differ between the GD and NGD cells of the F_1 follicle of the E-line hens. The protein expression of ZPC also did not differ between GD and NGD perivitelline layer for the E- line hens, but in the F-line hens the NGD expression of ZPC in the perivitelline layer was not greater ($p = 0.07$) than GD expression. For the cell culture results, the protein expression results had the same numerical trends as the expression profile as the mRNA profile, but the statistical differences seen with the mRNA were not present. In the broiler breeder hens, there were significant differences at the protein level that were detected between the genetic strains that were not seen at the mRNA level, and there were significant differences in ZPC mRNA expression between the GD and NGD granulosa cells in 3 of the genetic lines that were not detected at the protein level. At this time without further research there is not an obvious biological explanation to account for the different results between the mRNA and protein expression data except that mRNA expression data does not always correlate with protein expression levels.

Correlation of mRNA and Protein Expression Levels

Previous research has indicated that our canonical understanding of how mRNA and protein expression levels are related necessitates re-evaluation. As reviewed by Abreu et al., (2009), studies in animals demonstrate that the correlation coefficient (R^2) between mRNA and protein can range anywhere from 0.09 and 0.46. Thus, only 9-46 % of protein expression levels could be directly explained by the mRNA expression levels alone. Therefore, at the very most, 56% of the protein level expression observed must be explained by other factors. Vogel et al., (2010) further supported these findings, whereby, they could only account for 67% of the protein expression levels using mRNA and sequence signatures. Taken together, these observations suggest that mRNA expression levels should be used as a general correlation to protein expression levels, but should not be used as a direct predictor for protein expression levels.

Importance of Research and Summary

An undesired effect from the genetic selection for rapid growth and meat yield in poultry has been an associated decline in fertility (Barbato 1999, Brillard 2004). Much of the research in poultry fertility focuses on the male and in particular on the quantity and quality of sperm produced by the male (Holsberger et al., 1998, Donoghue et al., 1999, Hammerstedt 1999). While sperm quality is important, understanding what constitutes female fertility is also important. Two important components of female fertility are sperm storage capacity and the amount of sperm receptors at the GD of the IPVL. Actually determining the capacity of sperm storage by examining and counting sperm storage tubules of female poultry is rarely done and the secondary sperm storage tubules in the infundibulum have yet to be characterized. Bakst et al., (2010) examined sperm storage tubule function and capacity in the same population of hens from the four genetic lines of broiler breeder hens utilized in the current ZP research. Bakst et

al., (2010) did not find differences in the sperm storage tubules between the four genetic lines, highlighting that any potential female fertility differences amongst the lines would not exist at the sperm storage level, but potentially with differences in sperm receptor number on ovulated ovum.

The current research strongly implicates that ZPB2 may play a critical role in the preferential binding of sperm at the GD region of the ovulated ovum. Thus, determining if ZPB2 is a component of the sperm receptor complex in future research is of high priority. In addition, the protein expression of ZPB2 in early follicular development suggests it may have an additional functional role in maintaining prehierarchical follicle viability. Investigating both roles of ZPB2 may demonstrate its usefulness in developing a powerful genetic marker for enhanced fertility. Additionally, further research is needed to determine if greater expression of ZPB1 in the perivitelline layer is actually a detriment to sperm binding as was suggested by its expression level in eggs from the poor fertility F-line turkey hens. Additionally with ZPB1, continued efforts should be undertaken to understand how it is transported to the developing follicle, as transport mechanisms may shed light onto an array of key functions involving fertility.

To highlight the potential fiscal outcome from this research, consider the U. S. broiler industry. In 2015, 9 billion broiler birds were produced, generating 39.62 billion pounds of meat (National Chicken Council). The wholesale price of chicken in 2015 was 0.905 $ per pound. Therefore, the broiler industry made approximately 35.86 billion dollars alone from meat sales in 2015. If the industry was to utilize a genetic tool that improved fertility, and through that tool, hatchability was improved by only 1% in 2015, that would have produced 90 million extra chicks. Furthermore, if taking into account the average mortality (4.8%) for a 48 day grow out

on broiler farms in 2015, the industry would have yielded 85.68 million more broilers, translating into approximately 534.6 million pounds more of broiler meat. That added meat yield would have made the broiler industry approximately 437.9 million dollars in additional revenue in 2015. In a world that by 2050 is projected to contain 9.6 billion individuals, the global food supply and land area will become a major issue requiring that the food industry focus on maximizing efficiency.

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