DIFFERENTIAL EXPRESSION OF THE mRNA FOR ZONA PELLUCIDA PROTEINS IN AVIAN SPECIES.

by

Andrew Parks Benson

(Under the Direction of Adam J. Davis)

ABSTRACT

The inner perivitelline layer (IPVL), the avian equivalent of the mammalian zona pellucida, is an extracellular glycoprotein coat that surrounds the hen's ovulated oocyte. In order for syngamy to occur, sperm must bind and subsequently penetrate through the IPVL. In the chicken, three different glycoprotein components of the IPVL (ZPC, ZPB1, ZPD) have been suggested as being the avian sperm receptor. In addition to these ZP proteins, three other ZP genes have been characterized in the chicken genome (ZPA, ZPB2, and ZPX). The disparity in research reports concerning the avian ZP proteins necessitates additional research concerning the expression and regulation of avian ZP proteins.

The mRNA expression of all six ZP genes was tested between two genetic lines of turkey hens that differ in fertility. Hepatic ZPB1 mRNA expression was significantly greater in the high fertility line (E line) than in the lower fertility line (F line). In addition, due to the prevalence of sperm penetrations of the IPVL overlying the germinal disc (GD) of the oocyte, the mRNA expression for granulosa derived ZP genes was compared between GD and non germinal disc (NGD) granulosa cells. ZPC mRNA expression was significantly higher in the NGD region of F line hens; however, ZPB2 mRNA expression was significantly greater in the GD region of both lines.

The mRNA expression of the ZPC and ZPD was analyzed in broiler breeder F_1, F_3 and small yellow (SY) follicle granulosa cells cultured with steroid hormones (estrogen and testosterone) and gonadotropins (LH and FSH). Expression of both ZPC and ZPD mRNA was induced in SY granulosa cell cultured with T, LH, and FSH. F_3 granulosa cells cultured with E2 and T had increased ZPD mRNA expression while FSH stimulated both ZPC and ZPD mRNA expression in F_3 granulosa cells.

The results suggest that the lower rates of fertility for the F line versus the E line may be related to the lower expression of ZPB1, and lower expression of ZPC in the GD region.

Key Words: Zona Pellucida Proteins, Inner Perivitelline Layer, Turkeys, Fertility, Germinal Disc, Granulosa, Genetic Lines, Broiler Breeder Hens

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Andrew Parks Benson

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Andrew Parks Benson

Major Professor:	Adam J. Davis
Committee:	Mark Compton Brian Fairchild Julie Northcutt Jeanna Wilson

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
August 2006

DEDICATION

This dissertation is dedicated to my parents, Jimmy and Guy Dean Benson, and my wife Larissa.

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CHAPTER 1

INTRODUCTION TO THE ZONA PELLUCIDA LAYER

General Introduction to Fertilization

Fertilization is the process involving the union of two haploid germ cells, egg and sperm, to form a new individual. The process of fertilization includes several ordered steps that lead to the formation of a pluripotent zygote or one-cell embryo (as reviewed by Talbot *et al.* 2003). Fertilization is initiated in the oviduct of avian and mammalian species with the binding of free-swimming sperm to the ovulated egg's extracellular glycoprotein coat, the zona pellucida (ZP). The process culminates with the union of male and female genetic material to form a single cell embryo or zygote. Between the initiation and conclusion of fertilization, several recognizable events take place, including the acrosomal reaction of the sperm (Wassarman, 1999), sperm penetration through the egg's zona pellucida (Stein *et al*. 2004), and in some species, the cortical reaction (Baker and Whitaker, 1978) and zona reaction (Jaffe, 1976) which prevent polyspermy. On a molecular basis, it is the interaction of the sperm and zona pellucida that is of inherent significance to the final steps of the fertilization process since it represents the initial interaction of gametes during the development of a new individual.

The Zona Pellucida

All vertebrate eggs are surrounded by an extracellular matrix or coat of glycoproteins. This extracellular coat that surrounds vertebrate oocytes is involved in sperm binding and also gives structural support to the ovulated oocyte as it travels down the oviduct. Fertilization has

attracted the interest of scientists from a wide range of disciplines using different animal models for their respected studies. An unfortunate consequence of these scientists' different perspectives is that different names have been assigned to functionally equivalent structures. As a result, the glycoprotein coat that surrounds a freshly ovulated oocyte has a different name in different vertebrate species. The glycoprotein coat is called the chorion in fish, the vitelline envelope in amphibians, the zona pellucida in mammals, and the perivitelline layer or perivitelline membrane in birds (Spargo and Hope, 2003). Despite the differences in terminology, these glycoprotein coats perform similar functions during fertilization and have remarkably similar ultrastructures. For the sake of simplicity, these glycoprotein coats are often now collectively referred to as the zona pellucida (Spargo and Hope, 2003). The zona pellucida of each species studied is constructed from conserved individual proteins that have a common protein domain or ZP domain (Bork and Sander, 1992; Spargo and Hope, 2003). For this reason, the individual glycoproteins which make up the zona pellucida in different species are known as ZP proteins.

ZP Glycoprotein Nomenclature

The ZP glycoproteins of various vertebrates are classified into ZP gene families according to their amino acid sequence similarities. The zona pellucida was traditionally believed to be constructed from only three major glycoproteins. Regretfully, as was the case in the naming of the extracellular glycoprotein coat for different species, the proteins and genes which make up this coat are also victims of different nomenclature systems. The initial nomenclature system named the glycoprotein components ZP1, ZP2, and ZP3 in the order of highest to lowest apparent molecular weight of the ZP proteins in the mouse (Bleil and Wassarman, 1980a). Following the cloning of the ZP1, ZP2, and ZP3 genes in the mouse, Harris *et al*. (1994)

proposed another nomenclature system in which the ZP proteins were named in order of their gene transcript size, from longest to shortest. Unfortunately, in the nomenclature system proposed by Harris *et al*. (1994), the mouse genes would have been ordered ZP2, ZP1, and then ZP3. In order to avoid the unnecessary confusion of swapping the names of mouse ZP1 and ZP2, Harris *et al*. (1994) proposed a letter-based system where ZP2 became ZPA, ZP1 became ZPB, and ZP3 became ZPC. These nomenclature systems have led to confusion since they were, and still are, used interchangeably. Further classification was required when Hughes and Barrat (1999) concluded that the human genome contained two ZPB genes. These two genes were classified as paralogous genes, and a new classification or nomenclature system needed to be proposed to account for this discovery. A unified system of nomenclature was proposed by Spargo and Hope (2003) that addressed the existing indistinctness. The Spargo and Hope system groups the ZP genes into subfamilies named alphabetically, ZPA, ZPB, and ZPC, in order of coding sequence length. Group level paralogues in the same subfamily are numbered in order of their coding sequence length, so that the human genome now contains the following ZP genes: ZPA, ZPB1, ZPB2, and ZPC (Lefievre *et al*., 2004).

The similarity in the primary structure of ZP glycoproteins establishes a common evolutionary relationship among the zona pellucida components from diverse species. Since all vertebrates studied have ZP genes, at least one ZP gene evolved during the earliest stages of vertebrate evolution (Spargo and Hope, 2003). Swanson and Vacquier (2002) concluded that in comparison to other genes, genes involved in reproduction are highly divergent and rapidly evolving. As a result of this rapid evolution, which is believed to be involved in the speciation process, six different subfamilies of ZP genes have now been identified in various vertebrates (Spargo and Hope, 2003; Smith *et al.* 2005). Therefore, the currently known ZP protein

subfamilies named according to the Spargo and Hope nomenclature system are ZPA, ZPB1, ZPB2, ZPC, ZPD, and ZPX. The chicken is the only known species to have a representative of all six ZP gene subfamilies present in its genome, and most species seem to have just a few (3-4) of the ZP gene subfamilies present in their genomes (Smith *et al.* 2005).

The ZP Glycoproteins

The size of the individual ZP glycoproteins across species exhibit heterogeneity due to extensive post-translational modifications including glycosylation and sulfation (Prasad *et al.* 2000). ZP glycoproteins exhibit both N-linked and O-linked glycosylation. In N-linked glycosylation, an oligosaccharide chain is attached by oligosaccharyl transferase to an asparagine amino acid occurring 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-acetylgalactosaminyltransferase. The oligosaccharides of the ZP proteins are also both sialyated 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). The heterogeneity of the ZP glycoproteins across species can be best illustrated by the following example. In the mouse, the zona pellucida contains ZPA, ZPB1, and ZPC, which have different average molecular masses of 200, 120, and 83 kDa, respectively (Bleil and Wassarman, 1980a), yet in the closely related rat, the molecular weight of ZPA is 140 kDa and the molecular weight of ZPB1 is 185 kDa (Akatsuka *et al.* 1998).

Although the molecular weights and the degree of post-translational modifications differ among the ZP proteins, ZP proteins do posses trademark features. Sequence analyses of all ZP genes reveal that they all have a common domain called the ZP domain (Bork and Sander, 1992).

This ZP domain consists of a 260-amino acid sequence characterized by 8 conserved cysteine residues (Bork and Sander, 1992). In addition to this common ZP domain, the three major ZP families in mammals (ZPA, ZPB, ZPC) are further related by the presence of an N-terminal hydrophobic signal sequence (Bork and Sander, 1992), a furin processing site (Dunbar *et al.* 1994) and a C-terminal hydrophobic transmembrane domain (Prasad *et al.* 2000). The preservation of these structural features implies that the three dimensional structure of the different ZP protein family members across species is similar. For example, although the amino acid sequence of the three mouse ZP proteins (ZPA, ZPB1, and ZPC) are only 39-48% similar to the three amino acid sequences of the corresponding ZP proteins in the frog, there is sufficient conservation in the overall protein structure that individual mouse ZP proteins expressed in frog eggs integrate into the surrounding frog zona pellucida (Doren *et al.* 1999).

ZP Glycoproteins in the Formation of the Three-Dimensional Zona Pellucida

In the mouse, ZPA and ZPC are thought to be organized into long filaments that are interconnected by ZPB1 giving rise to a three-dimensional matrix that forms the ZP (Green, 1997). Likewise, Rankin *et al.* (1999) reported that the oocytes of ZPB1 knockout mice are surrounded by a ZP that is thinner and has more poorly defined peripheral borders compared with that of a normal mouse oocyte. This study implies that mouse ZPB1 is not necessary for ZPA and ZPC assembly but is crucial for the structural integrity of the mouse ZP. The ZP domain, which is located at the C terminus of ZPA and ZPC proteins, is essential for the polyemerization of mouse ZP proteins into the zona pellucida (Jovine *et al*. 2002). However, the structural model of the mouse ZP cannot be directly applied to other species since the human ZP is known to consists of four ZP glycoproteins (Lefievre *et al.* 2004) while the frog has five ZP glycoproteins in its egg envelope (Lindsay *et al.* 2002).

ZP Glycoprotein Sites of Synthesis

Although the overall structures of the ZP proteins are conserved across species, there are differences among species in the site of ZP protein production. The oocyte of the mouse is solely responsible for the production of all three of the identified mouse ZP proteins (Epifano *et al.* 1995). Conversely, in the cow (Kolle *et al.* 1998) and rabbit (Lee, 2000), the ZP proteins are differentially expressed by both the oocyte and granulosa cells at different stages of follicular development. In general, the synthesis of the ZP proteins in some species are transiently expressed in granulosa cells during early stages of folliculogenesis, but in more mature follicles, the oocyte is solely responsible for ZP protein expression (Kolle *et al*. 1998; Lee *et al*. 2000). Thus, the granulosa cells contribute, at least partially, to synthesis and assembly of the ZP matrix in some mammals (Sinowatz *et al.* 2001). The sites of ZP synthesis can also vary within in a class of vertebrates. The ZP proteins of the trout (Hyllner *et al.* 1991), salmon (Oppen-Berntsen *et al*. 1999), and white flounder (Lyons *et al.* 1993) are synthesized in the liver and transported by the bloodstream to the ovary to be deposited in the chorion or zona pellucida of the developing follicles. In contrast, in other fish species, like the carp (Mold *et al.* 2001) and zebrafish (Conner *et al.* 2003), the growing oocyte is responsible for the synthesis of the ZP glycoproteins.

Sperm-ZP Adhesion

Just as the sites of synthesis differ among vertebrate species, the ZP glycoprotein component responsible for sperm binding varies from species to species. Bleil and Wassarman (1980b) identified ZPC as the initial adhesion or binding ligand for sperm in the mouse zona pellucida. Additionally, they reported that sperm binding to ZPC initiated acrosome exocytosis (Bleil *et al*. 1983). Mouse ZPA has been implicated as a contributor in the sperm-ZP interaction by serving

as a secondary receptor for sperm during the fertilization process in mice (Bleil and Wassarman 1988). ZPC has also been experimentally shown to be involved in sperm binding in amphibians (Vo and Hedrick, 2000). However, in the cow, ZPB1 exhibits the strongest sperm-binding activity among the identified bovine ZP glycoproteins (Yonezawa *et al*. 2001). ZPB1 has also been implicated as the sperm binding protein in rabbits since recombinant rabbit ZPB1 binds to the anterior portion of the sperm head overlying the acrosomal cap, and since antibodies to rabbit ZPB1 inhibit sperm binding to the zona pellucida of ovulated rabbit ova (Prasad *et al.* 1996). ZPB1 was also, at one time, implicated as the sperm binding ZP in the pig (Sacco *et al.* 1989), but this binding activity in now attributed to a ZPB1-ZPC heterodimer (Yurewicz *et al.* 1998).

As was addressed earlier, ZP glycoproteins exhibit extensive heterogeneity in molecular weight and charge primarily due to extensive post-translational modifications involving both N-linked and O-linked glycosylation. Experiments on mouse sperm-ZP interactions have shown that the sperm-binding activity of the mouse zona pellucida is related to O-linked oligosaccharides located near the C-terminus of mouse ZPC protein (Florman and Wassarman, 1985). Conversely, in pigs N-linked carbohydrates, and not O-linked carbohydrates, present on the ZP are involved in the sperm-ZP interaction (Yonezawa *et al*. 1995). It therefore appears that the carbohydrate moieties of the ZP glycoproteins, as well as the ZP glycoprotein's amino acid backbone (Chapman *et al.* 1998), may be involved in the species-specificity of sperm-ZP interaction.

Regulation of ZP Protein Synthesis

The mechanisms regulating ZP gene expression are largely unknown for many of the species whose ZP genes have been cloned. The promoter region of the ZP genes in mice all have a binding site for the transcription factor zona activating protein (ZAP-1) that has been implicated

in the positive regulation of ZP gene expression (Millar *et al*. 1993). It has also been suggested that in the mouse all of the known ZP genes (ZPA, ZPB1, and ZPC) are developmentally regulated in coordinate, since mouse ZPA, ZPB1, and ZPC mRNA expression increases together during the early stages of oogenesis and subsequently decline together (Epifano *et al*. 1995).

There is also little to no research addressing the hormonal regulation of the ZP genes. Estradiol administration was found to increase the 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). Due to estrogens profound impact on hepatic ZP expression in some fish species, ZP protein expression can be used as an indicator of environmental contamination of estrogen-like substances (Oppen-Berntsen *et al.* 1999). In chickens, estrogen also stimulates hepatic production of ZPB1 (Bausek *et al*. 2000). In addition, Pan *et al*. (2001) reported that quail ZPC production is stimulated by testosterone in cultured granulosa cells.

Practical Applications

As scientists continue to elucidate the complex process of sperm and egg binding at the molecular level, the information already generated is being utilized in the development of novel approaches to control fertility. ZP glycoproteins, due to their critical role of binding sperm during fertilization, have been proposed as candidate antigens for the development of an immunocontraceptive vaccine aimed at the interruption of the sperm-egg recognition. The feasibility of using antibodies against ZP glycoproteins for immunocontraception was demonstrated almost thirty years ago when hamsters injected with heat-solubilized mouse ZP generated antibodies which bound their own ZP proteins and induced partial infertility (Gwatkin *et al*. 1977). Since the potential of this technique was first discovered by Gwatkin *et al*. (1977), active immunization studies in a variety of animal species, using either native or recombinant ZP

proteins, has demonstrated the contraceptive capability of this method in inducing both reversible and irreversible infertility (Wood *et al*. 1981; Mahi-Brown *et al*. 1982; Gulyas *et al*. 1983). However, there is a mounting body of evidence to suggest that antibodies directed against the ZP glycoproteins cause infertility not only by an antibody-mediated disruption of the sperm- egg interaction but also via a harmful effect on ovarian function itself (Paterson *et al.* 2000). This 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). The mechanism responsible for the loss of ovarian function is currently under intense investigation since it is one of the major obstacles for the application of ZP immunocontraception as a reversible contraceptive method for humans (Gupta *et al.* 2004).

In contrast to humans, exhaustion of the primordial follicle population may be advantageous in the administration of immunocontraceptive vaccines for wildlife population management. The effective use of immunocontraceptive vaccines for providing a humane alternative for population control has been demonstrated in several species including feral horses, white-tail deer (Kirkpatrick *et al.* 1997), and feral dogs (Srivastava *et al.* 2002). These finding are extremely valuable since some species, like white-tail deer, are extremely adaptable and have successfully inhabited and overpopulated many suburbs, urban parks, and government campuses, where lethal control is no longer safe or legal.

Summary

The zona pellucida is a glycoprotein, extracellular matrix that surrounds freshly ovulated oocytes. The number of glycoproteins that make up this extracellular matrix are very limited, and in most species the matrix is made up of just 3 or 4 proteins called zona pellucida proteins.

Across all species examined, only six zona pellucida proteins have been identified, and the chicken is the only animal identified thus far in which genes for all six of the zona pellucida proteins are present. The zona pellucida proteins have a sperm binding capacity but the specific zona pellucida protein(s) responsible for sperm binding varies from species to species.

CHAPTER 2

AVIAN FEMALE REPRODUCTIVE PHYSIOLOGY

The Avian Ovary

The ovary of a mature hen consists of a visually evident hierarchy of follicles arranged according to size and time to ovulation. In the chicken, there is commonly four to six large yellow yolk – filled follicles termed hierarchical follicles that are approximately 12 -40 mm in diameter. These follicles are accompanied by several 5 to 12 mm follicles in which yellow yolk deposition has begun and a large number of small white follicles that are less than 5 mm in diameter. The large yolk filled follicles are named according to size. The largest follicle is termed the F_1 follicle and will ovulate within the next 24 hours. The next largest follicle is named the F_2 follicle and it will ovulate 24-26 hours after the ovulation of the F_1 follicle. The remaining large yolk filled follicles are named accordingly (F_3-F_n). With each ovulation the hierarchical follicles advance up the hierarchy and a new follicle enters into the hierarchy from the pool of small yellow follicles. In addition, some of the larger white follicles will start the uptake of yellow yolk and become small yellow follicles. A vast majority of the small yellow and large white follicles will never advance into the hierarchy but will instead undergo atresia by apoptosis (Gilbert *et al*. 1983)

Profile of Avian Follicular Tissues: Granulosa and Theca Cell Layers

Each preovulatory follicle consists of distinct tissue layers that surround the yolk– filled oocyte. In each follicle, the developing oocyte is first surrounded by its plasma membrane, then

the zona pellucida or inner perivitelline layer (IPVL), followed by the granulosa cell layer, a basement membrane, and theca tissue layers. The theca tissue is highly vascularized, in contrast to the avascular granulosa cell layer, and facilitates the transfer of yolk precursors from plasma to the developing follicles in the ovary (as reviewed by Schneider *et al.* 1998).

In general terms, follicular maturation can be characterized by the accumulation of yolk and the development of endocrine capabilities within the follicular tissues (Huang and Nalbandov, 1979). Follicular maturation is regulated by two pituitary glycoprotein hormones, luteinizing hormone (LH) and follicle stimulating hormone (FSH). The theca cells of the small yellow and white follicles are steroidogenically competent and secrete androgens and estrogens via the Δ^5 steroidogenic pathway (Lee *et al.* 1998). However, the granulosa cells of these small follicles are steroidogenically incompetent since they lack P_{450} side chain cleavage (P_{450}SCC) enzyme activity, which catalyzes the initial step in the metabolic pathway that converts cholesterol to steroids (Li and Johnson, 1993). Li and Johnson (1993) proposed that follicle recruitment into the hierarchy is coupled to the initial expression of LH receptor (LHR) mRNA and the acquisition of functional P_{450}SCC enzyme activity within the granulosa layer. Expression of P_{450}SCC mRNA and the initiation of the activity of this enzyme *in vitro* is induced by FSH (Li and Johnson, 1993). Furthermore, only one follicle from the cohort of small yellow follicles has an up-regulated level of FSH receptors and it is likely the one that advances into the hierarchy (Woods and Johnson, 2005). Finally, FSH treatment attenuates the onset of apoptotic cell death in prehierarchical follicle granulosa cells (Johnson *et al.* 1996). Taken together, these results suggest that the prehierachial follicle that is more responsive to FSH avoids atresia by apoptotic cell death and is recruited into the avian follicular hierarchy.

Once the selection of a follicle occurs, granulosa cells begin the transition from being predominantly FSH dependent to becoming primarily LH dependent (Calvo and Bahr, 1983). The granulosa cells of the hierarchical follicles acquire P_{450}SCC activity (Kato *et al.* 1995) and begin to produce progesterone via the Δ^4 steroidogenic pathway in response to LH. However, significant secretion of progesterone in response to LH occurs only from the F_1 follicle in the hen prior to ovulation (Huang and Nalbandov, 1979). As reviewed by Etches (1996), plasma progesterone produced by the F_1 follicle triggers secretion of luteinizing hormone releasing hormone (LHRH-1) from the hypothalamus. LHRH-1 travels to the anterior pituitary through the hypothalamus-pituitary portal vascular system and initiates the secretion of LH from the anterior pituitary. LH and progesterone engage in a positive feedback loop which generates the preovulatory surge of both LH and progesterone 4 to 6 hours before ovulation of the F_1 follicle.

Germinal Disc Region

The germinal disc (GD) represents less than 1% of the mature follicle volume, yet it can be easily distinguished visually as a white disc around 3 to 4 mm in diameter on the surface of the yolk. The GD functionally and structurally resembles the mammalian oocyte since just prior to ovulation the GD contains the 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), and thus, the female pronucleus and subsequently any zygote that forms are surrounded by a fluid that more closely resembles normal physiological fluids than the highly lipid yellow yolk (Perry *et al*. 1978). The germinal disc region is considered the growth center of avian preovulatory follicles since granulosa cells proximal to the GD region are more differentiated and produce greater amounts of progesterone in response to LH (Tischkau *et al.* 1997). In addition, DNA synthesis is 2-fold

higher in granulosa cells within the GD region when compared to granulosa cells from the non-germinal disc (NGD) regions (Tilly *et al.* 1992)

The morphology of the plasma membrane of the oocyte at the GD area is considerably different from the other areas of the oocyte (Bakst and Howarth, 1977a). Bakst and Howarth (1977a) found that the plasma membrane of the oocyte is discontinuous and yolk material comes into direct contact and even breaches the plasma membrane of the oocyte away from but not above the GD region. In addition, Bakst (1978) reported that microvillous projections, possibly originating from the plasma membrane of the oocyte, are more numerous and longer in the GD region than those located in the NGD regions.

Ovulation and the Avian IPVL

The theca and granulosa cell layers are left behind on the ovary as a post-ovulatory follicle when the follicle ovulates. Therefore at ovulation, the oocyte consists of the yolk mass, surrounded by the plasma membrane (or oolemma) of the ovum which is covered by the IPVL on its outer surface (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 engulfs the ovulated oocyte.

Based on observations from over 130 years ago by Tauber (1875), the infundibulum was proposed as the site of fertilization, and this was later confirmed through electron microscopy (Okamura and Nishiyama, 1978). At the time of fertilization the plasma membrane of the oocyte is only surrounded by the IPVL. Therefore, chicken spermatozoa must penetrate through the IPVL before gaining access to the perivitelline space and plasma membrane of the oocyte. Initial observations of the IPVL's fibrous meshwork suggested that chicken sperm might merely pass through the fibrous meshwork since the holes in the meshwork of the IPVL are around 2 μm in

diameter and individual sperm are around 0.5 μM in diameter at their broadest point (Bellairs *et al.* 1963). Bellairs *et al.* (1963) did not observe any substance between the fibers of the IPVL to impede sperm entry to the oocyte. Conversely, later studies discovered that the spaces between the ZP fibers were filled with granular material that prevented simple penetration or diffusion of the sperm through the IPVL (Bakst and Howarth, 1977a).

Subsequent research established that sperm penetration of the IPVL was dependent on an acrosome reaction. Okumura and Nishiyama (1978) reported that the fibrous meshwork of the IPVL dissolved under the head of sperm creating a hole around 9 μm in diameter. Furthermore, electron microscopy reveled that all sperm passing through the IPVL were found within sperm holes following hydrolysis of the IPVL (Okamura and Nishiyama, 1978). Kuroki and Mori (1997) reported that sperm penetration of the IPVL could be hampered by trypsin protease inhibitors, and concluded that the protease inhibitors were preventing acrosin released by the sperm from digesting a hole in the IPVL. The IPVL isolated from ovulated oocytes (Koyanagi *et al*. 1988), prevovulatory follicles (Steele *et al*. 1994), and laid eggs (Robertson *et al*. 1997) displays a similar ability to bind sperm and initiate the acrosome reaction which causes the hydrolysis of sperm holes in the IPVL.

Because the formation of the sperm holes in the IPVL require that sperm first bind to the IPVL and consequently undergo the acrosome reaction, sperm holes in the IPVL are indicative of a successful sperm:IPVL interaction. Formation of sperm holes in the IPVL occur within 2 ½ minutes of *in vitro* co-incubation with sperm, and over time these sperm holes will enlarge as the hydrolytic enzymes released after the induction of the acrosome reaction continue to digest the IPVL (Robertson *et al*. 1997).

Relationship between Sperm Hole Formation and Fertility

Although only one sperm is required for fertilization, the probability of syngamy increases if multiple sperm penetrate the egg in avian species. In chickens and turkeys, the probability of fertilization is 100% when six or more sperm penetrate the IPVL over the GD region and about 50% when 3 sperm penetrate the IPVL over the GD region (Bramwell and Howarth 1997; Wishart, 1997). These findings are consistent with the notion that a minimum number of sperm entering the oocyte may be required for fertilization which would result in a greater probability that syngamy will occur (Wishart and Staines, 1999). The fate of the "extra" sperm that did not join 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). In fact, Wishart (1997) demonstrated that embryonic development proceeds normally in eggs that contain only one sperm hole in IPVL over the GD region.

Excessive numbers of supernumerary sperm might interfere with normal embryonic development and possibly cause embryonic mortality. Bekhtina (1968) suggested that greater than 200 sperm penetrating the site of fertilization would result in early embryonic mortality, yet Bramwell *et al.* (1995) found more than 200 sperm holes in fertile chicken eggs that did not have excessive early embryonic mortality. Although there is a disagreement in the exact number of maximal sperm penetration holes permissible before early embryonic mortality occurs, it seems logical that there would be a maximum number of sperm penetrations and associated release of hydrolytic enzymes, before abnormal development occurs. In fact, Van Krey *et al*. (1966) reported an increased frequency of early embryonic death from intramagnal inseminations and the associated increase in the numbers of sperm which interacted with the oocyte. Nevertheless,

it is apparent that the purpose of the observed physiological polyspermy in birds is simply to increase the chances of syngamy occurring (Birkhead *et al.* 1994).

Sperm Holes or Sperm Disks

Sultana *et al.* (2004) identified two distinct configurations in the IPVL of fertile quail eggs by electron microscopy: a disk in which the 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 identified disks in the IPVL were classified as being formed when sperm only partially dissolved the IPVL. In a latter study, Rabbani *et al.* (2006) 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. In fact, using electron microscopy, Rabbani *et al.* (2006) found that only 5 % of the ring shaped configurations in the IPVL from fertile quail eggs were true sperm holes. Although these "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 new findings by Sultana *et al*. (2004) and Rabbani *et al*. (2006) also 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

There have been several studies involving the interaction of sperm with the IPVL over the blastodisc or GD region versus the interaction of sperm with the IPVL overlying other regions of the oocyte or nongerminal disc (NGD) regions. A majority of the studies across many species of birds (Howarth and Digby, 1973; Ho and Meizel, 1975; Bramwell and Howarth, 1992a; Birkhead *et al.* 1994; and Wishart, 1997) indicate that sperm holes are preferentially formed over

the GD region of the oocyte. It should be noted that Steele *et al.* (1994) reported during an assay where fragments of the IPVL from different regions of freshly ovulated eggs were incubated with sperm *in vitro*, sperm hole formations appeared with similar frequency in the IPVL from over the GD region and NGD region. The reason that Steele *et al.* (1994) obtained such different results from the other research reports is unclear, but these unusual results of Steele *et al.* (1994) have not been duplicated to date.

The Window of Fertilization

Fertilization is accomplished in the infundibulum before the oocyte arrives at the magnum of the oviduct (Kaupp, 1918; Olsen and Neher, 1948). The ovulated oocyte spends around 15 minutes in the infundibulum of the oviduct (Warren and Scott, 1935), and this time frame between ovulation and the oocyte reaching the magnum is commonly referred to as the "15 minute window of fertilization."

Subsequent research established why the fertilization window only exists for 15 minutes. Bellairs *et al.* (1963) reported that ovulated oocytes recovered from the distal infundibulum and upper magnum were covered with a new layer of extracellular fibers that he named the outer perivitelline layer (OPVL). 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 follicle moves down the infundibulum and the upper magnum regions of the oviduct (Bellairs *et al.* 1963; Bain and Hall, 1969). The OPVL 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 OPVL varies in thickness from about 8.5 μm to as little as 3 μm (Bellairs *et al.* 1963). The addition of the OPVL around the IPVL was subsequently discovered to account for the cessation

of the 15 minute fertilization window since sperm are no longer able to bind or penetrate through the IPVL following OPVL deposition (Howarth and Digby, 1973; Bakst and Howarth, 1977b).

Polyspermy and the OPVL

Unlike mammals, but similar to reptiles and some amphibians, avian species, as previously mentioned, exhibit physiological polyspermy, which means several sperm penetrate the IPVL and plasma membrane of the oocyte. Bakst and Howarth (1977b) suggested that physiological polyspermy arises in birds and other species which produce large, yolky eggs since the germinal disc comprises a rather small target for the sperm on the surface of the oocyte. Due to this observed polyspermy in birds, there is obviously not a mechanism analogous to the mammalian cortical reaction present in bird oocytes to prevent multiple sperm from hydrolyzing holes in the IPVL. The prevention of pathological polyspermy and subsequent loss of zygote viability in birds, however, is typically prevented by the deposition of the outer perivitelline layer (OPVL).

As the OPVL is deposited around the IPVL, sperm which are free in the lumen at this area of the oviduct become trapped in the proteinaceous material secreted by the epithelial cells of the oviduct to form the OPVL. The sperm which are recovered from the OPVL are trapped in the fibers of the OPVL (Bobr *et al.* 1964) and do not appear to have undergone the acrosome reaction (Bakst and Howarth, 1977b), so these sperm are distinct from those sperm which have penetrated the IPVL. In ovipositioned chicken eggs, there are close to 10 times more sperm trapped in the OPVL than there are holes in a equivalent area of the IPVL (Wishart, 1997), yet in quail and other birds, the numbers appear similar (Birkhead *et al*. 1994).

Sperm trapped in the OPVL are unable to hydrolyze the IPVL because the OPVL contains protease inhibitors, such as ovomucin and ovoinhibitor (Matsushima, 1958), which prevent the activity of the proteases released during the acrosome reaction. Wishart and Fairweather (1999)

reported that preparations of the OPVL can inhibit the IPVL-induced sperm acrosome reaction *in vitro*. Thus, sperm trapped in the OPVL probably do not initiate the acrosome reaction since they are unable to bind to the IPVL and be activated, and because sperm just contacting the IPVL as the OPVL is deposited on top of them may also be incapable of hydrolyzing a hole through the IPVL because of the protease inhibitors present in the OPVL.

Summary

The freshly ovulated yolk – filled avian oocyte is surrounded by its plasma membrane and the IPVL. During the fertilization process, sperm must bind to the IPVL which activates the acrosome reaction that results in a hole being digested in the IPVL through which the sperm can pass to gain access to the plasma membrane of the oocyte. Sperm preferentially bind to the IPVL that overlies the germinal disc region of the oocyte. This preferential binding at the germinal disc region helps to insure subsequent syngamy. Finally, the deposition of the OPVL around the IPVL prevents any further sperm penetrations of the IPVL.

CHAPTER 3

THE ZONA PELLUCIDA PROTEINS OF AVIAN SPECIES

Identification of the Avian ZP Proteins

Structurally, the IPVL is about 2.7 μm thick, although it is thinner over the GD (Bellairs *et al*. 1963). The IPVL consists of an open lattice of cylindrical fibers, about 500 nm in diameter, which run parallel to the surface of the yolk (Bellairs *et al*. 1963). The 500 nm diameter filaments of the chicken IPVL are much wider than the 7 nm diameter filaments observed in the zona pellucida of the mouse (Wassarman, 1990; Takeuchi 2001). Electophoretic separation of IPVL homogenates followed by Coomassie brilliant blue staining revealed very few potential protein components; however, the molecular masses of these relatively few proteins varied widely among individual research reports (Kido and Doi, 1988; Howarth, 1992; Steele *et al.* 1994). The differences in the results of these studies appear to be based on the widely variable protocols used in obtaining and processing the IPVL samples. Subsequent research, involving protein purification and molecular biology techniques, has thus far identified two 42 kDa glycoproteins and one 95 kDa glycoprotein that are components of the chicken IPVL (Waclawek *et al*. 1998; Bausek *et al*. 2000; Okumura *et al*. 2004).

Waclawek *et al.* (1998) and Takeuchi *et al*. (1999) isolated a 42 kDa protein from the IPVL and then obtained the N-terminal amino acid sequence of this protein. Based on sequence homology, the isolated protein was subsequently identified as the chicken homologue of mammalian ZPC. The mRNA transcript for chicken ZPC was detected by Northern analysis

only in the granulosa cells of 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). Interestingly, Western blot analysis of the IPVL isolated from freshly laid eggs revealed that chicken ZPC undergoes postovulatory modifications since anti-chicken ZPC antibodies only detected a single 34 kDa protein in these samples (Waclawek et al. 1998). The size reduction is independent of sperm contact with the IPVL since this size modification of chicken ZPC 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 from the infundibulum. Waclawek *et al*. (1998) reported that the size reduction was independent of oligosaccharide side chain modifications in the chicken. In contrast, Pan *et a*l (2000) reported that, in addition to the proteolytic processing, the size modification resulted in some modification of N-linked and O-linked oligosaccharide chains in quail ZPC.

Bausek *et al.* (2000) subjected the extracts of chicken IPVL to SDS-PAGE under reducing conditions and identified a 95 kDa protein component of the IPVL which they subsequently isolated and cloned from a liver cDNA library. Based on the significant homology of the coding sequence of their isolated clone and mammalian ZPB1 sequences, Bausek *et al.* (2000) classified their cone as chicken ZPB1. Antisera to chicken ZPB1 bound a 97 kDa protein in immunoblot samples from the IPVL and liver but not from granulosa samples separated on SDS-PAGE gels under reducing conditions (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 97 kDa peptide formed through intermolecular disulfide bonds (Bausek *et al.* 2000; Takeuchi *et al*. 2001; Okumura *et al*. 2004).

Okumura *et al.* (2004) successfully cloned the avian homologue of ZPD from preovulatory follicular granulosa cells. Antisera to chicken ZPD bound an abundant 42 kDa protein on immunoblots prepared from IPVL (Okumura *et al*. 2004). Interestingly, chicken ZPC had been previously classified as a 42 kDa protein (Waclawek *et al.* 1998; Takeuchi *et al*. 1999). Immunoblotting analyses by Okumura *et al.* (2004) revealed that the two 42 kDa peptides, ZPC and ZPD, co-migrate under reducing conditions, but the two ZP proteins separate under non-reducing conditions due to a drastic and unexplained increase in ZPC mobility in the presence of a reducing agent.

In addition to ZPB1, ZPC, and ZPD, three other ZP proteins have been characterized in the chicken genome. Tsuda and Matsuda deposited a cDNA sequence for ZPB in GenBank in 1999 (Accession # AB025428). Based on this sequence Bausek *et al.* (2000) made a cDNA probe of ZPB for Northern analysis and detected expression of this message only in small stroma embedded follicles in the ovary. Subsequently, the reported GenBank sequence for ZPB was renamed ZPB2 by Sargo and Hope (2003) in their naming classification system. Smith *et al.* (2005) identified and subsequently mapped two additional ZP genes in the chicken genome: chicken ZPA and ZPX. Although chicken ZPA and ZPX were classified as part of the chicken genome project, expression analysis of these two ZP proteins has not been completed. Interestingly, the chicken is the only known species to have a representative of all six ZP gene families present in its genome. 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 ZPA on chromosome 14, ZPB1 on chromosome 5, ZPB2 on chromosome 6, ZPC on chromosome 10, ZPD on chromosome 11, and ZPX on chromosome 3 (Smith *et al*. 2005).

ZP Sites of Synthesis and Expression

In addition to being the only known vertebrate to have a representative of all six ZP families, the chicken is also unique since it is the only species known to have expression of ZP glycoproteins in both gonadal and extra-gonadal tissues. In all mammals studied thus far, ZP glycoproteins are either synthesized by the oocyte itself or by the somatic tissues directly surrounding the developing oocyte. However, the chicken and quail were found to synthesize ZPB1 in the liver and then subsequently transport this glycoprotein to the developing follicles in the ovary (Bausek *et al.* 2000; Sasanami *et al.* 2003a). This pattern of synthesis and transport is not novel in the chicken or quail since avian yolk components are also synthesized in the liver and subsequently transported to the developing follicles (Lazier, 1978; Nadin-Davis *et al.* 1980). Hepatic expression of the ZP glycoproteins has been reported in some species of teleosts such as the rainbow trout (Oppen-Berntsen *et al.* 1992), white flounder (Lyons *et al.* 1993), and medaka (Sugiyama *et al.* 1998). However, in these vertebrates, the liver expresses all of the ZP glycoproteins, but in the chicken only ZPB1 is expressed in the liver, while ZPB2, ZPC and ZPD are produced by the ovary (Waclawek et al. 1998; Bausek et al. 2000; Okumura et al. 2004).

Hormonal Regulation of Avian ZP Proteins

Very little is known about the hormonal regulation of the ZP proteins in avian species. Expression studies have revealed information on the transcriptional regulation of avian ZPB1

and ZPC biosynthesis. ZPB1 is synthesized by the liver of reproductively active female chickens (Bausek *et al.* 2000) and quail (Sasanami *et al.* 2003a) but not sexually active males. However, estrogen treatment of roosters and diethylstilbestrol treatment of male quail 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; Sasanami *et al.* 2003a). Estrogen's 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 of environmental estrogen exposure. Unlike ZPB1, estrogen did not have a significant effect on the transcriptional regulation of ZPC (Pan *et al*. 2001).

Pan *et al*. (2001) reported that ZPC production is stimulated by testosterone in cultured quail granulosa cells. FSH was also found to stimulate the production of ZPC in cultured avian granulosa cells (Pan *et al*. 2003).

Secretion of ZP Proteins and Assembly of the IPVL

Since ZPC is secreted by the granulosa cells, several studies have looked at the intracellular trafficking and highly polarized secretion of avian ZPC. Because avian granulosa cells are arranged on the surface of the oocyte as a single layer in mature follicles, granulosa cell production of ZPC provides an excellent model for studying the vectorial secretion of proteins (Sasanami *et al.* 2003b). Immunohistochemical staining indicates the exclusive presence of chicken ZPC on the apical side of granulosa cells (Waclawek *et al.* 1998). Waclawek *et al.* (1998) proposed that the hydrophobic domain near the carboxy-terminus of ZPC may serve as a glycosylphosphatidylinositol (GPI) anchor which directs the newly synthesized ZPC to the apical side of the granulosa cells. Both quail and chicken ZPC have been shown to undergo post-translational proteolytic processing, which is a common characteristic of GPI – anchored

proteins (Jovine *et al.* 2005). In fact, C-terminal proteolytic processing of proZPC is a prerequisite event for quail ZPC secretion since blockage of this cleavage results in the accumulation of proZPC in the endoplsmic reticulum (Sasanami *et al.* 2003b). In addition, secretion of quail ZPC does not require N-linked glycosylation (Sasanami *et al*. 2003c) which is a requirement of ZP secretion in other species (Roller and Wassarman, 1983).

Once secreted, the avian ZP glycoproteins interact and assemble to form the three-dimensional network of coarse fibers referred to as the IPVL. However, little is known about the mechanism by which these ZP proteins are assembled to form the avian IPVL. Some insight into the assembly process has been provided by the research of Ohtsuki *et al*. (2004). They reported that granulosa cell cultures secrete ZPC in a soluble form, but ZPC become insoluble when IPVL homogenates are added to the cell culture media. In addition, Ohtsuki *et al*. (2004) were able to demonstrate that ZPC secreted from cultured granulosa cells specifically interacts with ZPB1. Obviously additional studies are needed to characterize the formation of the avian IPVL especially in light of the fact that ZPD has recently been reported to be a major component of the IPVL (Okumura *et al.* 2004).

Sperm Binding to ZP Glycoproteins

Species specific sperm-egg binding interactions have been shown to be a significant obstacle that prevents interspecies fertilization (Hartman *et al.* 1972); however, in closely related species, such as bovine and sheep, this does not represent such a stringent barrier (Slavik and Fulka, 1990). Cross-reactivity between chicken sperm and the IPVL from closely related species has been demonstrated in terms of sperm hole formation following co-incubations of chicken sperm with turkey and guineafowl IPVL (Steele *et al.* 1994). In an earlier study, Bramwell and Howarth (1992b) used a sperm competition assay to illustrate the cross-reactivity of rooster, tom,

and drake sperm with IPVL homogenates from chicken, turkey, and duck oocytes. In a more extensive study, Stewart *et al*. (2004) reported that the average interaction (binding) between chicken sperm and several other avian species as a proportion of the interaction between chicken sperm and chicken IPVL. The average interaction was 100 % or greater for Galliformes (quail, turkey, pheasant and guineafowl), 44 % within Anseriformes (goose, duck and swan) and less than 30 % in Passeriformes (Zebra Finch, Bald Eagle, Emperor Penguin). These findings indicate that the core structural characteristics of the sperm binding component of the IPVL have been conserved in avian species and this may explain why hybridizations between species occur more frequently in avian species.

Research on avian sperm-egg recognition has been consistent in implicating the carbohydrate moieties of the ZP glycoproteins as a necessary structural characteristic for successful sperm binding and penetration of the IPVL. Howarth (1992) found that the chicken IPVL lost its sperm receptor activity following the removal of both N- and O-linked oligosaccharides from the IPVL. More specifically, Robertson *et al*. (2000) reported that removal of N-linked, but not O-linked sugars, inhibit the avian sperm-IPVL interaction. Furthermore, Horrocks *et al.* (2000) reported that the acrosomal reaction in chickens is induced by N-linked glycans which have a terminal *N*-acetyl-glucosamine residue. The number of potential N-glycosylation sites in the chicken ZPD, ZPB1, and ZPC proteins are four, three and one, respectively (Okumura *et al.* 2004). Which ZP glycoprotein component of the IPVL carries the carbohydrate moiety responsible for sperm-IPVL binding has yet to be determined.

There is no paradigm in vertebrates regarding the ZP glycoprotein that interacts with and activates the sperm since ZPC, ZPB1 or a combination of ZP proteins have all been identified as the sperm receptor in different vertebrate species (Bleil and Wassarman, 1980b; Yurewicz *et al.*

1998; Yonezawa *et al*. 2001). Therefore, in avian species, ZPB1 ZPC and ZPD have all been examined as potential sperm receptors. Antiserum against quail ZPC inhibits sperm binding and perforation of the IPVL (Mori *et al*. 1998; Pan *et al.* 1999). Bausek *et al.* (2004) also has presented evidence which suggested that ZPC may in fact play a role in the sperm-egg interaction since chicken ZPC binds with far more affinity than ZPB1 to two specific proteins from chicken sperm that had been solubulized and separated by electrophoresis. Both chicken ZPC and ZPB1 are able to bind individually to the acrosomal region of rooster sperm (Bausek *et al.* 2004). Therefore, 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.

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 more intimately involved in the sperm-IPVL interaction. In addition, antiserum directed against ZPB1 significantly reduces the number of sperm holes generated in the IPVL (Bausek *et al.* 2004; Takeuchi *et al.* 2001). Finally, Okumura et al. (2004) reported that dimeric ZPB1 was capable of stimulating the sperm acrosome reaction during an *in vitro* sperm activation assay.

The most recently discovered IPVL protein component, ZPD, was reported to play a role in the sperm-egg interaction immediately following its identification (Okumura *et al.* 2004). Upon the discovery of this loosely bound IPVL component, chicken ZPD was discovered to stimulate *in vitro* sperm acrosome reactions more than ZPC and monomeric ZPB1. It is notable that chicken ZPD was discovered to have the most potential N-glycosylation sites of any of the

known avian ZP glycoproteins since N-linked glycans have been shown to be vital in avian sperm-egg interactions (Robertson *et al*. 2000; Horrocks *et al*. 2000)

Summary

Considerable progress has been made in the last few years in distinguishing the ZP glycoproteins which make up the avian IPVL, yet there exists contradicting *in vitro* research in identifying the avian equivalent of the mammalian sperm receptor. Clearly, there is evidence suggesting that ZPB1, ZPC, and ZPD may all play a role in sperm binding and induction of the acrosome reaction. More research is needed to establish the exact interactions involved in sperm binding and penetrating the IPVL.

CHAPTER 4

STATEMENT OF PURPOSE

The sperm binding protein(s) in the avian IPVL has not been identified and very little information is known about the quantitative differences in the expression of the ZP glycoproteins in avian species. As of yet, no research has been conducted to determine if differential ZP gene expression exist between the granulosa cells of the germinal disc region and the non-germinal disc regions of the preovulatory follicles even though it has been proposed by several researchers that the unidentified avian sperm receptor is concentrated in the small area of the IPVL overlying the germinal disc. Although there is a positive relationship between the number of sperm holes in the IPVL and fertility, no research has been conducted to see if there is a correlation between genetic line fertility and ZP protein mRNA expression. There is also a need to further examine the hormonal regulation of the known avian ZP protein genes. Furthermore, there is no data on the expression or existence of ZP proteins in the turkey hen (*Meleagris gallopavo*). Therefore, the overall objectives of this dissertation are: (1) to determine if the known chicken ZP genes are expressed in the turkey, and if so, determine their site of synthesis, (2) to determine if differential expression of the mRNA for the ZP proteins exists between two genetic lines of hens which are known to significantly differ in fertility and between GD and NGD granulosa cells, (3) to determine the endocrine effect of gonadotropins and gonadal steroids on the mRNA expression of granulosa ZP proteins.

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CHAPTER 5

EXPRESSION OF THE MRNA FOR ZONA PELLUCIDA PROTEINS B1, C AND D IN TWO GENETIC LINES OF TURKEY HENS THAT DIFFER IN FERTILITY[1]

[1]Benson, A. P., V. L. Christensen, B. D. Fairchild and A. J. Davis
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Abstract

The avian inner perivitelline layer contains zona pellucida protein-B1 (ZPB1), zona pellucida protein-C (ZPC) and zona pellucida protein-D (ZPD). These three proteins may be involved in sperm binding to the IPVL. ZPB1 is produced by the liver and transported to the developing preovulatory follicle, while ZPC and ZPD are synthesized and secreted by the granulosa cells of the preovulatory follicle. The mRNA expression of ZPB1, ZPC, and ZPD was investigated in two lines of turkey hens selected for over 40 generations for either increased egg production (E line) or increased body weight (F line). Total RNA was extracted from the liver and from 1 cm^2 sections of the granulosa layer around the germinal disc (GD) and a nongerminal disc (NGD) area of the F_1 and F_2 follicles of six, 48 week-old hens from each genetic line. Northern analysis was performed using chicken cDNA probes for all three ZP proteins. Hepatic expression of the mRNA for ZPB1 was significantly ($p < 0.05$) greater in turkey hens from the E line than the F line. The expression of the mRNA for ZPC and ZPD in the two largest preovulatory follicles was equal between the two lines of turkey hens. There were no differences in the expression of ZPD between the GD and NGD regions of the F_1 and F_2 follicles for both genetic lines. Although, there was no difference in the expression of ZPC between the GD and NGD regions of the two largest follicles in E line hens, expression of ZPC was greater in the NGD region compared to the GD region in the two largest follicles obtained from the F line hens. The results suggest that the higher rates of fertility previously observed in eggs from the E line hens versus the F line of hens may be related to differential expression of the potential sperm binding proteins ZPB1 and ZPC.

Key words: zona pellucida proteins, turkey hens, fertility, mRNA expression

Introduction

The freshly ovulated eggs of vertebrates are surrounded by an insoluble extracellular matrix, which is referred to as the zona pellucida in mammals and the inner perivitelline layer (IPVL) in avian species (Spago and Hope, 2003). This glycoprotein coat is the interface between the ovum and the environment of the oviduct where fertilization occurs in avian species (Bellairs *et al*. 1963). In birds, spermatozoa in the infundibulum region of the oviduct bind with this glycoprotein coat and undergo the acrosome reaction which facilitates their penetration of the inner perivitelline layer (Okamura and Nishiyama, 1978).

In avian species the ovary contains large yolk – filled preovulatory follicles arranged in a hierarchy according to size. The largest follicle, which will typically be ovulated in 24 hours, is designated as the F_1 follicle, and the second largest follicle, which will be ovulated 24-26 hours after the F_1 follicle, is the F_2 follicle, and so on. Visually apparent on the surface of the yolk of each one of the large preovulatory follicles is a white colored, disc-shaped area referred to as the germinal disc. Although the germinal disc region is less than 1 % of the total volume of the avian oocyte, it houses the female pronucleus and cellular organelles (Perry *et al*. 1978). In addition, the granulosa cells surrounding the germinal disc (GD) region and nongerminal disc (NGD) regions of the preovulatory follicles in the ovary are morphologically distinct from one another (Perry *et al*. 1978), and differential mRNA expression of granulosa derived proteins have been previously reported between GD and NGD granulosa cells (Yao and Bahr, 2001). Penetration of the IPVL by avian sperm takes place at a significantly higher frequency in the GD region than in NGD regions of the oocyte (Howarth and Digby, 1973; Bakst and Howarth, 1977;

Bramwell and Howarth, 1992; Birkhead *et al.* 1994; Kuroki and Mori, 1997; Wishart, 1997), which may indicate that this area of the IPVL contains a higher concentration of sperm binding sites.

The glycoprotein components of the inner perivitelline layer are called zona pellucida proteins or ZP proteins since the IPVL is the avian equivalent of the mammalian zona pellucida. At present, at least one representative of the five subfamilies of genes within the zona pellucida gene family has been identified in avian species (ZPA, ZPB, ZPC, ZPD and ZPX) (Waclawek *et al*. 1998; Takeuchi *et al*. 1999; Bausek *et al*. 2000; Sasanami *et al.* 2003a; Sasanami *et al*. 2003b; Okumura *et al*. 2004; Smith *et al*. 2005). A total of six ZP genes have been identified in birds since the ZPB subfamily contains two paralogous genes, ZPB1 and ZPB2 (Bausek *et al*. 2000; Spargo and Hope, 2003). Based on immunohistochemistry and Western blotting techniques, ZPB1, ZPC, and ZPD, are known to exist as glycoprotein components of the avian IPVL (Bausek *et al.* 2000; Waclawek *et al.* 1998; Okumura *et al*. 2004). In both the chicken and quail, ZPB1 is synthesized by the liver and then subsequently transported to the developing preovulatory follicles (Bausek *et al.* 2000; Sasanami *et al.* 2003). In contrast, ZPC and ZPD are both synthesized by the granulosa cells of the large preovulatory follicles (Waclawek *et al.* 1998; Takeuchi *et al.* 1999; Pan *et al*. 2001; Okumura *et al.* 2004).

Research in the chicken suggests that ZPB1, ZPC, and ZPD may all be involved in sperm binding or activation at the IPVL. Antibodies directed against quail ZPC (Pan *et al*. 1999) or chicken ZPB1 (Takeuchi *et al.* 2001; Bausek *et al.* 2004) inhibit the *in vitro* formation of sperm holes in the IPVL. In addition, Bausek *et al.* (2004) reported that chicken ZPC and ZPB1 both individually bind to rooster sperm and specifically to the acrosome region of the sperm when

these components are incubated together *in vitro*. Chicken ZPD, the most recently identified IPVL component, and dimeric ZPB1 were reported to induce *in vitro* rooster sperm activation (Okumura *et al.* 2004).

Commercially appealing carcass characteristics in poultry, such as rapid body growth, are inversely correlated with favorable reproductive traits such as egg production, hatchability, fertility and high sperm – IPVL binding (Barbato, 1999; Pollock, 1999). The Ohio Agriculture Research and Development Center maintains several genetic lines of turkey hens and two of these lines of hens differ in growth rate, egg production and fertility (Nestor and Noble, 1995; Christensen *et al.* 2005). Since ZPB1, ZPC, and ZPD have been reported to be important in sperm binding and subsequent fertility, birds that express reduced levels of these proteins may have lower fertility rates. Therefore, the goal of the present research was to determine if the genetic lines of turkey hens which differ in fertility also differ in their level of ZPB1, ZPC, or ZPD mRNA expression.

Materials and Methods

Animals

Two lines of turkey hens differing greatly in body weight and egg production were used in the present experiments. E line or egg line has been selected for 180 day egg production for over 53 generations and F line or growth line has been selected for 16 week body weight for over 44 generations. The E line originated from a randombred control established by crossing four popular large white turkey strains (McCartney *et al.* 1968). The F line was developed from a randombred control line that was started in 1966 from reciprocal crosses of two commercial strains of turkeys (McCartney *et al.* 1968; Nestor *et al*. 1969). Higher rates of egg production,

fertility and sperm penetration of the IPVL have been reported for the E line hens than for the F line hens (Nestor and Noble, 1995; Christensen *et al.* 2005).

E line and F line poults were hatched at the Ohio Agriculture Research and Development Center and immediately 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

Turkey hens (44-58 weeks of age) were killed by electrocution 2-4 hours prior to ovulation. In experiment 1, the four largest follicles (F_1-F_4) were removed from 6 E line and 6 F line hens for isolation of the granulosa cell layer. The granulosa cell layer was manually separated from the theca cell layers of each follicle (Huang and Nalbondov, 1979). In experiments 2, 3, and 4, 1 cm^2 sections of the granulosa layer surrounding the germinal disc (GD) area and a nongerminal disc (NGD) area, on the opposite side of the follicle to the GD area, of the F_1 and F_2 follicles were collected from six hens 2-4 hours prior to ovulation from each genetic line of turkey hens. In order to obtain enough RNA for subsequent Northern analysis, the granulosa samples from the GD and NGD regions were pooled from two birds for each follicle size. All isolated granulosa cell samples were frozen and stored at -80°C in 1 mL of guanidine isothiocyanate solution for subsequent RNA extraction.

In addition to the granulosa samples, liver samples were also collected from each of the turkey hens in experiments 2, 3, and 4. Approximately 300 mg of liver was removed from the left lobe of the liver from each hen and placed in 1 ml of RNA*later* (Ambion, Austin, TX). The liver samples were then kept at 4°C for subsequent RNA extraction.

Reverse Transcriptase Polymerase Chain Reaction

Primers for reverse transcriptase polymerase chain reaction (RT-PCR) were designed based on reported chicken cDNA sequences for ZPC (GenBank accession number D89097) and ZPD (GenBank accession number AB114441). The forward primer for ZPC was 5'-CACACAACACCGTCACCTTC – 3', while the sequence of the reverse primer was 5'-CCCAGGTGTTTCTGGCTTTA – 3'. These primers predicted a 675- base pair PCR product, which corresponds to bases 320-994 of the Genbank sequence. The forward ZPD primer was 5'-ACACGCTCTACGTCATGCTG – 3', while the sequence for the reverse ZPD primer was 5'-GATGGCTCTCCACTCTCAGG – 3'. These primers predicted a 407 base pair PCR product, which corresponds to bases 744-1151 of the Genbank sequence. Both the ZPC and ZPD primers were provided by the University of Georgia Molecular Genetics and Instrumentation Facility (MGIF). Reverse transcription was performed as previously described (Davis and Johnson, 1998) with total RNA extracted from the granulosa tissue of an F_1 follicle from a 35 week old broiler breeder hen. Polymerase chain reactions (PCR) were also conducted as previously described (Davis and Johnson, 1998) with an annealing temperatures of 58 °C. To produce a sufficient quantity of the cDNA for labeling for Northern analysis, the PCR products were cloned into pCR II vectors using the TA cloning kit (Invitrogen, San Diego, CA). The PCR products were also sequenced by MGIF to confirm that they matched the predicted chicken ZPC and ZPD sequences.

RNA Extraction and Northern Blot Analysis

Total RNA was extracted from the granulosa and liver samples using a guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). Twenty-five micrograms (μg) of total RNA for each liver sample and 7.5 μg of total RNA for each granulosa sample was electrophoresed on an agarose/formaldehyde gel and then transferred to a nylon membrane as previously described (Davis and Johnson, 1998). There were 3 blots (one each from experiments 2, 3, and 4) generated for the analysis of the expression of ZPB1 with each blot containing RNA isolated from six samples from each of the genetic lines. The expression of ZPC and ZPD in the four largest follicles from each genetic line was examined using 6 blots. Each blot contained the RNA isolated from the four largest follicles of one individual bird from each genetic line (experiment 1). Finally, the expression of ZPC and ZPD in the GD region versus the NGD region of the F_1 and F_2 follicles was examined by utilizing 3 blots for each follicle size (experiments 2, 3, and 4). Each blot for a given follicle size contained the RNA isolated from 3 GD and 3 NGD samples from each of the genetic lines.

A chicken ZPB1 cDNA clone [generously provided by Dr. Nina Bausek from the University and Biocenter of Vienna (Bausek *et al*. 2000)] and the RT-PCR generated chicken ZPC and ZPD cDNA clones were prepared and labeled with ^{32}P for Northern blot analysis as previously described (Davis and Johnson, 1998). The hybridization and densitometry procedures also followed those described previously (Davis and Johnson, 1998). To verify and correct for equality of RNA loading and transfer, a final hybridization of the blots was performed using the chicken glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene for the Northern blots used in the ZPB1 and ZPD analyses. Since the transcript size of GAPDH is similar in size to ZPC, mouse 18s DECA templates (Ambion, Austin, TX) for random priming were labeled with ^{32}P

and hybridized with the ZPC blots to correct for equality of RNA loading and transfer. After each of the hybridizations, the membranes were subjected to a stringent wash and exposed to x-ray film as described previously (Davis and Johnson, 1998). The ZPB1 films were exposed for 24 h, the ZPD films for 12 hours, the GAPDH films for 4-6 h, the ZPC films for 1 h, and the 18S films for 2-10 minutes. The relative mRNA expression of ZPB1, ZPC, or ZPD was determined for the samples of each blot by calculating the signal intensity for each sample relative to the strongest signal which was assigned a value of 1. Before calculation of relative ZPB1, ZPC, or ZPD mRNA levels, GAPDH or 18S mRNA expression was used to correct the ZPB1, ZPC, or ZPD values for equality of RNA loading and transfer for each blot.

In order to determine the kilobase (kb) size of turkey ZPB1, ZPD, and ZPC mRNA transcripts, 25 μg of total RNA from liver and 7.5 μg of total RNA from granulosa cells were electrophoresed adjacent to a 0.28-6.8 kb RNA ladder (Promega, Madison, WI) for subsequent Northern blot analysis. For comparison purposes, 7.5 μg of total RNA extracted from Japanese quail and broiler breeder hen F_1 granulosa cells were also included on the gel as was a 25 μg sample of total RNA extracted from the liver of a broiler breeder hen.

Statistics

Data from each experiment were subjected to ANOVA according to the General Linear Model (GLM) procedure. Tukey's multiple comparison procedure (Neter *et al.* 1990) was used to detect significant differences among genetic lines and granulosa cell layer location. Differences were considered significant when p values were < 0.05. All statistical procedures were completed with the Minitab statistical software package (Release 13, State College, PA).

Results

ZPB1 mRNA expression

In the Northern analysis of total RNA derived from turkey liver, the ZPB1 cDNA probe hybridized to a major band at approximately 3.5 kb (Figure 5.1); which was larger than the transcript of about 3.2 kb detected in chicken liver RNA (Figure 5.1). Hepatic ZPB1 mRNA expression was not significantly different between turkey hens from the two genetic lines in experiment 2; however, ZPB1 mRNA expression tended ($p = 0.07$) to be higher in the E line hens (Figure 5.2). In the two subsequent experiments, hepatic ZPB1 mRNA expression was significantly ($p < 0.05$) higher in the E line hens than in the F line hens (Figure 5.2).

ZPC and ZPD mRNA expression

The ZPC cDNA probe hybridized to a major band at approximately 1.8 kb in the Northern analysis of total RNA derived from F_1 granulosa cells from turkey, quail, and chicken (Figure 5.3). Similar to ZPC, no size difference was detected in the transcript size of ZPD mRNA extracted from turkey, quail, and chicken granulosa cells (Figure 5.3). The detected ZPD transcript in all species was about 1.5 kb.

The mean ± SEM (n = 6 replicate birds) relative density of ZPC mRNA for the F_1, F_2, F_3 and F_4 follicles in the E line hens was 0.706 ± 0.186, 0.749 ± 0.095, 0.676 ± 0.064 and 0.715 ± 0.115, respectively. For the F line hens the mean ± SEM (n = 6 replicate birds) relative density of ZPC mRNA for the F_1, F_2, F_3 and F_4 follicles was 0.865 ± 0.057, 0.971 ± 0.016, 0.762 ± 0.084 and 0.759 ± 0.011, respectively. The mRNA expression of ZPC did not differ between the four largest follicles within either the E line or F line hens. In addition, there was no differences in the expression of ZPC mRNA for the individual sized follicles between each genetic line of hens; however, the overall expression of ZPC mRNA was significantly greater for the four

largest follicles from the F line hens when compared to the overall expression of ZPC mRNA for the four largest follicles from the E line hens (Figure 5.4).

The mean ± SEM (n = 6 replicate birds) relative density of ZPD mRNA for the F_1, F_2, F_3 and F_4 follicles in the E line hens was 0.860 ± 0.072, 0.816 ± 0.123, 0.811 ± 0.065 and 0.886 ± 0.087, respectively. For the F line hens the mean ± SEM (n = 6 replicate birds) relative density of ZPD mRNA for the F_1, F_2, F_3 and F_4 follicles was 0.854 ± 0.092, 0.917 ± 0.040, 0.898 ± 0.086 and 0.885 ± 0.047, respectively. Similar to ZPC mRNA expression, the mRNA expression of ZPD did not differ between the four largest follicles within either the E line or F line hens. In addition, there was no difference in the expression of ZPD mRNA for the individual sized follicles between each genetic line of hens, and in contrast to ZPC mRNA expression, the overall expression of ZPD mRNA in the four largest follicles did not differ between the two genetic lines of turkey hens (Figure 5.4).

In the two largest follicles from the E line hens, the mRNA expression of ZPC was not significantly different between the GD and NGD granulosa cells (Figure 5.5). In contrast, the mRNA expression of ZPC was significantly greater in the NGD granulosa cells than GD granulosa cells from each of the two largest follicles in the F line hens (Figure 5.5).

The mRNA expression of ZPD did not differ between the GD and NGD granulosa cells from the F_1 or F_2 follicles for either genetic line of turkey hens. The mean ± SEM relative density of ZPD mRNA for the GD and NGD granulosa cells of the F_1 follicle were 0.619 ± 0.063 and 0.774 ± 0.072, respectively for E line and were 0.667 ± 0.073 and 0.593 ± 0.062, respectively for F line. For the F_2 follicle the mean ± SEM relative density values for the GD and NGD granulosa cells were 0.660 ± 0.081 and 0.642 ± 0.100, respectively for the E line and were 0.592 ± 0 .074 and 0.454 ± 0.047, respectively for the F line.

Discussion

The zona pellucida proteins which assemble the zona pellucida in mammals and the IPVL in avian species play an important role in fertilization. Over the last 10 years, studies have been conducted that analyze the different glycoproteins which form the avian IPVL; however, until now, there has been no research on the differential expression of any of these zona pellucida genes between genetic lines of birds that have different rates of fertility or between granulosa cell location within preovulatory follicles.

Higher numbers of sperm hole penetrations in the IPVL overlying the GD region of the oocyte and higher fertility rates have been reported for the E line turkey hens than for the F line hens (Christensen *et al*. 2005). The current results suggest that the lower rate of sperm penetration of the IPVL and the subsequent lower rate of fertility observed in eggs from the F line versus the E line of turkey hens may be related to the lower hepatic mRNA expression of ZPB1, and the lower expression of ZPC in the germinal disc region of preovulatory follicles from F line hens.

The large size of the hen's yolk-filled oocyte, in relation to the small germinal disc portion of the oocyte, may necessitate that sperm preferentially bind or be attracted to the GD region of the oocyte for successful fertilization. In fact, the fertility of eggs from both chickens and turkeys is related to the number of sperm hole penetrations in the IPVL overlying the GD region (Bramwell *et al.* 1995; Wishart, 1997), and in oocytes from both species, there are approximately 25 times more sperm holes per square mm in the IPVL overlying the GD region than from the NGD region (Wishart, 1997). Therefore, since far more sperm bind to the GD region in birds, it is

assumed that there is a higher concentration of sperm receptors associated with the IPVL overlying the GD region (Howarth, 1990; Bramwell and Howarth, 1992; Kuroki and Mori, 1997).

The lower expression of ZPC mRNA in the GD region granulosa cells of the two largest preovulatory follicles of the F line hens is noteworthy given that ZPC has been implicated in sperm binding in both chicken and quail (Pan *et al.* 1999; Bausek *et al.* 2004). Chicken ZPC is secreted toward the apical side of the granulosa cells (Waclawek *et al.* 1998), and thus it seems likely the ZPC content of the IPVL simply mirrors the production and secretion of ZPC by the granulosa cells directly underneath it. Western blot analyses need to be completed to determine if the F line hens have less ZPC in the IPVL overlying the GD region than in the NGD regions of preovulatory follicles. Finally, the higher expression of ZPC mRNA observed in the NGD granulosa cells probably accounts for the higher overall expression of ZPC in the four largest follicles of the F line hens when compared to the E line hens, since these follicles have a far greater population of NGD granulosa cells than GD granulosa cells.

The fact that ZPB1 mRNA expression was greater in E line hens was also interesting since it has been implicated in sperm binding in the chicken (Bausek *et al.* 2004; Takeuchi *et al.* 2001). The distribution of ZPB1 in the IPVL of the preovulatory follicles needs to be examined, especially since this protein is produced in the liver and transported to the developing follicles. Western blot analyses and immunohistochemistry studies could determine if there is preferential deposition of ZPB1 in the IPVL above the GD, and if more ZPB1 protein is present in the IPVL of preovulatory follicles from E line hens than F line hens.

Previous reports have indicated chicken and quail ZPC mRNA expression increased as the large preovulatory follicles matured (Waclawek *et al.* 1998; Takeuchi *et al*. 1999; Pan *et al.*

2001). In contrast, the present study did not find any difference in the amount of ZPC mRNA expressed in the four largest follicles within either E line or F line turkey hens, which indicates that, unlike quail and chicken ZPC, turkey ZPC mRNA expression is not developmentally regulated in the largest preovulatory follicles. The mRNA for the newest ZP glycoprotein, ZPD, was mentioned to be expressed by chicken granulosa cells during the final stage of follicular development (unpublished results from Okumura *et al*. 2004). In the current research, turkey ZPD mRNA was detected in the granulosa cells of developing follicles, and there was no difference in the expression of turkey ZPD mRNA between the four largest follicles.

The size of the mRNA transcript for ZPC detected by Northern analysis in chicken and quail granulosa RNA samples has differed in previous reports (Waclawek *et al.* 1998; Takeuchi *et al.* 1999; Pan *et al.* 2001). In the current research a 1.5 kb ZPC transcript was detected in an F1 granulosa sample obtained from a turkey, broiler breeder and quail hen. The size of this transcript is in close agreement with a previous report in quail (Pan *et al*. 2001) and one of the previous reports in chicken (Takeuchi *et al.* 1999).

Modern turkey and broiler breeder hens are selected commercially for growth rate and specific carcass characteristics. This selection for higher yielding meat-type birds has exacerbated fertility problems in breeder flocks (Brillard, 2004). The reproductive efficiency costs of these selection programs has been dramatic, especially for the commercial turkey industry, where all hens now have to be artificially inseminated since males are so large in size that natural mating is impossible (Donoghue, 1999; Foote 2002). The traditional approach to increase fertility has been to focus on the reproductive performance of the adult male birds, with little attention given to female birds (Donoghue, 1999). Various techniques have been developed to evaluate avian semen quality, and these methods have been employed to select the males most

suitable for breeding (Holsberger *et al.* 1998; Donoghue, 1999; Hammerstedt, 1999). The current results indicate that ZPB1 and ZPC mRNA expression have the potential to be used as genetic markers for selecting female lines of poultry hens for greater fertility.

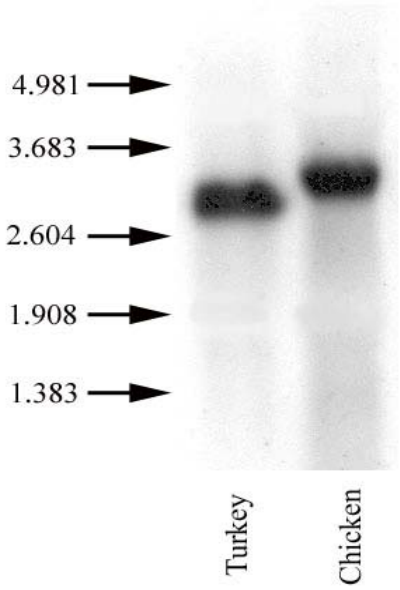

Figure 5.1. Autoradiogram from a Northern analysis of ZPB1 with total RNA (25 μg) from turkey and chicken liver. A 0.28 – 6.8 kb RNA ladder was run in the lane adjacent to the turkey liver sample, and the kilobase sizes of the RNA bands of the ladder are shown with arrows.

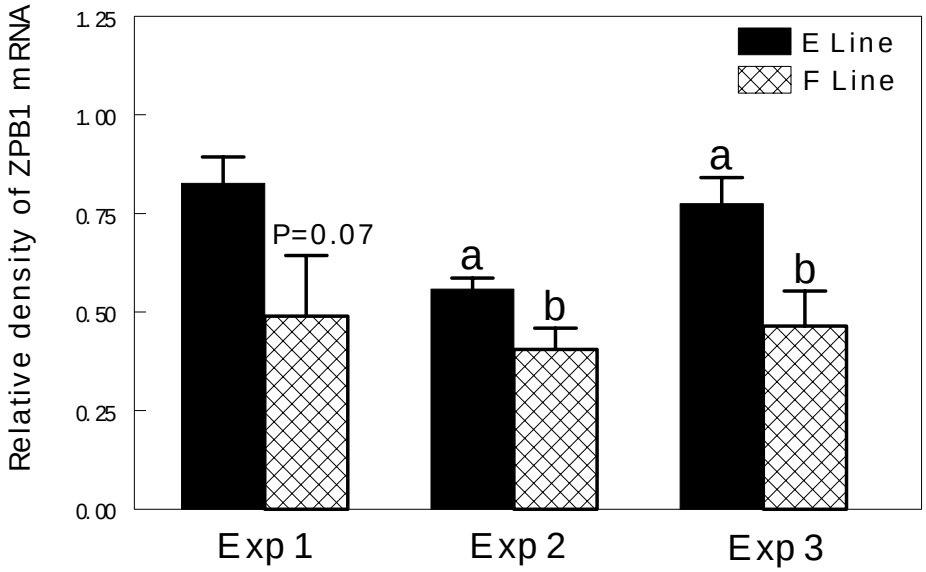

Figure 5.2. The relative density of hepatic ZPB1 mRNA of turkey hens from two different genetic lines for three separate experiments. Values are means ± SEM, n = 6 replicate birds. Means for each experiment with different letters differ, $p < 0.05$.

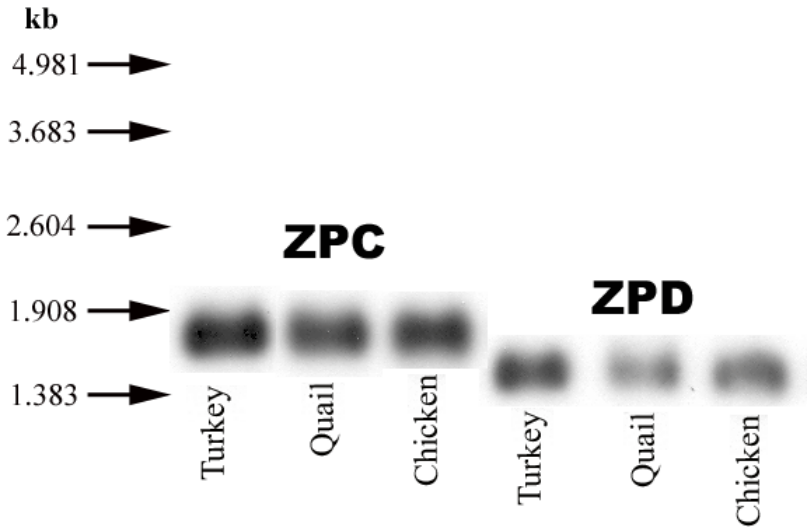

Figure 5.3. Autoradiogram from the Northern analysis of ZPC and ZPD with total RNA (7.5 μg) from the granulosa cells of an F_1 follicle from a turkey, quail and chicken hen. A 0.28 – 6.8 kb RNA ladder was run in the lane adjacent to the turkey hen sample and the kilobase sizes of the RNA bands of the ladder are shown with arrows.

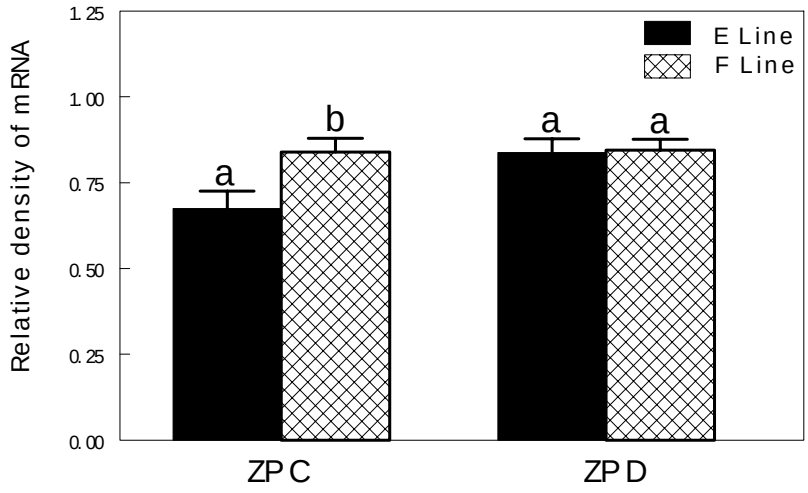

Figure 5.4. The overall relative density of ZPC and ZPD mRNA of the four largest preovulatory follicles from two different genetic lines of turkey hens. Values are mean ± SEM, n = 6 replicate birds with the value for each bird consisting of the mean expression of ZPC across the F_1, F_2, F_3, and F_4 follicles. Means for each ZP protein with different letters differ, $p < 0.05$.

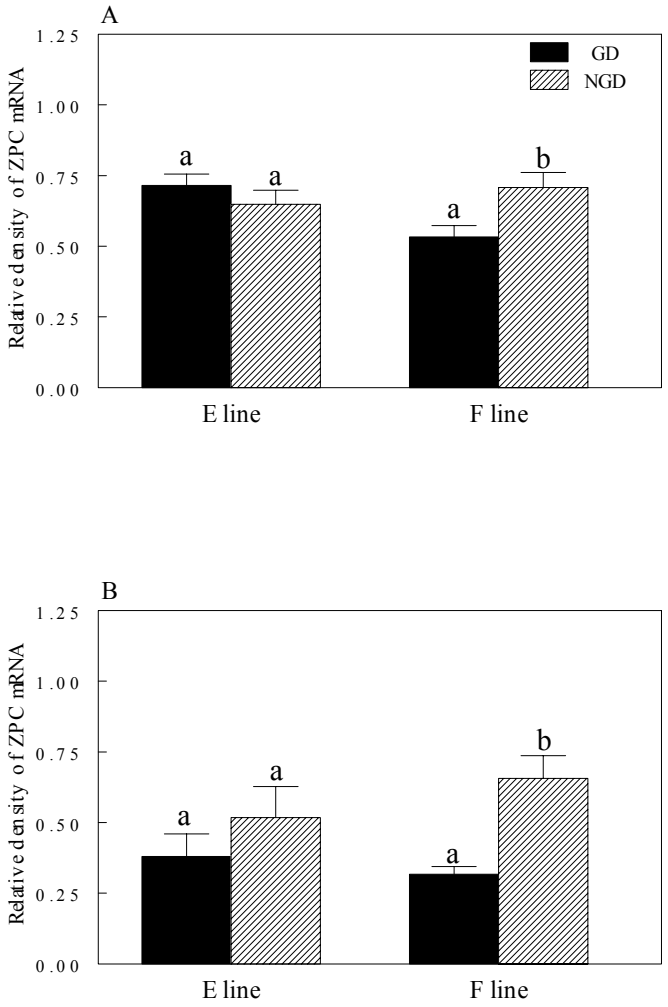

Figure 5.5. The relative density of ZPC mRNA of germinal disc (GD) and nongerminal disc (NGD) granulosa cells from the F_1 follicle **(A)** and F2 follicle **(B)** from two different genetic lines of turkey hens that differ in fertility. Values are means ± SEM, n = 9 replicate samples each consisting of granulosa cells from two hens. Means for granulosa cell location for each genetic line with different letters differ, $p < 0.05$

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CHAPTER 6

EXPRESSION OF THE MRNA FOR ZONA PELLUCIDA PROTEINS A, B2 AND X IN TWO GENETIC LINES OF TURKEY HENS THAT DIFFER IN FERTILITY[1]

[1]Benson, A. P., V. L. Christensen, B. D. Fairchild and A. J. Davis
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Abstract

The freshly ovulated ovum in avian species is surrounded by a protein layer called the inner perivitelline layer (IPVL), which is equivalent to the zona pellucida in mammals. For successful fertilization, sperm must attach and penetrate the IPVL. The IPVL contains the zona pellucida family of proteins and members of this family have been reported to possess sperm binding activity and to stimulate sperm penetration of the zona pellucida. In the domestic chicken six distinct zona pellucida genes have been identified (ZPA, ZPB1, ZPB2, ZPC, ZPD and ZPX). Previously our laboratory characterized the mRNA expression of ZPB1, ZPC, and ZPD by Northern blot analysis in two lines of turkey hens selected for over 40 generations for either increased egg production (E) or increased body weight (F). In the present research, the expression of the mRNA for ZPA, ZPB2, and ZPX was investigated in these two lines of turkey hens.

Individual granulosa layers of three 48-wk old hens were isolated from the F_1 to F_4 follicles in E line and from the F_1 to F_{10} follicles in F line. In addition, a 1 cm^2 section of the granulosa layer around the germinal disc (GD) and a nongerminal disc (NGD) area of the F_1 and F_2 follicles was isolated from 12 hens from each line. Total RNA was extracted from the samples and DNase treated for two step real-time PCR analyses of ZPA, ZPB2, and ZPX. Taqman minor groove-binding probes and primers for detecting ZPA, ZPB2, ZPX, and GAPDH (endogenous control) were designed based on the nucleotide sequences of PCR – generated turkey cDNA clones for these three ZP proteins. Expression of ZPA, ZPB2, and ZPX was detected in all follicle sizes from both genetic lines. No significant differences in ZPA and ZPX mRNA expression were detected between the GD and NGD granulosa cells. However, the expression of the mRNA for ZPB2 was significantly greater in the GD granulosa cells when compared to the NGD granulosa

cells in F_1 and F_2 follicles from both E line and F line hens. The results suggest that higher expression of ZPB2 in the germinal disc area may be important for the preferential binding of sperm to this region of the IPVL.

Key Words**:** zona pellucida proteins, turkeys, fertility, inner perivitelline layer

Introduction

The ovary of a mature hen consists of a visually evident hierarchy of follicles arranged according to size and time to ovulation. The large yolk filled follicles are named both according to size and time to ovulation (F_1-F_n) with the largest follicle designated the F_1 follicle ovulating within 24 hours and the second largest follicle designated the F_2 follicle ovulating 24-26 hours following the ovulation of the F_1, and so on. In each follicle, the developing oocyte is first surrounded by the oolema, followed in order by a glycoprotein coat termed the inner perivitelline layer (IPVL), granulosa cell layer, basement membrane and the theca cell layers. Visually apparent on the surface of the yolk of each one of the large preovulatory follicles is a white colored, disc shaped spot referred to as the germinal disc which houses the female pronucleus and organelles (Perry *et al*. 1978).

At the time of ovulation, the theca and granulosa cell layers are left behind on the ovary as a post-ovulatory follicle. Therefore, the freshly ovulated yolk – filled oocyte is surrounded by only its oolema and IPVL when it is captured by the infundibulum of the oviduct. Spermatozoa in the infundibulum bind with the IPVL and undergo the acrosome reaction which facilitates their penetration through the IPVL for subsequent contact with the oolema (Okamura and Nishiyama, 1978).

Based on immunohistochemistry and Western blotting techniques, three ZP proteins, ZPB1, ZPC, and ZPD, have been identified as glycoprotein components of the chicken IPVL (Waclawek *et al.* 1998; Takeuchi *et al.* 1999; Bausek *et al.* 2000; Okumura *et al.* 2004). In the chicken and quail, ZPB1 is synthesized by the liver and then subsequently transported to the developing preovulatory follicles (Bausek *et al.* 2000; Sasanami *et al.* 2003). In contrast, ZPC and ZPD are both synthesized by the granulosa cells of the large preovulatory follicles

(Waclawek *et al.* 1998; Takeuchi *et al.* 1999; Okumura *et al.* 2004). Previously our laboratory reported that the mRNA for both turkey ZPC and ZPD are expressed by the granulosa cells of the developing follicles of the ovary and that turkey ZPB1 mRNA is expressed in the liver (Benson *et al.* 2004; Benson *et al.* 2005).

In addition to ZPB1, ZPC, and ZPD, three other ZP proteins (ZPA, ZPB2, and ZPX) have been identified in the chicken. Smith *et al.* (2005) identified and subsequently mapped ZPA and ZPX in the chicken genome, but did not examine the expression of these two ZP proteins. Tsuda and Matsuda deposited a cDNA sequence for chicken ZPB in GenBank in 1999 (Accession # AB025428). Based on this sequence Bausek *et al.* (2000) made a cDNA probe of ZPB for Northern analysis and they detected expression for ZPB in only the small stroma embedded follicles of the hen ovary. Subsequently, chicken ZPB was renamed ZPB2 by Spargo and Hope (2003) in their new ZP protein naming classification system.

The Ohio Agriculture Research and Development Center maintains several genetic lines of turkey hens and two of these lines (E and F line) of hens significantly differ in both growth rate and fertility (Nestor and Noble, 1995; Christensen *et al*. 2005). Previously, our laboratory reported that hepatic ZPB1 mRNA expression was lower in the genetic line of turkey hens that exhibited lower fertility (Benson *et al*. 2004). Furthermore, the mRNA expression of ZPC in the granulosa cells surrounding the GD was lower than in granulosa cells not associated with the GD in the low fertility line, but this was not observed in the high fertility line of turkey hens (Benson *et al.* 2004). These research findings were significant given the fact that both ZPB1 and ZPC have been implicated as sperm binding proteins in avian species (Pan *et al*. 1999; Takeuchi *et al.* 2001; Bausek *et al.* 2004), and given the fact that several studies across many species of birds indicate that the formation of sperm holes in the IPVL is preferentially formed over the GD

region of the oocyte (Howarth and Digby, 1973; Bakst and Howarth, 1977; Bramwell and Howarth, 1992; Birkhead *et al.* 1994; Kuroki and Mori, 1997; Wishart, 1997). Therefore, the goals of the present research were to determine if granulosa cells express the mRNA for ZPA, ZPB2 and ZPX and to determine if there is differential expression of these three ZP proteins between GD associated granulosa cells and those not associated with the GD.

Materials and Methods

Animals

Two lines of turkey hens differing greatly in body weight and egg production were used in the present experiments. E line or egg line has been selected for 180 day egg production for over 53 generations and F line or growth line has been selected for 16 week body weight for over 44 generations. The E line originated from a randombred control established by crossing four popular large white turkey strains (McCartney *et al.* 1968). The F line was developed from a randombred control line that was started in 1966 from reciprocal crosses of two commercial strains of turkeys (McCartney *et al.* 1968; Nestor *et al*. 1969). Higher rates of egg production, fertility and sperm penetration of the IPVL have been reported for the E line hens than for the F line hens (Nestor and Noble, 1995; Christensen *et al.* 2005).

E line and F line poults were hatched at the Ohio Agriculture Research and Development Center and immediately 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

Turkey hens (44-58 weeks of age) were killed by electrocution 2-4 hours prior to ovulation. In experiment 1, the four largest follicles (F_1-F_4) were collected from 3 E line hens; however, since F line turkey hens have more ova in rapid development than the E line hens (Nestor *et al.* 1970), the ten largest follicles (F_1-F_{10}) were collected from 3 F line hens. The granulosa cell layer was manually separated from the theca cell layers of each follicle (Huang and Nalbondov, 1979). In experiment 2, 1 cm^2 sections of the granulosa cell layer surrounding the germinal disc (GD) area and a nongerminal disc (NGD) area, on the opposite side of the follicle to the GD area, of the F_1 and F_2 follicles were collected from twelve hens from each genetic line 2-4 hours prior to ovulation. In order to obtain enough RNA for subsequent Northern analyses, the granulosa samples from the GD and NGD regions were pooled from two birds for each follicle size. All isolated granulosa cell samples were frozen and stored at -80°C in 1 mL of guanidine isothiocyanate solution for subsequent RNA extraction.

Reverse Transcriptase Polymerase Chain Reaction

Primers for reverse transcriptase polymerase chain reaction were designed based on reported chicken cDNA sequences for ZPA (GenBank accession # AB197938), ZPB2 (GenBank accession # NM_2048791), and ZPX (GenBank accession # XM_419964). The forward primer for ZPA was 5'-GAGGAGACTGTGGCAATGGT – 3', while the sequence of the reverse primer was 5'-GAAGATGGGCTGCTGTAGGT – 3'. These primers predicted a 532- base pair PCR product, which corresponds to bases 1397-1929 of the Genbank sequence. The forward ZPB2

primer was 5'-TTGGAGCTGTGTTCTTCTTGG – 3', while the sequence for the reverse ZPB2 primer was 5'-GGTTGTAACAACAGCCTCGC – 3'. These primers predicted a 510 base pair PCR product, which corresponds to bases 35-545 of the Genbank sequence. The forward primer for ZPX was 5'-TACAGCAGCAGGAACAGCAT-3', while the sequence for the reverse ZPX primer was 5'-GCCTTGACCAGTTGAATGTG-3'. These primers predicted a 461 base pair PCR product, which corresponds to bases 283-744 of the Genbank sequence. Each of the above primer sets were provided by the University of Georgia Molecular Genetics and Instrumentation Facility (MGIF). Reverse transcription was performed as previously described (Davis and Johnson, 1998) with total RNA extracted from the granulosa tissue of an F_1 follicle from a 44 week old F line turkey hen. Polymerase chain reactions (PCR) were also conducted as previously described (Davis and Johnson, 1998) with annealing termperatures of 50 °C for ZPB2 and 52 °C for both ZPA and ZPX. To produce a sufficient quantity of the cDNA for labeling for Northern analysis, the reverse transcriptase PCR products were cloned into pCR II vectors using the TA cloning kit (Invitrogen, San Diego, CA). Sequence analysis of the PCR generated cDNA clones for ZPA, ZPB2, and ZPX were preformed by the University of Georgia MGIF.

RNA Extraction and Two-Step Real-Time Polymerase Chain Reaction

Total RNA was extracted from the granulosa and liver samples using a guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). Northern analyses indicated that ZPA, ZPB2 and ZPX mRNA could not be detected in granulosa samples by this method. Therefore, to prepare for subsequent real time PCR, potential genomic DNA contamination was removed from each sample using the TURBO-DNA-*free* kit (Ambion, Austin, TX) according to the manufacturer's protocol.

Primers and TaqMan probes specific for turkey ZPA, ZPB2, ZPX, and GAPDH were generated using Primer Express software version 2.0 (Applied Biosystems, Foster City, CA). The ZPA, ZPB2 and ZPX assays (Table 6.1) were designed for use in both chickens and turkeys based on the generated nucleotide sequences in turkey and the submitted Genbank chicken nucleotide sequences for these genes. The GAPDH primer/probe set (Table 6.1) was specific for turkey and designed based on a turkey GAPDH cDNA sequence (GenBank accession # U94327). All assays except ZPX were designed based on the chicken genome to span an exon / exon junction. Each probe was labeled at the 5' end with FAM (6-carboxyfluorescein) as the reporter dye and at the 3' end with TAMRA (6-carboxy-*N,N,N',N'*-tetramethylrhodamine) as the quencher dye. Primer and probe sets were validated for real-time PCR by determining the optimal amplification efficiency and primer/probe concentrations as described by the manufacturer (Applied Biosystems).

Reverse-transcription cDNA synthesis reactions were performed using the TaqMan Reverse Transcription kit (Applied Biosystems) following the manufacturer's protocol. For two-step real-time PCR, 150 ng of cDNA was used for each sample. The reactions were preformed in a 25 µl volume of reaction buffer containing1x TaqMan Universal PCR Master Mix (Applied Biosystems) and 900 nM of either ZPA, ZPB2, ZPX or GAPDH primer pairs and 25 nM of the appropriate probe. The reactions were completed in an ABI 7500 Thermocycler (Applied Biosystems). The thermocycler conditions were 10 minutes at 95 °C and 40 cycles each of 15 seconds at 95 °C and 1 minute at 60 °C. The C_T (the cycle number at which the fluorescence exceeds the threshold level) was determined for each reaction (run in duplicate) using the Sequence Detection software (version 1.2.2, Applied Biosystems), and quantification was completed using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). Briefly, the ZPA, ZPX and

ZPB2 C_Ts were determined for each sample, then normalized to the GAPDH C_T from the same sample (GAPDH C_T subtracted from the ZP protein C_T yields the ΔC_T). These values were then compared to control levels using the $2^{-\Delta\Delta C_T}$ method and data was expressed as the fold-difference relative to sample with the highest expression.

Statistics

Data from each experiment were subjected to ANOVA according to the General Linear Model (GLM). Tukey's multiple comparison procedure (Neter *et al.* 1990) was used to detect significant differences among genetic lines, follicle size and granulosa cell layer location. Differences were considered significant when p values were < 0.05. All statistical procedures were completed with the Minitab statistical software package (Release 13, State College, PA).

Results

Cloning Turkey ZPA, ZPB2, and ZPX

The RT-PCR reactions with the primers based upon the submitted cDNA sequences of the chicken ZP proteins yielded a 532, 510 and 461 bp product for turkey ZPA, ZPB2 and ZPX, respectively. The homology of the turkey nucleotide sequences with the corresponding chicken ZP sequences was 99% for both ZPA and ZPX and 94% for ZPB2.

ZPA, ZPB2 and ZPX Expression in F line and E line Hens

Although ZPA, ZPB2 and ZPX mRNA could not be detected by Northern analyses in granulosa samples from both genetic lines of turkey hens (data not shown), the mRNA for all three ZP proteins was detected using real-time PCR. There were no significant differences in the mRNA expression of ZPA or ZPX between the ten largest follicles in F line hens (Figure 6.1) or in the four largest follicles of E line hens (Figure 6.2). In contrast, ZPB2 mRNA was significantly greater ($p<0.05$) in the two smallest follicles (F_9-F_{10}) versus the six largest follicles

(F_1-F_6) of F line hens (Figure 6.3). No significant differences in ZPB2 expression were detected in the follicles of the condensed hierarchy of the E line hens (Figure 6.3).

ZPA, ZPB2, and ZPX Expression in GD and NGD Granulosa Cells

ZPA and ZPX mRNA expression did not significantly differ between F_1 GD granulosa cells and NGD granulosa cells in either the E line or F line. The mean ± SEM relative expression of ZPA mRNA for the GD and NGD granulosa cells of the F_1 follicle was 0.630 ± 0.129 and 0.508 ± 0.113, respectively for E line, and was 0.648 ± 0.152 and 0.440 ± 0.142, respectively for F line. The mean ± SEM relative expression of ZPX mRNA for the GD and NGD granulosa cells of the F_1 follicle was 0.502 ± 0.122 and 0.723 ± 0.097, respectively for E line, and was 0.697 ± 0.168 and 0.518 ± 0.149, respectively for F line. Conversely, ZPB2 mRNA expression in both genetic lines was significantly greater ($p<0.05$) in the GD region granulosa cells than in the NGD region granulosa cells in the F_1 follicle (Figure 6.4), and this difference was also detected in the F_2 follicle (Figure 6.4).

Discussion

The turkey is the first known species to express the mRNA for all six known ZP gene subfamilies. Unlike turkey ZPB1, ZPC and ZPD mRNA expression (Benson *et al*. 2004; Benson *et al.* 2005), the mRNA expression of turkey ZPA, ZPB2 and ZPX could not be detected by Northern blot analysis in the current research. Therefore, real-time PCR was utilized to detect the lower mRNA expression levels of ZPA, ZPB2 and ZPX in granulosa cells from the largest hierarchical follicles.

Of the five ZP proteins expressed by the granulosa cells of turkey hierarchical follicles, only the expression of ZPB2 is significantly higher in the GD granulosa cells than in NGD granulosa cells. The higher 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. Sperm binding and penetration of the avian IPVL preferentially occurs at the germinal disc region (Howarth and Digby, 1973; Ho and Meizel, 1975; Bramwell and Howarth, 1992; Birkhead *et al.* 1994; and Wishart, 1997). Furthermore, Waclawek *et al.* (1998) reported that the transport of another granulosa derived ZP protein, ZPC, is secreted towards the apical side of granulosa cells which suggests that the composition of the IPVL directly above the granulosa cells may simply reflect what is secreted by the granulosa cells below it. Therefore, it would be interesting to determine if ZPB2 protein expression is more prevalent in the IPVL overlying the GD region by Western blot analysis or immunohistochemistry.

In contrast to the reported increase of chicken and quail ZPC mRNA expression during follicular maturation (Waclawek *et al.* 1998; Takeuchi *et al*. 1999; Pan *et al.* 2001), turkey ZPB2 mRNA expression decreased with increasing follicular maturation when examined in the extended hierarchy of the F line hens. Bausek *et al.* (2000) could not detect ZPB2 mRNA expression in large preovulatory follicles, but were able to detect ZPB2 mRNA expression in very small stroma embedded follicles in the chicken by Northern analysis. Thus it appears that ZPB2 protein expression might occur and play a role in early follicular development in 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 ZP proteins play a role in early follicular maturation and in maintaining viability.

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, however, 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). If ZPB2 is determined to be responsible for the preferential binding of sperm to the GD region then ZPB2 expression has the potential to be used as a genetic marker for selecting female lines of turkey hens for greater fertility.

Table 6.1. Oligonucleotide primer pairs and probes for real time PCR

Product	Primer	Oligonucleotide Primer and Probe Sequence	Product Size (Base Pairs)
ZPA	Forward	5'-CAT CGC CAT TGC TGA AGG A-3'	
	Reverse	5'-GCA CAG GAT CTC AAG GGA CAG T-3'	66
	Probe	5'-AGC ACT GTT AGC CTG AAC-3'	
ZPB2	Forward	5'-TGT GCT GAC TGC TTG GGA TAC T-3'	
	Reverse	5'-AGA GAC CAC AGC CAG AAT CAT TCT-3'	68
	Probe	5'-AGA GAC CAC AGC CAG AAT CAT TCT-3'	
ZPX	Forward	5'-TGG AGG GCT GGC TAT GGA-3'	
	Reverse	5'-GGC ACT CGC AGC AGA ATT CT-3'	56
	Probe	5'-TCA ACA GCA CAG AAA C-3'	
GAPDH	Forward	5'-CCT AGG ATA CAC AGA GGA CCA GGT T-3'	
	Reverse	5'-AGG TGG AGG AAT GGC TGT CA-3'	66
	Probe	5'-CTC CTG TGA CTT CAA TG-3'	

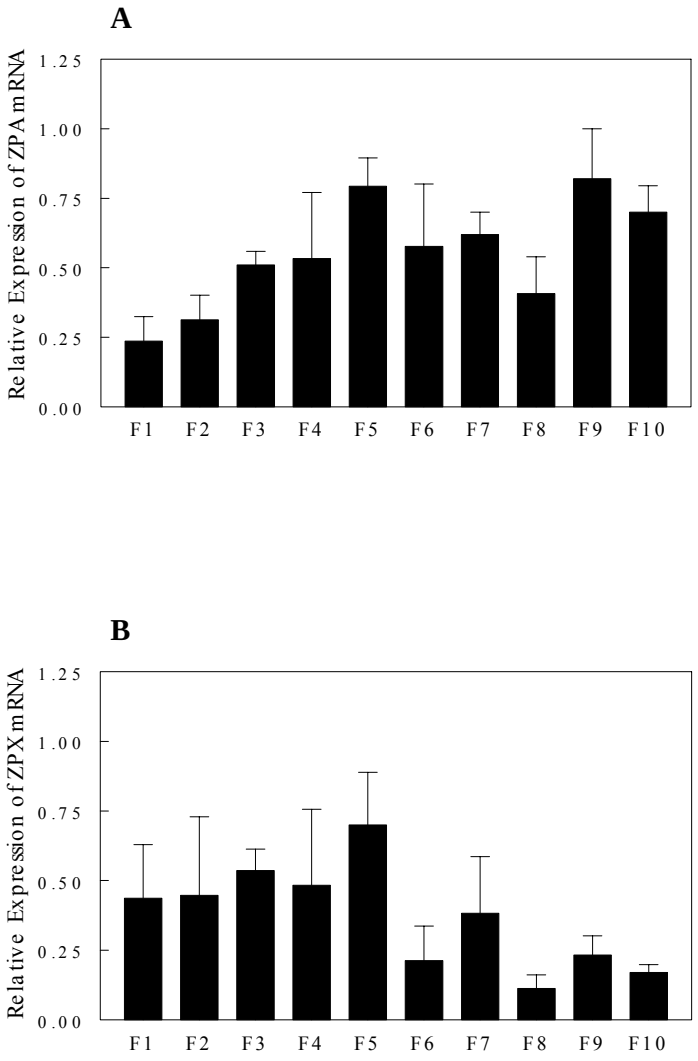

Figure 6.1. Relative expression of ZPA mRNA **(A)** and ZPX mRNA **(B)** as determined by real time PCR using total RNA isolated from the granulosa cells of the ten largest follicles from F line turkey hens. Data were normalized to GAPDH and expressed as the mean fold difference $\Delta\Delta C_T \pm$ SEM, n = 3. No statistical differences were detected for the relative expression of ZPA or ZPX between the different follicle sizes.

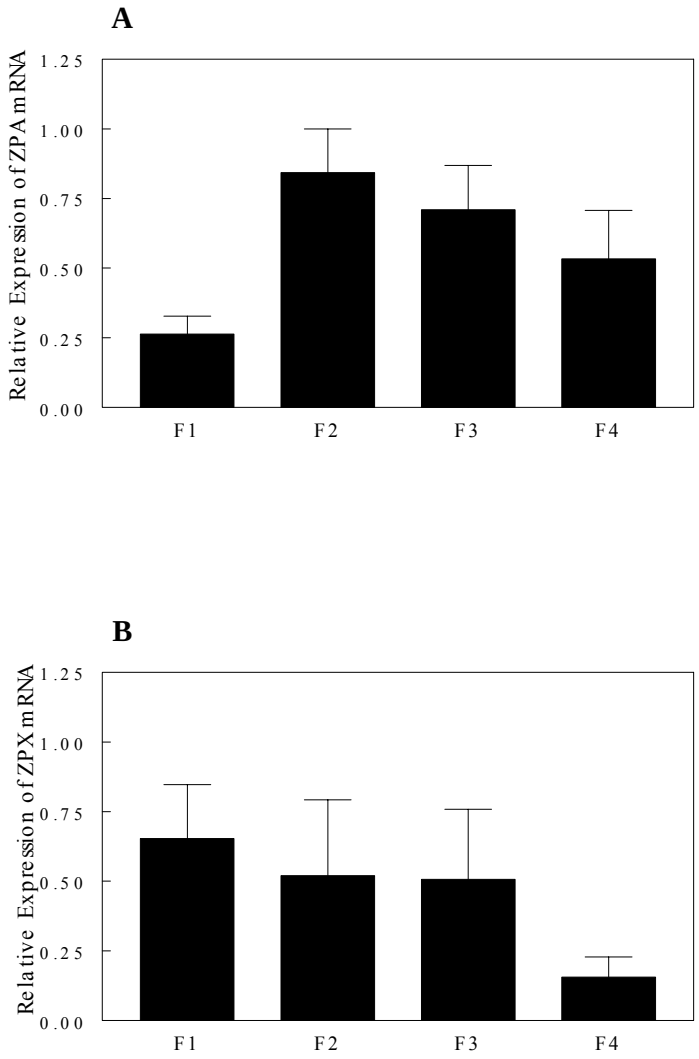

Figure 6.2. Relative expression of ZPA mRNA **(A)** and ZPX mRNA **(B)** as determined by real time PCR using total RNA isolated from the granulosa cells of the four largest follicles from E line turkey hens. Data were normalized to GAPDH and expressed as the mean fold difference $\Delta\Delta C_T \pm$ SEM, n = 3. No statistical differences were detected for the relative expression of ZPA or ZPX between the different follicle sizes.

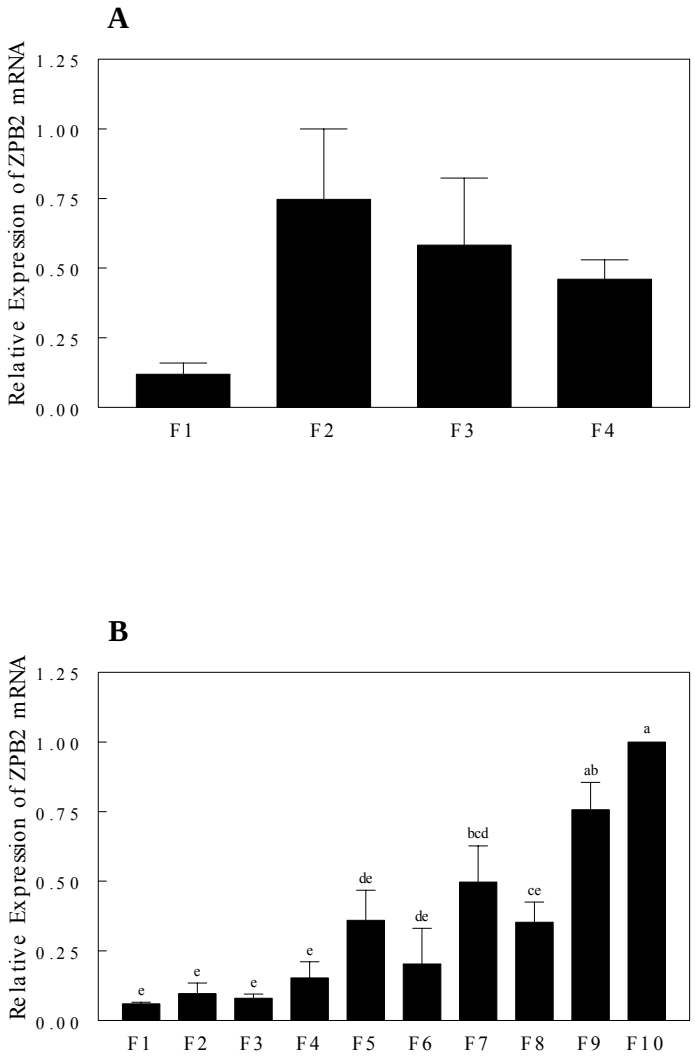

Figure 6.3. Relative expression of ZPB2 mRNA as determined by real time PCR using total RNA isolated from the granulosa cells of the four largest follicles in E line turkey hens **(A)** and the ten largest follicles from F line turkey hens **(B)**. Data were normalized to GAPDH and expressed as the mean fold difference $\Delta\Delta C_T \pm$ SEM, n = 3. Means for follicle sizes with different letters differ, $p < 0.05$.

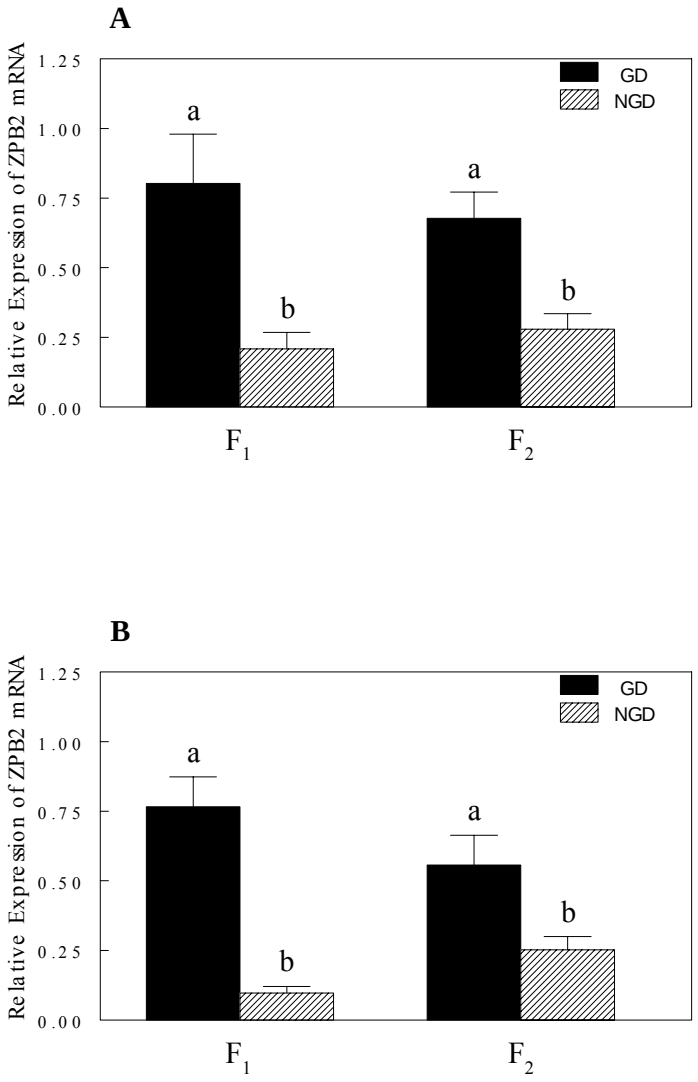

Figure 6.4. The relative expression of ZPB2 mRNA of germinal disc (GD) and nongerminal disc (NGD) granulosa cells from the F_1 and F2 follicles from E line hens **(A)** and from F line hens **(B)**. Data for each follicle size were normalized to GAPDH and expressed as the mean fold difference $\Delta\Delta C_T \pm$ SEM, n = 6. Means for granulosa cell location within a follicle size with a different letter differ, $p < 0.05$

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CHAPTER 7

GONADOTROPIN AND STEROID HORMONE REGULATION OF ZONA PELLUCIDA PROTEINS C AND D MESSENGER RNA IN AVIAN GRANULOSA CELLS[1]

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[1]Benson, A. P., M. E. Freeman, J. B. Hoffman and A. J. Davis
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Abstract

The freshly ovulated ovum in avian species is surrounded by a protein layer called the inner perivitelline layer (IPVL), which is equivalent to the zona pellucida in mammals. For successful fertilization, sperm must attach and penetrate the IPVL. Previous studies have established that two of the IPVL components, ZPC and ZPD, are synthesized by the granulosa cells of the hierarchical follicles of the chicken ovary. In the current study, gonadotropin (LH and FSH) and steroid hormone (estrogen and testosterone) regulation of both ZPC and ZPD mRNA expression was investigated in cultured chicken granulosa cells, which were isolated from the F_1, F_3, or small yellow follicles (SYF) from three broiler breeder hens for each replicate experiment. Isolated and dispersed granulosa cells from each follicular size were cultured in the absence or presence of 50 ng/ml of culture media of LH or FSH (4 replicate experiments), or in the presence or absence of 1×10^{-6} M testosterone or 17 β-estradiol (4 replicate experiments). For all experiments, cultures were terminated at 24 hours followed by RNA extraction and subsequent Northern blot analysis of ZPC and ZPD. Neither ZPC nor ZPD mRNA were detected in freshly isolated granulosa cells from (SYF), however, both ZPC and ZPD mRNA expression was detected in untreated SYF granulosa cells cultured for 24 hours. Furthermore, ZPC and ZPD mRNA expression was further up-regulated in SYF granulosa cells cultured in the presence of LH, FSH, or testosterone. F_3 granulosa cells cultured with FSH have higher ZPC and ZPD mRNA expression levels than untreated control cells. The mRNA expression of ZPD is higher in F_3 granulosa cells cultured with testosterone and estrogen. The addition of LH to F1 granulosa cells lowered the mRNA expression of ZPC but not ZPD. These results indicate that

gonadotropins and steroid hormones may play vital roles in regulating the expression of the mRNA for ZPC and ZPD in the granulosa cells of developing preovulatory follicles in the hen.

Key Words: zona pellucida proteins, inner perivitelline layer, gonadotropins, granulosa, chicken, steroid hormones

Introduction

The ovary of a mature hen consists of a visually evident hierarchy of follicles arranged according to size and time to ovulation. In the feed-restricted broiler breeder hen there is commonly four to six large yellow yolk – filled follicles termed hierarchical follicles that range from 12 to 40 mm in diameter. These hierarchical follicles are named according to size with the largest follicle designated as the F_1 follicle and the second largest one designated as the F_2 follicle and so on for the remaining smaller hierarchical follicles. The development of these hierarchical follicles is closely regulated with a period of 24 to 26 hours between each successive ovulation. The hierarchical follicles are accompanied by, and develop from, a pool of several 5 to 12 mm follicles, termed the small yellow follicles (SYF), in which yellow yolk deposition has begun and a large number of white follicles that are less than 5 mm in diameter.

When the F_1 follicle is ovulated the yolk-filled oocyte is surrounded by the inner perivitelline layer (IPVL). The IPVL is a glycoprotein coat that surrounds the oolema and thus is the interface between the ovum and the environment of the oviduct where fertilization occurs in avian species (Bellairs *et al*. 1963). In birds, spermatozoa in the infundibulum region of the oviduct bind to the IPVL and undergo the acrosome reaction which permits sperm penetration of the IPVL (Okamura and Nishiyama, 1978).

The glycoprotein components of the IPVL are called zona pellucida proteins or ZP proteins since the IPVL is the avian equivalent of the mammalian zona pellucida. The mRNA for six ZP proteins (ZPA, ZPB1, ZPB2, ZPC, ZPD and ZPX) has been detected in avian species (Waclawek *et al*. 1998; Takeuchi *et al*. 1999; Bausek *et al*. 2000; Sasanami *et al.* 2003a; Sasanami *et al*. 2003b; Okumura *et al*. 2004; Benson *et al*. 2006a, 2006b). Based on immunohistochemistry and Western blotting techniques, ZPB1, ZPC, and ZPD, are known to exist as glycoprotein

components of the chicken IPVL (Waclawek *et al.* 1998; Bausek *et al.* 2000; Okumura *et al*. 2004). In the chicken and quail, ZPB1 is synthesized by the liver and then subsequently transported to the developing preovulatory follicles (Bausek *et al.* 2000; Sasanami *et al.* 2003a). In contrast, ZPC and ZPD are both synthesized by the avian granulosa cells of the large preovulatory follicles (Waclawek *et al.* 1998; Takeuchi *et al.* 1999; Pan *et al*. 2001; Okumura *et al.* 2004).

The mechanisms regulating ZP gene expression are largely unknown for many of the species whose ZP genes have been cloned. The promoter region of the ZP genes in mice all have a binding site for the transcription factor zona activating protein (ZAP-1) that has been implicated in the positive regulation of ZP gene expression (Millar *et al*. 1993). It has also been suggested that in the mouse all of the known ZP genes (ZPA, ZPB1, and ZPC) are developmentally regulated in coordinate, since mouse ZPA, ZPB1, and ZPC mRNA expression increases together during the early stages of oogenesis and subsequently decline together in later stages of oogensis (Epifano *et al*. 1995). Like the mouse ZP genes, previous reports in quail and chicken have suggested that there is developmental regulation of the expression of the mRNA for ZPC, since follicular expression of ZPC significantly increases with follicular maturity (Waclawek *et al.* 1998; Takeuchi *et al*. 1999; Pan *et al.* 2001).

Injecting fish with estradiol was found to increase the hepatic expression of the ZP proteins in female fish as well as young male fish (Hyllner et al. 1991; Larsson *et al*. 1994; Murata *et al.* 1994). Due to estrogens profound positive impact on hepatic ZP expression in some fish species, ZP protein expression can be used as an indicator of environmental contamination of estrogen-like substances (Oppen-Berntsen *et al.* 1999). In chickens, estrogen also stimulates hepatic

production of ZPB1 (Bausek *et al*. 2000). In addition, FSH and testosterone both stimulate ZPC production in cultured quail granulosa cells (Sasanami *et al.* 2003b; Pan *et al.* 2001).

Although chicken ZPC and ZPD have both been suggested to play critical roles in sperm binding to the IPVL and in their subsequent penetration of the IPVL (Pan *et al.* 1999; Bausek *et al*. 2004; Okumura *et al*. 2004), the hormonal regulation of the synthesis of both of these granulosa derived proteins during follicular maturation is not well characterized. Therefore, the gonadotropin (LH and FSH) and steroid (estrogen and testosterone) hormone regulation of chicken ZPC and ZPD mRNA expression was investigated in cultured granulosa cells isolated from broiler breeder hen preovulatory follicles in different stages of follicular maturation.

Materials and Methods

Animals

Individually caged Cobb slow-feathering broiler breeder hens were used in the present experiments. The hens were maintained on 15 hours of light, were fed according to broiler breeder guidelines and had free access to water. For experiments 1, 2, and 3 the hens were killed by cervical dislocation 2-4 hours prior to ovulation at 58-62, 50-55, and 58-62 weeks of age, respectively. The Instructional Animal Care and Use Committee of the University of Georgia approved all animal procedures.

Experiment 1

The four largest follicles (F_1-F_4), small yellow follicles (SYF) (>5-10mm diameter), and large white follicles (LWF) (>2-5 mm diameter) were removed from six individual birds and placed in ice-cold Krebs solution. The granulosa cell layer was manually separated from the theca cell layers of each hierarchical follicle (Huang and Nalbondov, 1979), and the theca and granulosa cells were enzymatically separated in the LWF and SYF as previously described

(Davis *et al.* 2000). The granulosa and theca samples from two individual birds were pooled to generate three replicate samples of theca and granulosa tissue for each follicle size. All isolated granulosa cell and theca cell samples were frozen and stored at -80 °C in 1 mL of guanidine isothiocyanate solution for subsequent RNA extraction.

Cell Culture

The F_1, F_3 and small yellow follicles were removed from three hens for each experiment. Granulosa cells were isolated from each of the follicles as previously described (Davis *et al.* 2000) and subsequently cultured in 6-well tissue culture plates as previously described (Davis *et al.* 2000) except that the lipoprotein supplement was not added to the M199 culture media. Cell number and viability were estimated using a hemocytometer with trypan blue exclusion. Cell viability was greater than 95%.

Experiment 2

Ovine LH (Lot AFP8468A) or human recombinant FSH (Lot AFP5551B) (both generously provided by Dr. A. F. Parlow of the National Hormone and Peptide Program, Torrance, CA) were added at doses of 0 or 50 ng/ml of culture media. The cultures were terminated 24 h after the cells were plated. After termination, the medium was saved for RIA and the granulosa cells were collected in solution D (Chomczynski and Sacchi, 1987) and frozen at -80 °C for future RNA extraction. Each treatment consisted of three wells and the cells were combined from these three wells prior to RNA extraction. This experiment was replicated three more times (n = 4).

Experiment 3

The same protocol that was used in experiment 2 was followed in experiment 3, except the isolated and dispersed granulosa cells were cultured in the absence or presence of 1×10^{-6} testosterone (Steraloids, Newport, RI) or 17 β-estradiol (Sigma, St. Louis, MO).

RNA Extraction and Northern Analysis

Total RNA was extracted from all samples using a guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). For experiment 1, 25 μg of total RNA and for experiments 2 and 3, 7.5 μg of total RNA, for each sample was used in Northern analysis for chicken ZPC, ZPD and 18S mRNA as previously described (Davis and Johnson, 1998; Benson *et al.* 2006a). The hybridization and densitometry procedures were completed as previously described (Davis and Johnson, 1998; Benson 2006a).

Radioimmunoassay (RIA)

Media progesterone concentrations were determined by radioimmunoassay (RIA) for experiments 2 and 3. The progesterone RIA was completed with a Coat-A-Count Progesterone kit (Diagnostic Products Corporation, Los Angeles, CA) following the manufacture's protocol.

Statistics

Data from each experiment were subjected to ANOVA according to the General Linear Model (GLM) procedure. Tukey's multiple comparison procedure (Neter *et al.* 1990) was used to detect significant differences between samples. Differences were considered significant when *p* values were less than 0.05. All statistical procedures were completed with the Minitab statistical software package (Release 13, State College, PA).

Results

ZPC and ZPD mRNA expression in broiler breeder hen follilces

The mRNA expression for both ZPC and ZPD was undetectable by Northern analysis in the theca tissue samples from the F_2, F_3 and F_4 follicles and in the granulosa and theca samples from small yellow, and large white follicles. ZPC and ZPD mRNA expression was detected in the F_1 theca cells (Figure 7.1). There was a significant increase in the granulosa cell expression of the

mRNA for both ZPC and ZPD as the F_4 follicle matured to a F_3 follicle, and the expression of ZPD mRNA was highest in the granulosa cells of the F_1 follicle (Figure 7.1).

LH and FSH effect on ZPC and ZPD mRNA expression

Neither ZPC nor ZPD mRNA can be detected by Northern analysis in freshly isolated granulosa cells from SYF (Figure 7.1), but the mRNA expression of both ZPC and ZPD was detected by Northern analysis in untreated granulosa cells following 24 hours of culture in M199 (Figures 7.2-7.4). Furthermore, both ZPC and ZPD mRNA expression was increased in SYF granulosa cells cultured with LH in comparison to the corresponding expression in untreated cell cultures (Figures 7.2 and 7.3). In contrast, ZPC mRNA expression in the F_1 granulosa cells cultured with LH was reduced in comparison to the untreated F_1 cultured granulosa cells (Figure 7.3). The addition of LH to F_3 granulosa cell culture had no effect on ZPC and ZPD mRNA expression (Figures 7.2 and 7.3).

The addition of FSH to granulosa cells cultured from the SYF increased the expression of ZPD (Figure 7.3) but not ZPC (Figure 7.2). Moreover, the expression of both ZPC and ZPD mRNA was significantly increased in F_3 granulosa cells cultured with FSH in comparison to untreated control cells (Figures 7.2 and 7.3). The addition of FSH to the F_1 granulosa cell cultures had no effect on ZPC and ZPD mRNA expression (Figures 7.2 and 7.3)

Progesterone accumulation in the media of the granulosa cells cultured with LH was higher than the progesterone accumulation in the untreated cell cultures for all follicle sizes except the SY (Table 7.1). The addition of FSH to the granulosa cell culture media significantly increased the accumulation of progesterone for the granulosa cells from all follicle sizes (Table 7.1).

The effect of testosterone and 17-β estradiol on ZPC and ZPD mRNA expression

The addition of testosterone to the granulosa cell cultures from SYF increased the expression of both ZPC and ZPD mRNA (Figures 7.5 and 7.6). Testosterone increased the mRNA expression of ZPD but not ZPC in F_3 granulosa cell cultures. The addition of testosterone to the F_1 granulosa cell cultures had no effect on ZPC and ZPD mRNA expression (Figures 5 and 6).

The addition of 17-β estradiol to the culture media had no effect on the mRNA expression of ZPC and ZPD in cultured granulosa cells from the F1 or SY follicles (Figures 7.5 and 7.6), but it did increase the expression of ZPD mRNA in the cultured F_3 granulosa cells (Figure 7.6).

Progesterone accumulation in the media of granulosa cells cultured with testosterone was significantly higher than in control granulosa cell cultures (Table 7.2). In contrast, the addition of 17-β estradiol to the cultured granulosa cells had no effect on progesterone production (Table 7.2).

Discussion

In the present study, the only significant difference for ZPC mRNA expression in granulosa cells during follicular maturation of the four largest chicken preovulatory follicles was between the F_3 and F_4 follicles. In contrast to previously reported increases in avian ZPC mRNA expression during the final 48 hours of follicular development (Waclawek *et al*. 1998; Pan *et al*. 2001), the present study did not detect any significant increase in the expression of ZPC mRNA between the F_1 and F_2 follicles. However, the expression of ZPC mRNA was increased to detectable levels in the theca cells during the final 24-48 hours of follicular maturation. In contrast to turkey ZPD mRNA expression (Benson *et al*. 2006a), the expression of the mRNA for chicken ZPD increased during the final 48 hours of follicular maturation. Interestingly, the expression of the mRNA for ZPD also increased to detectable levels by Northern analysis in the

theca cells of the F_1 follicle. The conflicting results suggest there may be subtle differences in the expression of the mRNA for ZPC and ZPD in avian species, or that the differences may result from differences in the exact timing of when the follicles are collected in the experimental birds relative to ovulation.

The detection of the mRNA for both chicken ZPC and ZPD in the F_1 theca cells is novel. Previous attempts in both the White Leghorn chicken and quail have failed to detect ZPC mRNA expression in theca cells (Takeuchi *et al*. 1999; Pan *et al*. 2001). Therefore, this is the first report of avian ZP mRNA being differentially expressed in two ovarian tissues during folliculogenesis. Differential expression of ZP proteins in morphologically distinct ovarian tissues during follicular maturation has been seen in several other species (Lee and Dunbar, 1993; Sinowatz *et al*. 1995; Kolle *et al*.1998; Totzauer *et al.* 1998; Bogner *et al.* 2004). However, in these species it was the oocyte and granulosa cells that were involved in the expression of the ZP proteins and not the granulosa and theca cell layers as seen in the current research. The expression of the mRNA for an avian ZP protein in two distinct tissues is not a novel concept since the chicken transcripts for ZPB1 mRNA have been detected in both the liver and adrenals (Bausek *et al.* 2000).

In contrast to a reported increase in quail ZPC mRNA expression (Pan *et al.* 2001) in F_1 and F_2 granulosa cells cultured for 24 h with 1μM testosterone, chicken ZPC mRNA expression did not increase in either F_1 or F_3 granulosa cells cultured for 24 hour with the same concentration of testosterone in the present experiment. However, this difference in results may be related to the developmental regulation of ZPC mRNA that was not observed in the current study but previously reported to occur in quail (Pan *et al.* 2001). Quail ZPC protein production increased significantly in F_1, F_2 and F_3 granulosa cells cultured with FSH for 66 hours with the strongest

induction occurring in the F_3 follicle (Sasanami *et al.* 2003b). In the current study, the increased expression of ZPC mRNA was also elevated in the F_3 granulosa cells cultured with FSH for 24 hours. However, there was no significant increase in the expression of ZPC mRNA in the F_1 granulosa cell cultured with FSH for 24 hour. The differences between these results may be due to the different lengths of time in which the granulosa cells were cultured with FSH.

This is the first report of the developmental regulation of the mRNA for chicken ZPD mRNA. Like ZPC mRNA expression, chicken ZPD mRNA expression is increased in a 24 h granulosa cell culture with 50 ng/ml of FSH. However, ZPD mRNA expression, and not ZPC mRNA expression, is increased in the F_3 granulosa cells cultured with either testosterone or 17-β estradiol. This difference may reflect a different regulatory mechanism for ZPC and ZPD mRNA expression in hierarchical follicles.

The expression of ZPC mRNA was significantly reduced in F_1 granulosa cells cultured with 50 ng/ml of LH for 24 hours. Since LH-R mRNA expression is greatest in the F_1 follicle (Johnson *et al*. 1996) and F_1 granulosa cells are more responsive to LH *in vitro* (Asem and Hertelendy, 1985), any effect of LH on the expression of ZP protein mRNA expression would be greater in the F_1 follicle than any other hierarchical follicle. The current results suggest that high levels of LH *in vivo* associated with the preovulatory LH surge could reduce ZPC mRNA expression in the F_1 follicle prior to ovulation. The down regulation of ZPC mRNA expression by LH just prior to ovulation would potentially make sense since the assembly of the IPVL should be completed or near completion just prior to ovulation. In addition, Waclawek *et al.* (1998) reported that ZPC mRNA could not be detected in granulosa cell enriched postovulatory follicle fractions.

The inability to detect ZPC mRNA expression by Northern analysis in the SYF granulosa cells is consistent with the reported lack of quail ZPC mRNA expression in the SYF (Pan *et al.* 2001). Waclawek *et al*. (1998) did detect ZPC mRNA expression in SYF granulosa cells from laying hens by Northern analysis, but the blot had to be exposed to film for 7 days versus 5 hours for the F_1-F_5 granulosa cell samples. However, it is interesting that both ZPC and ZPD mRNA expression is induced from undetectable levels to detectable levels by Northern analysis following 24 hour granulosa cell culture. This induction of both ZPC and ZPD mRNA expression during granulosa cell culture may reflect the removal of some inhibitory factor which prevents the abundant expression of both ZP genes in SYF. The inhibitory factor is not likely to be blood borne since there is not a similar inhibition of the mRNA expression of ZPC and ZPD in the hierarchical follicles. Thus, the inhibitory factor is likely derived from the theca cells of the SYF since removal of the theca cell layers for cell culture removes the inhibitory effect in the SYF and allows detection of both ZPC and ZPD mRNA in granulosa cells cultured for 24 hours.

Both 17-β estradiol and testosterone are secreted at high levels by the theca cells of the SYF (Senior and Furr, 1975; Robinson and Etches, 1986). Therefore, the paracrine activity of 17-β estradiol and testosterone would both be natural suspects for potentiating the inhibitory mechanism in preventing ZPC and ZPD mRNA expression *in vivo*. However, the mRNA expression of both ZPC and ZPD mRNA was not reduced in granulosa cells cultured with either 17-β estradiol or testosterone. In fact, the mRNA expression of both ZPC and ZPD increased in SYF granulosa cells cultured with 1×10^{-6} M testosterone.

Interestingly, Johnson and Brigdham (2001) reported that the LH receptor (LH-R) mRNA expression pattern in freshly isolated granulosa cells and cultured granulosa cells from the SYF was identical to what was observed for ZPC. They also suggested that the increase in the

expression of LH-R mRNA during SYF granulosa cell culture is possibly due to a release from an inhibitory mechanism which exists *in vivo*. In the same study, inhibition of transforming growth factor α (TGFα) signaling through mitogen-activated protein kinase (MAPK) increased LH-R mRNA expression in SYF granulosa cell cultures (Johnson and Brigdham, 2001). Transforming growth factor α (TGFα) signals through a MAPK pathway and inhibition of this pathway has been proposed as the trigger for granulosa cell differentiation (Woods and Johnson, 2005). Interestingly, TGFα was found to abolish chicken ZPC expression in a long-term granulosa cell culture (Schmierer *et al.* 2003) and also inhibit quail ZPC protein production during a 66 hour granulosa cell culture (Sansanami *et al*. 2003b). Furthermore, TGFα receptor protein expression decreases during follicular maturation (Onagbesan and Peddie, 1998). Thus, the decreased TGFα sensitivity as follicles mature may account for the increased granulosa cell expression of ZPC mRNA seen in the current research and in previous research (Waclawek *et al*. 1998; Takeuchi *et al.* 1999; Pan *et al*. 2001) as preovulatory follicles advanced in maturity.

In summary, the expression of the mRNA for both chicken ZPC and ZPD mRNA are regulated by both gonadotropins and sex steroid hormones in a manner dependent on follicular maturation. Expression of the mRNA for ZPC and ZPD can be detected in SYF following the removal of an undetermined inhibitory mechanism, and further studies involving paracrine and autocrine factors known for granulosa cell differentiation, such as TGFα, should be pursued to elucidate the inhibitory mechanism responsible for the undetectable *in vivo* expression of ZPC and ZPD mRNA in SYF. In addition, the reduction of ZPC mRNA expression in the F_1 follicle should be further pursued by *in vitro* Western analysis and immunohitochemistry to determine if LH reduces ZPC protein synthesis in the F_1 follicles. It will be interesting to see if the

expression of the other three granulosa expressed avian ZP proteins (ZPA, ZPB2 and ZPX) are regulated in a similar manner as reported in this study for chicken ZPC and ZPD.

Table 7.1. Progesterone concentrations 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) and 50 ng/ml culture media of LH or FSH.

	Progesterone concentration[1] (ng/ml)
F1	
Control	356 ± 35^{a}
LH	3600 ± 390^{b}
FSH	1800 ± 346^{c}
F3	
Control	134 ± 9^{a}
LH	1900 ± 179^{b}
FSH	1460 ± 154^{c}
SY	
Control	0.168 ± 0.08^{a}
LH	0.959 ± 0.46^{ab}
FSH	1.275 ± 0.54^{b}

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

Table 7.2. Progesterone concentrations 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) and 1 x 10^{-6} M of estrogen or testosterone.

	Progesterone concentration[1] (ng/ml)
F1	
Control	335 ± 39[a]
Estrogen	297 ± 40[a]
Testosterone	619 ± 68[b]
F3	
Control	150 ± 16[a]
Estrogen	137 ± 12[a]
Testosterone	679 ± 36[b]
SY	
Control	0.40 ± 0.1[a]
Estrogen	0.29 ± 0.1[a]
Testosterone	3.24 ± 0.49[b]

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

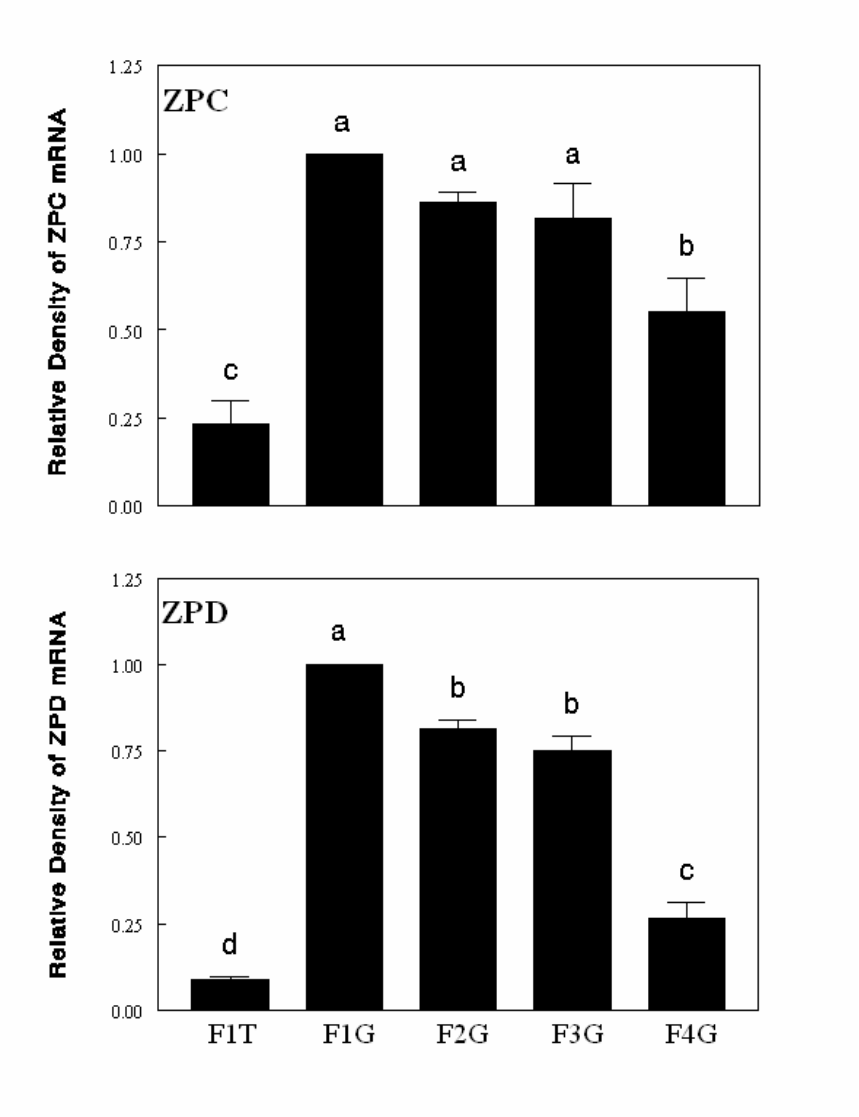

Figure 7.1. The relative density of ZPC and ZPD mRNA in the four largest preovulatory follicles. Values are means ± SEM, n = 3 replicate birds. The mRNA expression of both ZPC and ZPD was undetectable by Northern analysis in the theca tissue samples from the F_2, F_3 and F_4 follicles and from the granulosa and theca samples from the small yellow and large white follicles and therefore are not shown. Means for follicle samples with different letters differ, $p < 0.05$.

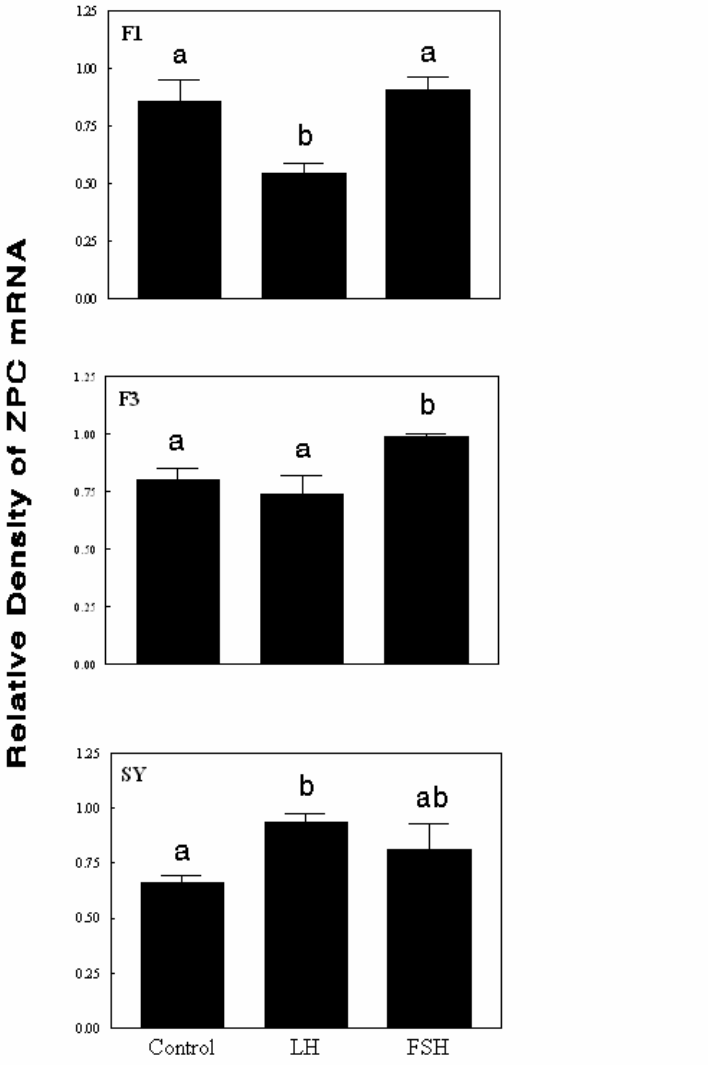

Figure 7.2. The relative density of ZPC mRNA in granulosa cells from the F_1, F_3 and small yellow (SY) follicles cultured for 24 hours in the presence of 0 (control) and 50 ng/ml culture media of LH or FSH. Values are means ± SEM, n = 4 replicate experiments. Means for a follicle size with different letters differ, $p < 0.05$.

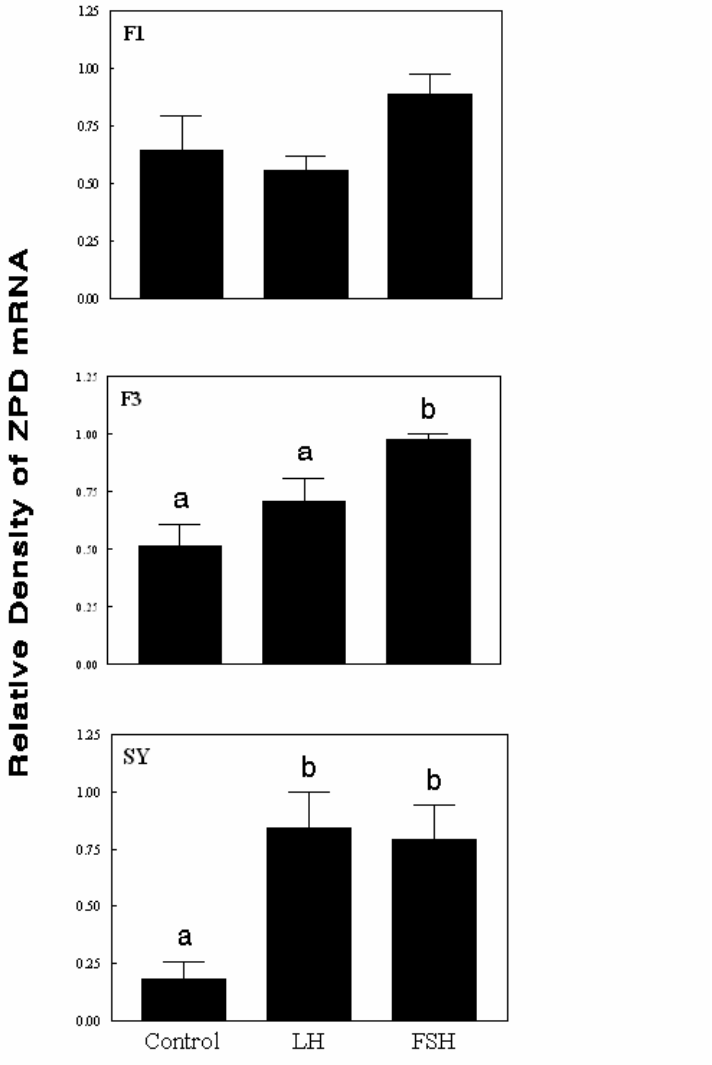

Figure 7.3. The relative density of ZPD mRNA in granulosa cells from the F_1, F_3 and small yellow (SY) follicles cultured for 24 hours in the presence of 0 (control) and 50 ng/ml culture media of LH or FSH. Values are means ± SEM, n = 4 replicate experiments. Means for a follicle size with different letters differ, $p < 0.05$.

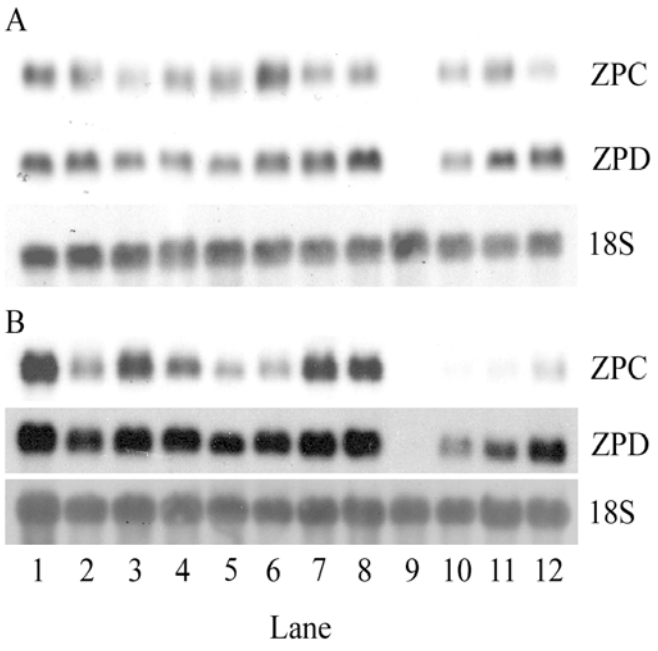

Figure 7.4. Autoradiograms from the Northern analyses of ZPC and ZPD showing one replicate experiment form the culture of F_1, F_3, and small yellow (SY) follicle granulosa cells with 0 and 50 ng/ml culture media of LH or FSH **(A)** or 0 and 1 x 10^{-6} M 17-β estradiol or testosterone **(B)**. Total RNA (7.5 μg) was loaded for each sample. Samples from F_1, F_3 and SY granulosa cells are in lanes 1-4, 5-8 and 9-12, respectively. For each follicle size the order of samples for section **(A)** is freshly dispersed cells (time zero), control (0 ng/ml), 50 ng/ml LH and 50 ng/ml FSH, respectively and for section **(B)** is time zero, control, 1 x 10^{-6} M 17-β estradiol and 1 x 10^{-6} M testosterone, respectively.

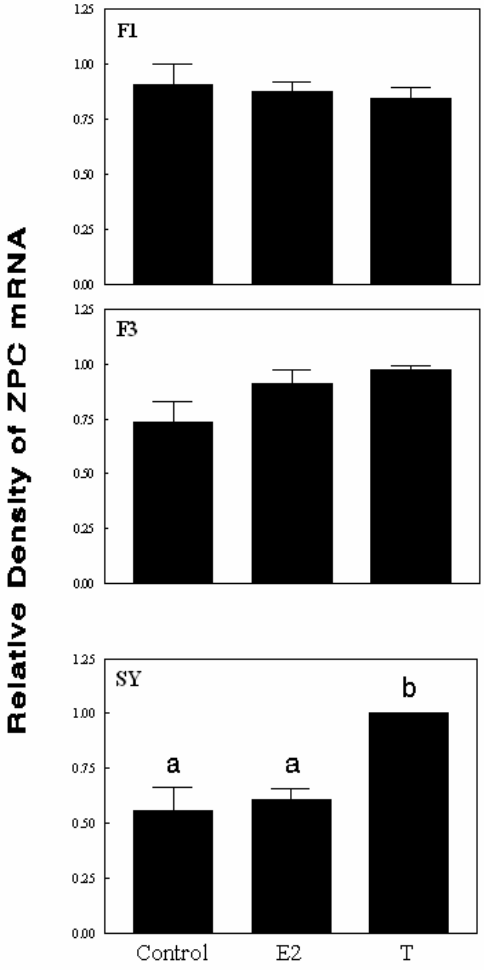

Figure 7.5. The relative density of ZPC mRNA in granulosa cells from the F_1, F_3 and small yellow (SY) follicles cultured for 24 hours in the presence of 0 (control) and 1×10^{-6} M 17-β estradiol (E2) or testosterone (T). Values are means ± SEM, n = 4 replicate experiments. Means for a follicle size with different letters differ, $p < 0.05$.

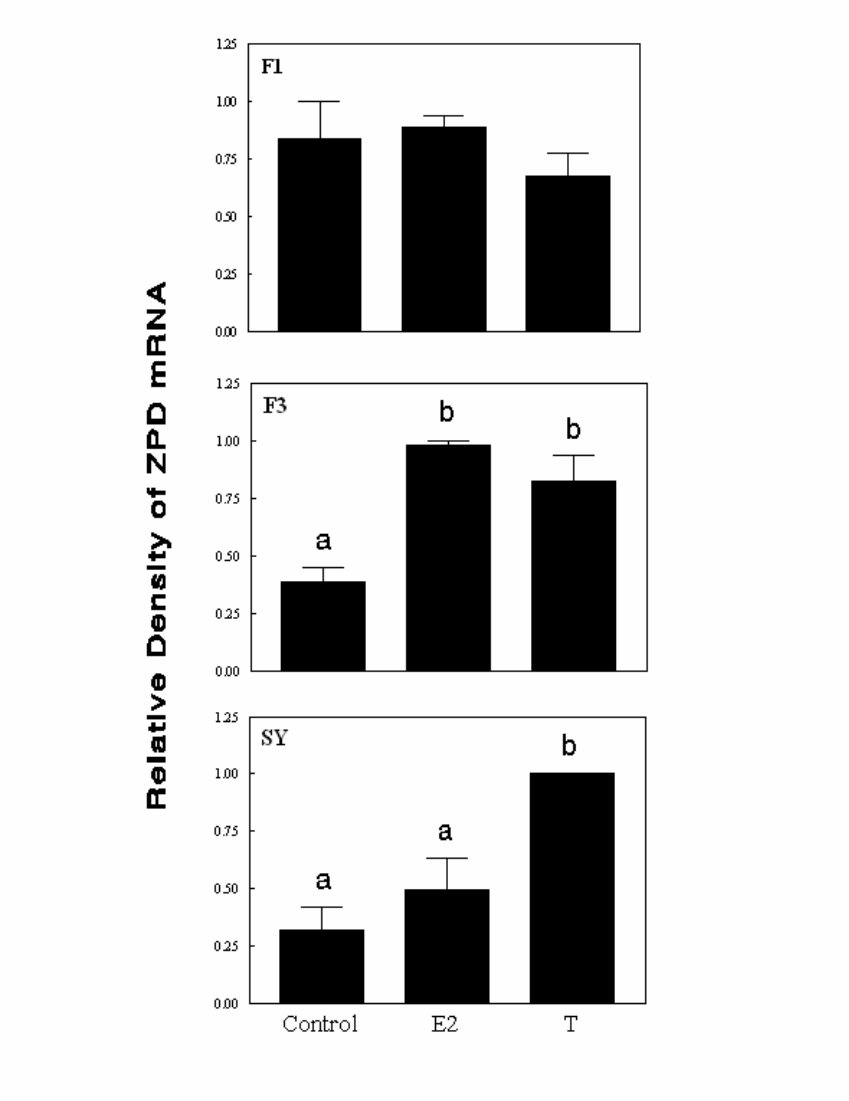

Figure 7.6. The relative density of ZPD mRNA in granulosa cells from the F_1, F_3 and small yellow (SY) follicles cultured for 24 hours in the presence of 0 (control) and 1×10^{-6} M 17-β estradiol (E2) or testosterone (T). Values are means ± SEM, n = 4 replicate experiments. Means for a follicle size with different letters differ, $p < 0.05$.

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CHAPTER 8

GENERAL CONCLUSIONS

There is no paradigm in vertebrates regarding the ZP glycoprotein that binds and activates the sperm to penetrate the ZP since ZPC, ZPB1 or a combination of ZP proteins have all been identified as the sperm receptor in different vertebrate species (Bleil and Wassarman, 1980; Yurewicz *et al.* 1998; Yonezawa *et al*. 2001). A total of six genes for ZP proteins have been identified in avian species (ZPA, ZPB1, ZPB2, ZPC, ZPD and ZPX) (Waclawek *et al*. 1998; Takeuchi *et al*. 1999; Bausek *et al*. 2000; Sasanami *et al.* 2003a; Sasanami *et al*. 2003b; Okumura *et al*. 2004; Smith *et al*. 2005), and previous research has shown that avian ZP proteins may play a role in sperm binding and in the induction of the acrosome reaction (Pan *et al.* 1999; Takeuchi *et al.* 2001; Bausek *et al.* 2004; Okumura *et al.* 2004). However, there is still no conclusive answer regarding which avian ZP protein(s) serve as the sperm receptor, and the disparity in research reports concerning the avian ZP proteins necessitated additional research concerning the expression and regulation of avian ZP proteins.

Since the ZP proteins are known to consist of conserved structural domains (Bork and Sander, 1992; Dunbar, 1994; Prasad, 2000) and given that the IPVL is simply a meshwork of interwoven ZP proteins, immunohistochemistry and Western blotting could not be used to accurately quantitate the differences in the expression of ZP proteins. The possibility of cross-reactivity among antibodies produced against ZP proteins and the lack of a constant entity in the IPVL to serve as an internal control for sample protein content can be avoided with mRNA expression studies. Northern blot analysis and real-time PCR both allow for the detection of

quantitative differences since there are known internal standards or housekeeping genes such as GAPDH and 18s mRNA in the cells that synthesize the ZP proteins. Thus, Northern blot analysis and real-time PCR were utilized to determine the differential mRNA expression of the avian ZP genes in the current research.

Since ZP proteins play a vital role in the process of fertilization (as reviewed by Wassarman, 1999), one of the goals of the current research was to determine if differential expression of the mRNA for all six avian zona pellucida genes occurs in two lines of turkey hens which differ significantly in fertility. Furthermore, since sperm binding has been reported to occur at higher levels in the IPVL overlying the germinal disc (GD) region (Howarth and Digby, 1973; Ho and Meizel, 1975; Bramwell and Howarth, 1992; Birkhead *et al.* 1994; and Wishart, 1997), another goal of the research was to investigate the mRNA expression of the granulosa derived ZP proteins between GD and NGD granulosa cells in these turkey hens.

Since there was no data on the expression or existence of ZP proteins in the turkey hen (*Meleagris gallopavo*), the mRNA expression of all six of the known chicken ZP genes had to be established in turkey hens. Three ZP proteins (ZPB1, ZPC and ZPD) previously reported to be glycoprotein components of the chicken IPVL by immunohistochemistry and Western blotting techniques (Waclawek *et al.* 1998; Takeuchi *et al.* 1999; Bausek *et al.* 2000; Okumura *et al.* 2004) had higher mRNA expression than the other three ZP proteins (ZPA, ZPB2 and ZPX) in turkey hens. In fact, the mRNA expression of turkey ZPA, ZPB2 and ZPX could not be detected by Northern blot analysis in the current research. Therefore, real-time PCR was utilized to detect the lower mRNA expression levels of ZPA, ZPB2 and ZPX in granulosa cells from the largest hierarchical follicles. There was no significant increase in the mRNA expression of any of the

five granulosa produced avian ZP proteins during follicular maturation in the turkey hen. However, ZPB2 mRNA expression was found to decrease during follicular maturation.

The hepatic expression of ZPB1 mRNA was significantly greater in the high fertility line (E line) than in the low fertility line (F line). In addition, the mRNA expression of ZPC was greater in the NGD granulosa cells of F line hens. Interestingly, antibodies directed against both ZPB1 and ZPC have been reported to inhibit avian sperm-IPVL binding (Pan *et al.* 1999; Takeuchi *et al.* 2001; Bausek *et al.* 2004). Thus, the results suggest that the higher rates of fertility previously observed in eggs from the E line hens versus the F line of hens may be related to differential expression of the potential sperm binding proteins ZPB1 and ZPC.

Even though ZPB2 mRNA is not highly expressed in turkey granulosa cells, it is worth noting that of the 5 ZP proteins expressed by the granulosa cells of turkey hierarchical follicles, only ZPB2 mRNA expression is significantly higher in the GD granulosa cells than in NGD granulosa cells in both genetic lines of turkey hens. The higher 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 if proteins expression mirrors the mRNA expression.

Due to the notable differential expression of the mRNA for ZPB1, ZPB2, and ZPC, future studies involving these three ZP proteins should be pursued. For example, the distribution of ZPB1 in the IPVL of the preovulatory follicles needs to be examined, since this protein is produced in the liver and transported to the developing follicles. Western blot analyses and immunohistochemistry studies could determine if there is preferential deposition of ZPB1 in the IPVL above the GD, and if more ZPB1 protein is present in the IPVL of preovulatory follicles from E line hens than F line hens. Furthermore, it would also be of interest to use Western blot

and immunohistochemistry studies to determine if ZPB2 and ZPC protein expression mirror the observed granulosa cell mRNA expression in the current studies.

Although chicken ZPC and ZPD have both been suggested to play critical roles in sperm binding to the IPVL and in their subsequent penetration of the IPVL (Pan *et al.* 1999; Bausek *et al*. 2004; Okumura *et al*. 2004), the hormonal regulation of the synthesis of both of these granulosa derived proteins during follicular maturation is not well characterized. Therefore, the gonadotropin (LH and FSH) and steroid (estrogen and testosterone) hormone regulation of chicken ZPC and ZPD mRNA expression was investigated in cultured granulosa cells, and both ZPC and ZPD mRNA expression was found to be regulated by both gonadotropins and sex steroid hormones in a manner dependent on follicular maturation. Expression of the mRNA for ZPC and ZPD can be detected in SYF granulosa cells once they are removed an unknown *in vivo* inhibitory mechanism. Additional studies involving paracrine and autocrine factors known to influence granulosa cell differentiation, like TGFα, should be pursued to reveal the inhibitory mechanism responsible for the depressed expression of ZPC and ZPD mRNA in SYF *in vivo*. Additionally, the reduction of ZPC mRNA expression in the F_1 follicle should be further pursued by *in vitro* Western analysis and immunohitochemistry to determine if LH reduces ZPC protein synthesis in the F_1 follicles.

The selection for higher yielding birds has exacerbated fertility problems in breeder flocks (Brillard, 2004), and the traditional approach to increase fertility has been to focus on the reproductive performance of the adult male birds, with little attention given to female birds (Donoghue, 1999). The move toward higher yielding birds will continue because of market requirements, and greater effort will be required in breeder management to maintain reasonable fertility (Pollock, 1999). The current results indicate that ZPB1, ZPB2 and ZPC mRNA

expression have the potential to be used as genetic markers for selecting female lines of turkey hens for greater fertility. If ZPB1, ZPB2, and ZPC protein patterns reflect the observed mRNA expression, Western analyses of laid eggs for these potential sperm binding proteins could be used by the poultry industries as a non-lethal method to select for higher fertility in breeder hens.

The economic impact of improved fertility in turkey and broiler breeder hens would be substantial for US agriculture. The 2005 value of turkey production in the US is 3.23 billion dollars, an increase of 6 percent from 2004. Broiler production in the U.S. had a total dollar value of around 20 billion in 2005 and the state of Georgia contributed 2.9 billion dollars to that total (USDA NASS). Thus, an increase of fertility by only one percent, barring no additional changes in parameters such as hatchability and chick value, would be worth 29 million dollars annually to the production of poultry in the state of Georgia alone (2.9 billion dollars x 0.01 x $0.20 per chick). Furthermore, improving hen fertility even marginally (1 percent) through genetic selection based on ZP protein expression will reverse the current trend of poultry integrators encouraging farmers to build more grower and laying facilities to counteract the decline in average number of chicks produced per turkey or broiler breeder hen. By reducing the number of new facilities constructed and the number of turkey and broiler breeder hens needed to meet turkey poult and broiler chick production needs, natural resources can be conserved and the total environmental impact of poultry production facilities can be reduced.