THE mRNA AND PROTEIN EXPRESSION OF AVIAN ZONA PELLUCIDA PROTEINS

by

Mia Nyren Malloy

(Under the Direction of Adam J. Davis)

The freshly ovulated ovum in avian species is surrounded by the inner perivitelline layer (IPVL). For successful fertilization, sperm must attach and penetrate the IPVL. In the domestic chicken six distinct zona pellucida genes have been identified (ZPA, ZPB1, ZPB2, ZPC, ZPD and ZPX1). ZPB1 is produced by the liver and transported to developing follicles, while ZPA, ZPB2, ZPC, ZPD and ZPX1 are synthesized by follicular granulosa cells. In turkey hens, differences in mRNA expression of the ZP proteins are associated with preferential binding of sperm at the germinal disc (GD) region and with differences in fertility among genetic strains of hens.

The expression of ZPB2 mRNA was investigated by real time RT-PCR in theca and granulosa cells from the developing preovulatory follicles of broiler breeder hens. Expression of ZPB2 mRNA was highest in granulosa cells isolated from follicles with a diameter less than 2 mm, followed by 2-5 mm diameter and F_4 follicles. Expression of ZPB2 mRNA in the F_2 and F_3 follicle was lower than the F_4 follicle. ZPB2 mRNA was not detected in the granulosa cells isolated from 5-8 mm diameter, or 8-12 mm diameter follicles or in theca samples from hierarchical follicles. Theca cell expression of ZPB2 mRNA was confined to the theca cells from the smallest follicles.

The mRNA expression of ZPB1, ZPB2 and ZPC was analyzed in four broiler breeder hen genetic lines designated as B, G, O and R. Expression of ZPB1, ZPB2, and ZPC mRNA was

detected in all genetic lines. Expression of ZPB2 mRNA differed between NGD and GD granulosa cells. Hepatic ZPB1 mRNA levels were lower in line O than line B. In genetic strains B, G, and R, ZPC mRNA expression was higher in NGD than GD granulosa cells. However, in genetic strain O the expression of ZPC mRNA did not differ between NGD and GD granulosa cells.

Antibodies against chicken ZPB1, ZPB2 and ZPC were produced and validated for subsequent use in determining if differences in the mRNA expression of the ZP proteins are also present at the protein expression level.

Index Words: Inner Perivitelline Layer, Turkeys, Granulosa, Theca, Broiler Breeder Hens

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DEDICATION

This dissertation is dedicated to my husband Ryan and my parents, Christopher White, Christina White, and James Sargous.

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CHAPTER 1

AVIAN FEMALE REPRODUCTIVE PHYSIOLOGY

The Avian Ovary

The sexually active avian ovary contains a visually evident hierarchy of follicles arranged according to size and time until ovulation. In the laying hen, four to six large, yolk-filled follicles between 12-40 mm in diameter are referred to as the hierarchical follicles. The large, yellow hierarchical follicles are named according to size with the largest one, which will ovulate within 24 hours, designated as the F_1 follicle; the next largest follicle is the F_2 follicle and will ovulate in about 48 hours, and so on for the other follicles. The development of these hierarchical follicles is tightly regulated with an interval of 24-26 hours between each consecutive ovulation. After the F_1 follicle ovulates, succeeding follicles advance one place in the hierarchy and an additional follicle is recruited into the hierarchy from the prehierarchical follicles. The prehierarchical follicles are also categorized by size and there are several follicles in each category. Small yellow follicles (SYF) are 5 to 12 mm in diameter, the large white follicles (LWF) are 2- 5 mm in diameter, and the small white follicles (SWF) are less than 2 mm in diameter. With each ovulation, a follicle is recruited to the hierarchy from the pool of small yellow follicles. In turn, some of the small white follicles will begin the uptake of yellow yolk and advance to the pool of small yellow follicles. It is estimated that only 5% of the growing prehierarchical follicles will mature to reach a size of 6-8 mm in diameter (Gilbert et al. 1983b). The vast majority of follicles undergo follicular atresia with the individual cells dying by apoptosis (Johnson et al. 1996b).

Profile of Avian Follicular Tissues: Granulosa and Theca Cell Layers

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

Follicular maturation is described 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), and is mediated in part by the expression of LH and FSH receptors in granulosa tissue. FSH receptor expression in the hierarchy is highest in the granulosa cells of the small yellow follicles and the level of expression decreases with follicular maturation (Calvo and Bahr 1983, Ritzhaupt and Bahr 1987, You et al. 1996, Woods and Johnson 2005). Theca cells express much less FSH receptors than granulosa cells and the level of FSH receptor expression in the theca cells does not vary significantly with follicular maturation (Etches and Cheng 1981, Gilbert et al. 1985, You et al. 1996). In prehierarchical follicles, FSH promotes granulosa cell proliferation and maturation (Davis et al. 2000, 2001). FSH also helps maintain the follicular hierarchy through prevention of atresia (Palmer and Bahr 1992, Johnson et al. 1996b, 1999), induces LH receptor, steroidogenic acute regulatory protein (StAR) and P_{450} cholesterol side chain cleavage enzyme expression in granulosa cells for subsequent steroid production (Li and Johnson 1993, Johnson and Bridgham 2001, Johnson et al.

2004), and stimulates progesterone (P4) production (Calvo and Bahr 1983, Robinson et al. 1988, Davis et al. 1999, 2001, Johnson et al. 2004). Collectively, these results suggest that the prehierarchical follicle that is more responsive to FSH, avoids apoptotic cell death, and is then recruited into the avian follicular hierarchy. From the pool of small yellow follicles, it is hypothesized that only one follicle has an upregulated level of FSH receptors, specifically in the granulosa layer, and it is likely the one that advances into the hierarchy (Woods and Johnson 2005). However, this selective increase is the result, and not the cause, of follicular selection and very little is known about the nature of the mechanism responsible for up-regulating FSHR expression (as reviewed by Johnson and Woods 2009).

As the selected follicle is recruited into the follicular hierarchy, the follicle transitions from being predominantly FSH dependent to becoming primarily LH dependent (Calvo and Bahr 1983). LH receptor expression is highest in the granulosa cells of the large follicles, especially the F_1-F_3 follicles (Calvo et al. 1981, Calvo and Bahr 1983, Gilbert et al. 1983a, 1985, Johnson et al. 1996a). LH receptor mRNA expression in the theca tissue varies little with follicular development (Johnson et al. 1996a) and LH promotes steroidogenesis by the theca cells of prehierarchical and hierarchical follicles (Robinson et al. 1988, Kowalski et al. 1991). Decreased expression of the mRNA for the LH receptor in 3-5 mm diameter prehierarchical follicles is associated with atresia (Johnson et al. 1996a). LH promotes granulosa cell growth (Davis et al. 2000) and progesterone production (Davis et al. 1999, Johnson et al. 2004).

Steroidogenesis in the Hen Ovary

The vascularized theca cells surrounding the small yellow and white follicles are steroidically competent and secrete androgens and estrogens via the Δ^5 steroidogenic pathway (Lee et al. 1998) and these follicles are the primary source of plasma estrogen (Senior and Furr

1975, Lee and Bahr 1989, 1993). In contrast, the avascular granulosa cell layers of these small yellow and white follicles are steroidically incompetent due to a lack of P_{450} side chain cleavage enzyme activity (Li and Johnson 1993). This enzyme initiates steroidogenesis by converting cholesterol to pregnenolone (Lee and Bahr 1990, Tilly et al. 1991). Plasma concentrations of estrogen peak 4-6 hours before ovulation. Estrogen stimulates the hypothalamus and pituitary to express progesterone receptors (Wilson and Sharp 1976). Estrogen also stimulates the liver to produce lipid and vitellogenin for yolk formation (Deeley et al. 1975) and aids in calcium metabolism for shell formation and medullary bone deposition (Etches 1987). Estrogen increases the expression of its own receptor in the oviduct which increases estrogen's effects on oviductal functions (Takahashi et al. 2004). Estrogen also increases expression of the progesterone receptor in the ovary and oviduct, which promotes the formation of tubular secretary glands for albumen and shell secretion and the contractile activity of the myometrium (Yoshimura and Bahr 1991).

Both the granulosa and theca cells of the hierarchical follicles are steroidically competent. The granulosa cells express P_{450} side chain cleavage enzyme activity (Li and Johnson 1993, Kato et al. 1995) but very low levels of 17 α-hydroxylase and thus utilize the Δ^4 steroidogenesis pathway to produce progesterone (Etches and Duke 1984, Robinson et al. 1988, Lee and Bahr 1989, 1993, Johnson et al. 1996b, Lee et al. 1998). The theca cells of the hierarchical follicles also use the Δ^4 pathway to produce progesterone, which they metabolize to androgens. The theca cells of the hierarchical follicles, with the exception of those of the F_1 follicle, also metabolize the progesterone produced by the granulosa cells into androgens (Etches 1990). In the F_1 follicles, the theca cells do not metabolize the progesterone produced by the

granulosa cells (Marrone and Hertelendy 1985), and thus the F_1 follicle is the primary source of plasma progesterone (Huang and Nalbandov 1979).

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

Germinal Disc Region

The germinal disc (GD) appears as a 3-4 mm diameter white spot on the surface of the white yolk core of the preovulatory oocyte or yolk of the oviposited egg and represents less than 1% of the mature follicle volume. Just prior to ovulation, the avian GD resembles the mammalian oocyte both functionally and structurally with the GD containing 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). The composition of white yolk more closely resembles normal physiological fluids than the highly lipid yellow yolk (Perry et al. 1978) and thus provides a better environment for early zygote development. The germinal disc region is considered the growth center of the

avian preovulatory follicle because granulosa cells proximal to the GD region are more differentiated and produce greater amounts of progesterone in response to LH (Tischkau et al. 1997). In addition, DNA synthesis is 2-fold higher in granulosa cells within the GD region when compared to granulosa cells from non-germinal disc (NGD) regions (Tilly et al. 1992). There are also differential amounts of mRNA of granulosa-derived proteins that exist between GD and NGD granulosa cells (Yao and Bahr 2001). At the GD area, the plasma membrane of the oocyte is continuous while the plasma membrane in NGD areas is discontinuous so that yolk material is able to breach the plasma membrane in these areas (Bakst and Howarth 1977a).

Ovulation and the Avian IPVL

When the F_1 follicle is ovulated, the theca cell layer, basal lamina and granulosa cell layer are left behind on the ovary to form the postovulatory follicle, while the IPVL or ZP layer of the oocyte becomes the new external layer. Therefore at ovulation, the oocyte consists of the IPVL as the outermost layer, followed by the plasma membrane, or oolemma, surrounding the yolk mass (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.

Through electron microscopy, Okamura and Nishiyama (1978) confirmed that the infundibulum is the site of fertilization. At the time of fertilization, the plasma membrane of the oocyte is only surrounded by the IPVL and therefore, avian spermatozoa must bind and penetrate through the IPVL before gaining access to the plasma membrane of the oocyte. It was initially believed that sperm were able to pass through the loose fibrous meshwork of the IPVL because the spaces between the fibrous network are about 2 μm in diameter and sperm are about 0.5 μm in diameter (Bellairs et al. 1963). Bakst and Howarth (1977a) later reported that the apparent

holes in the meshwork of the ZP layer actually contained granular material that prevented simple penetration of the sperm through the IPVL.

The Acrosome Reaction

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

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

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

Relationship between Sperm Hole Formation and Fertility

Although only one sperm is required for fertilization, the probability of syngamy increases if multiple sperm penetrate the egg based on research in some avian species. In chickens and turkeys, the probability of fertilization is 100% when six or more sperm penetrate the IPVL over the GD region, yet about 50% when only 3 sperm penetrate the IPVL over the GD region (Bramwell and Howarth 1997, Wishart 1997). Wishart and Staines (1999) proposed that there may be a minimum number of sperm necessary to enter an oocyte in order for syngamy success in birds. The fate of the "extra" sperm 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). Wishart (1997) even demonstrated that embryonic development proceeds normally in eggs that contain only one sperm hole in the IPVL over the GD region. The prevailing hypothesis is that supernumerary sperm increase the probability of syngamy and early zygote development by activating the egg cell (Etches 1996).

There is most likely a threshold number of sperm supernumerary that, if surpassed, might interfere with normal embryonic development and possibly cause embryonic mortality. In chickens, this threshold has been proposed to be 200 sperm penetration holes with greater numbers resulting in early embryonic death (Bekhtina 1968, Bramwell et al. 1996, Hazary et al. 2000). However, one study suggested that the threshold is much higher after finding that more than 200 sperm holes did not have a significant effect in fertile chicken eggs compared to eggs with fewer than 200 sperm holes (Bramwell et al. 1995). Despite the disagreement in the exact number of maximum sperm penetration holes an ovum may endure before embryonic death

ensues, it does seem rational that there would be a threshold of sperm holes or genetic material before abnormal development occurs.

Sperm Holes vs. Sperm Disks

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

Preferential Binding of Sperm at GD Region

In chickens, turkeys, and quail, spermatozoa preferentially attach and digest the IPVL in a circular fashion preferentially over and around the GD area (Howarth and Digby 1973, Ho and Meizel 1975, Bramwell and Howarth 1992, Kuroki and Mori 1997, Wishart 1997). It should be noted, however that one study performed in chickens by Steele et al. (1994) found no sperm

binding preference towards the GD area compared to NGD areas. In this study, Steele and colleagues conducted an assay in which fragments from different regions of the IPVL of freshly ovulated eggs were incubated *in vitro* with sperm. A similar frequency of sperm hole formations were found in the GD and NGD regions, and the reason for the conflicting results is unclear, but these unusual results of Steele et al. (1994) have not been duplicated to date. One study performed on 27 various captive avian species, across many orders such as Galliformes, Anseriformes, Passeriformes, Gruiformes, Ciconiiformes, Charadriiformes, and Struthioniformes (Birkhead et al. 1994) and another performed in ostrich and emu (Malecki and Martin 2003) also indicate that sperm holes are preferentially formed over the GD region.

The Window of Fertilization

Fertilization occurs in the infundibulum before the oocyte arrives at the magnum of the oviduct (Kaupp 1918, Olsen and Neher 1948). The ovulated oocyte remains in the infundibulum of the oviduct for about 15 minutes (Warren and Scott 1935), and this period is commonly referred to as the "15 minute window of fertilization". This short window exists because a new fibrous protein layer which sperm cannot penetrate or bind (Howarth and Digby 1973, Bakst and Howarth 1977b) is deposited around the IPVL at the distal infundibulum (Bellairs et al. 1963). This layer is collectively named the outer perivitelline layer (OPVL), but is comprised of two distinct layers. The initial layer of the OPVL, termed the "middle continuous layer", is secreted and deposited around the IPVL in the distal infundibulum and is followed by a subsequent proteinaceous layer as the ovum enters the upper magnum (Bellairs et al. 1963, Bain and Hall 1969). The OPVL varies in thickness from about 8.5 μm to as little as 3 μm in the hen (Bellairs et al. 1963) and consists mainly of ovomucin, lysozyme, vitelline membrane outer protein I (VMOI) and vitelline membrane outer protein II (VMOII) (Back et al. 1982, Kido et al. 1992).

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

The OPVL and Polyspermy Prevention

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

During the deposition of the OPVL, the proteinaceous secretions from the epithelial cells of the oviduct trap free sperm residing in the lumen of the infundibular/magnal interface. The OPVL, along with the trapped sperm, are deposited on top of the outer IPVL on the oocyte. In the ovipositioned egg, these sperm are found evenly distributed through all regions of the OPVL of both chicken (Wishart 1987) and turkey (Wishart 1997) eggs. In oviposited chicken eggs, Wishart (1997) found 10 times more sperm trapped in the OPVL than the number of sperm that had hydrolyzed the IPVL. Birkhead et al. (1994) demonstrated that the number of sperm found in the OPVL and that had penetrated the IPVL were similar across several avian orders. Sperm

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

Summary

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

CHAPTER 2

INTRODUCTION TO THE ZONA PELLUCIDA LAYER

General Introduction to Fertilization

Fertilization is a precisely choreographed event that joins two highly differentiated haploid germ cells, to form a new, unique individual through a series of ordered steps. The egg and sperm merge to form a single celled pluripotent zygote (as reviewed by Talbot et al. 2003). Fertilization in mammalian and avian species occurs when the male's sperm swim up the female oviduct to meet and bind to the ovulated ovum's extracellular glycoprotein coat, the zona pellucida (ZP). Since sperm must bind and penetrate this glycoprotein coat in a species-specific manner, the zona pellucida is intimately involved in regulating the sperm-egg interaction.

The Zona Pellucida

The glycoprotein coat that surrounds all ovulated vertebrate eggs binds sperm and also acts as structural support (Greve and Wassarman 1985, Wassarman 1999) as the oocyte moves down the oviduct. Fertilization is species-specific and studies have found that if you remove the ZP in certain species such as the golden hamster, capacitated and acrosomally reacted human sperm can overcome this species-specific restriction and bind directly to the egg plasma membrane (Yanagimachi 1984). This finding indicated that the ZP coat may possess sperm receptors, and subsequent research across many species has confirmed specific sperm receptors within the ZP. Because of the importance of this protein coat in sperm binding, a great deal of research has been conducted on it. This research in varied species has lead to discrepancies in nomenclature and therefore the coat has a different name in each major vertebrate lineage. It is

called the chorion in fish, the vitelline envelope in amphibians, the perivitelline envelope in reptiles and birds, and the ZP in mammals (Spargo and Hope 2003). Despite the variation in terminology, its functional structure and involvement in fertilization across species is the same. For simplicity, the glycoprotein coat is often referred to as the ZP (Spargo and Hope 2003). In each species, the ZP is composed of several different proteins that all share a common protein domain called the ZP domain (Bork and Sander 1992, Spargo and Hope 2003). For this reason, the collective proteins that form the ZP are known as ZP proteins. The number of different ZP proteins that comprise the ZP varies across species.

ZP Glycoprotein Nomenclature

Originally, the zona pellucida was believed to be composed of three glycoproteins and they were classified into ZP gene families according to their amino acid sequence similarities. The naming of the ZP families, unfortunately, became more complex as proteins beyond the first three were identified, resulting in various ZP protein naming systems. The initial nomenclature, first described in mouse, had the ZP proteins named in order from highest to lowest apparent molecular weight: ZP1, ZP2, ZP3 (Bleil and Wassarman 1980b). From their ZP research in the cat, dog, pig, mouse, human and rabbit, Harris et al. (1994), proposed another nomenclature system ordering the ZP proteins from longest to shortest gene transcript size. This new method, however, put the mouse genes in order as ZP2, ZP1, and then ZP3. Instead of swapping the names, to lessen confusion, Harris et al. (1994) suggested a letter-based system where ZP2 became ZPA, ZP1 became ZPB, and ZP3 became ZPC. The new system of nomenclature was not universally accepted, which led to both the lettering and numbering systems being used simultaneously by different research groups. Classification methods were challenged again when Hughes and Barrat (1999) discovered a new genomic sequence, designated as ZP1, which

was orthologous to the mouse ZPB gene and paralogous to the human ZPB gene. In 2003, Spargo and Hope proposed a new, unified nomenclature system. The ZP genes were grouped into subfamilies based on the evolutionary relationships within the ZP gene family and then named alphabetically, ZPA, ZPB, ZPC, etc. in order of protein sequence length. Within these subfamilies, group-level paralogues were numbered in order of coding sequence length. Paralogues below group level of phylogeny were differentiated using an additional lowercase letter, also in order of coding sequence length (Spargo and Hope 2003). To simplify the current discussion, the nomenclature from Spargo and Hope (2003) will be used to refer to the ZP genes and proteins in all species.

The ZP Glycoproteins

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

different molecular masses of 200, 120, and 83 kDa, respectively (Bleil and Wassarman 1980b), yet in the rat, ZPA is 140 kDa and ZPB1 is 185 kDa and ZPC is 46 kDa (Akatsuka et al. 1998).

ZP Glycoprotein Sites of Synthesis

The tissue and cell location of ZP protein synthesis varies across species. In mammals, the origin of the ZP glycoproteins is still unresolved. Three theories have been proposed hypothesizing that the proteins are synthesized by (1) the oocyte alone, (2) the follicle cells alone, or (3) both the oocyte and the follicle cells. In the mouse, evidence indicates that mZPA, mZPB, and mZPC are exclusively synthesized by the growing oocyte and not the granulosa cells of the developing follicle (Epifano 1995). Both the oocyte and the surrounding follicular cells synthesize ZP proteins in human, monkey, rabbit, dog, pig and cow at different stages of follicular development (as reviewed by Sinowatz et al. 2001).

Most teleost fish synthesize ZP in either the liver or the ovary but in Gilthead Seabream, *Sparus aurata*, all four mRNA glycoprotein isoforms are transcribed in both the liver and ovary (Modig et al. 2006). In the chicken, ZPB1 is synthesized in the liver and transported via the bloodstream to the ovary (Bausek et al. 2000), while the rest of the identified chicken ZP proteins are made by the granulosa cells of the preovulatory follicles (Waclawek et al. 1998, Okumura et al. 2004).

The Hallmarks of ZP Glycoproteins

Despite 400 million years of evolution, egg coat proteins have retained some essential features in their primary structure that enable them to perform important functions during oogenesis, fertilization, and early development across vertebrates (Wassarman 1988, Wassarman et al. 2001, Monne et al. 2008). By definition, all ZP genes have a common domain called the ZP domain (Bork and Sander 1992). The mammalian ZP domain, located near the C terminus,

consists of ~260 amino acids and has 8 conserved cysteine residues that participate in intramolecular disulfide bonds. These bonds are responsible for the polymerization of these proteins into filaments that collectively form the zona pellucida protein matrix (Jovine et al. 2002). The conserved ZP domain has been identified within a variety of proteins and has led to the proposal that the sequence motif is involved in the assembly of protein filaments or matrices beyond the ZP. Mutations in the ZP domain of α-techtorin proteins, for example, result in defective techtoral membrane assembly. Deletion of the ZP domain in oocyte ZP proteins also prevents their incorporation into the ZP (Jovine et al. 2002). A high resolution, 2.3Å crystal structure of the ZP domain in mouse ZP3 has recently been resolved and will likely provide an important framework to study the ZP domain protein family (Monne et al. 2008).

Zona glycoproteins also have a consensus furin cleavage site (CFCS) located downstream of the ZP domain**.** It has been suggested that the CFCS plays a role in ZP protein secretion and assembly into the ZP matrix (Jovine et al. 2004, Litscher and Wassarman 2007) because once cleaved at the CFCS by proprotein convertase or furin, ZP proteins are then free from the endoplasmic reticulum within the oocyte and available for polymerization with other ZP proteins. To support this theory, recombinant ZP proteins synthesized from cDNAs mutated at the CFCS are not cleaved by furin-like enzymes and are therefore not secreted and accumulate in the endoplasmic reticulum of transfected cells, slowing the trafficking of those mutated ZP proteins throughout the secretory pathway (Williams and Wassarman 2001, Qi et al. 2002, Sasanami et al. 2003bb). However, transgenic mice with mutated CFCS still produced normal, fertile oocytes with intact ZP layers (Zhao et al. 2002). These results imply that there could be a secondary cleavage site to release the N-terminal portion of the ZP protein during intracellular trafficking (Zhao et al. 2002). More research will be needed to determine if this secondary

cleavage occurs and the relevance of cleavage at the CFCS prior to secretion of ZP glycoproteins and their incorporation into the ZP matrix.

Jovine et al. (2002) suggested that, like the ZP domain, the presence of a C-terminal transmembrane domain (TMD) is also required for ZP assembly. They demonstrated that mutant forms of ZP2 and ZP3, with the TMD region removed, were successfully secreted but not incorporated into the ZP matrix. It is now believed that the TMD is used to anchor the glycoproteins in secretory vesicles and the plasma membrane of the endoplasmic reticulum (Wassarman et al. 2005). Jovine and colleagues (2004) later discovered two other conserved and hydrophobic regions: the external hydrophobic patch (EHP) and internal hydrophobic patch (IHP). In their proposed mechanism for ZP protein assembly, Jovine *et al.* (2004) predict that the EHP functions as a control switch that prevents the premature polymerization of ZP precursors. When ZP glycoproteins are synthesized in the *trans*-golgi in the cytoplasm of the oocyte, premature assembly must be avoided while the proteins are still in the cytoplasm. This delay is achieved by the interaction of the EHP and IHP. Cleavage of ZP proteins by a furin-like enzyme leads to the loss of the EHP. After this cleavage, the glycoproteins can assemble into crosslinked filaments using the IHP within the ZP domain (Jovine et al. 2004).

Each ZP glycoprotein contains a hydrophobic N-terminal signal sequence that varies in length and targets nascent ZP glycoproteins to the secretory pathway (Wassarman et al. 2005). The signal peptide is cleaved off of the mature ZP glycoprotein, however, it is not known at which stage of ZP biosynthesis and assembly into the zona matrix, the signal peptide is removed.

The Assembly of a Three-Dimensional Zona Pellucida Matrix

In general, the ZP layer is a highly organized extracellular coat, consisting of long, interconnected fibrils or filaments that exhibit a structural repeat. The ZP is a pronounced

extracellular matrix and varies in thickness from less than 1 μm to more than 25 μm across studied mammalian species (Wassarman 1988). The 6 μm-thick mouse ZP has a sponge-like, porous structure, allowing relatively large molecules such as antibodies and small viruses to permeate through. In the mouse, it appears that ZPA and ZPC interact to form filaments that are, in turn, interconnected by disulfide-linked homodimers of ZPB1 (Greve and Wassarman 1985, Wassarman 1988). This mouse ZP assembly model is supported by several lines of evidence. The mouse ZP consists of equal amounts of ZPA and ZPC, with only 10% of the total mass being ZPB1 (Wassarman 1988). ZPB1-knockout mice have a thinner and more poorly defined ZP layer surrounding their oocytes and their fecundity is decreased compared to normal mice (Rankin et al. 1999). This latter study also implies that ZPB1 is not necessary for the interaction of ZPA and ZPC, but is vital for the structural integrity of the ZP matrix. In ZPA-null mice, ZPB1 interacts with ZPC to form a very thin ZP that breaks apart prior to the preovulatory phase, leaving ZPA-null mice sterile (Rankin et al. 2001). However, it is becoming increasingly apparent that the molecular mechanisms of mouse ZP assembly cannot be directly applied to other species because the human ZP consists of four ZP glycoproteins (Lefievre et al. 2004) while the frog vitelline envelope has five ZP proteins (Lindsay et al. 2002), and the chicken perivitelline envelope has at least six ZP glycoproteins (Hughes and Barratt 1999, Smith et al. 2005, Mann 2008).

Regulation of ZP Protein Synthesis

The mechanisms regulating the transcription on ZP genes are largely unknown for many species whose ZP genes have been cloned. Two ovary-specific DNA-binding proteins have been identified in the mouse; zona pellucida gene activating protein-1 (ZAP-1) binds to the conserved sequence 5'–CAC(G/C)TG-3' within 250 bp upstream of the mZPA and mZPC promotor

TATAA box (Millar et al. 1993), and oocyte-specific protein 1 (OSP-1) binds to the sequence 5'–GATAA-3' within the first 100 bp of the mZPC promoter (Schickler et al. 1992). Similar regulatory mechanisms controlling expression of the ZP genes may be conserved across mammals (Liang and Dean 1993, Millar et al. 1993, Buhi et al. 2000). Expression of mRNA for all three ZP proteins in mouse is developmentally regulated in coordinate as mouse ZPA, ZPB1, and ZPC mRNA expression increases together during the early stages of oogenesis and subsequently decline together (Epifano 1995).

There is also very little research on the hormonal regulation of ZP protein synthesis. Exogenous estradiol was found to increase hepatic expression of the ZP proteins in female fish as well as young male fish (Hyllner et al. 1991, Oppen-Burntsen et al. 1992, Larsson et al. 1994). Because estrogens affect hepatic 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). Bausek et al. (2000) reported that estradiol also stimulates hepatic production of ZPB1 in chickens. Quail ZPC production is stimulated by testosterone in cultured granulosa cells, but is unaffected by increasing concentrations of estradiol and progesterone (Pan et al. 2001).

Sperm-ZP Adhesion

The ZP layer acts as a protective barrier for the oocyte and sperm must hydrolyze a hole through the ZP before reaching the oocyte's plasma membrane. For successful fertilization to occur, the plasma membranes of sperm and egg must undergo several key steps before they fuse. The first step requires attachment of the sperm to the ZP layer of the ovulated oocyte, ensuring target specificity. This attachment occurs through protein-protein-mediated interactions between the plasma membrane of the sperm head and the ZP. Second, membrane apposition brings the

sperm and ZP closer together. At this point, the sperm is signaled to exocytose its acrosomal contents (Bleil and Wassarman 1983). This event, also named the acrosome reaction, is required for sperm in all species possessing sperm with an acrosome layer to become fusion competent. Lastly, lipid mixing occurs once the sperm inner acrosomal and oocyte plasma membranes fuse, leading to cytoplasmic continuity between the two cells (Stein et al. 2004) and sperm enter into the ovum.

The ZP glycoprotein component responsible for sperm recognition and binding and initiation of the acrosome reaction varies from species to species. Most of our understanding on the functional aspects of ZP glycoproteins during fertilization has been developed from the murine model. In the mouse, ZPC acts as the primary sperm receptor in the ZP and is responsible for inducing sperm acrosomal exocytosis (Bleil and Wassarman 1980a, 1983, 1986). Mouse ZPA acts as the secondary receptor for sperm and maintains the binding of the acrosome-reacted spermatozoa to the ZP (Bleil et al. 1988). The third protein component of the mouse oocyte ZP, ZPB, simply acts as a structural component of the ZP (Wassarman et al. 2001). Many studies done with mice have focused on the oligosaccharides linked to ZPC and have found O-linked oligosaccharides located near the C-terminus to be critical for sperm-egg recognition and initiating the sperm acrosomal reaction (Florman and Wassarman 1985, Chen et al. 1998, Kerr et al. 2004). Carbohydrates may not be the only factors involved in sperm-egg recognition. In both humans (Chapman et al. 1998, Chakravarty et al. 2005) and in mice (Ding et al. 2007) the ZP glycoprotein's polypeptide backbone also takes part in the species-specificity of the sperm-ZP interaction.

The human ZP is composed of four proteins ZPA, ZPB1, ZPB2, and ZPC (Hughes and Barratt 1999, Lefievre et al. 2004). Several studies indicate that ZPC can induce an acrosomal

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

The inability to clarify the specifics of sperm binding in humans may be due, in part, to research on the functional attributes of human ZP glycoproteins being hindered by the lack of human oocytes and thus the nonavailability of sufficient amounts of native purified human ZP proteins. Researchers have overcome this obstacle by resorting to recombinant DNA technology and the use of *Escherichia coli (E. coli)* and baculovirus expression systems. However the glycosylation of recombinant human ZP proteins are likely not exactly the same as native human ZP proteins and thus influence sperm binding artificially.

In non-human species, purified glycoproteins are comparatively more accessible and therefore the research is plentiful and has yielded results that support a great deal of variety in specific sperm-binding glycoprotein(s) across vertebrate species. Similar to humans, studies from various other species have suggested that more than one ZP protein is involved in binding to capacitated sperm and inducing the acrosome reaction. In the pig, a ZPB-ZPC heterodimer acts as the primary sperm receptor, binding with higher affinity to boar sperm-associated zona receptors than individual ZP subunits (Yurewicz et al. 1998). In addition, in contrast to mice, N-

linked carbohydrates, rather than O-linked carbohydrates, are involved in sperm binding to the ZP in pigs (Yonezawa et al. 1995). In the rabbit, both ZPB1 and ZPC have been found to bind sperm (Yamasaki et al. 1995), and baculovirus-expressed ZPB1 has been shown to bind to the sperm in a dose-dependent manner (Prasad et al. 1996). In *Xenopus laevis,* ZPA and ZPC are both individually able to bind sperm, yet ZPC appears to be the major ligand for sperm binding (Vo and Hedrick 2000). However, combining egg glycoproteins ZPA, ZPB, and ZPC in the natural ratio found in *Xenopus* egg envelopes, enhances sperm binding (Vo and Hedrick 2000). In the chicken, ZPC, ZPD and ZPB1 have each been reported to play a major role in initial interactions between sperm and egg (Bausek et al. 2004, Okumura et al. 2004).

Other vertebrate species appear to follow the previously discussed mouse model of having a single ZP protein acting as the sperm receptor and inducer of acrosome reaction. In the cow, ZPB1 exhibits the strongest sperm-binding activity among the three identified bovine ZP proteins (Yonezawa et al. 2001). In the quail, there is evidence that ZPB1 is responsible for activating the acrosomal reaction (Sasanami et al. 2007). Though most of our understanding on the functional aspects of ZP glycoproteins during fertilization has been developed from the murine model, it is clear that the sperm binding of ZP proteins vary greatly across species.

Practical Applications

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

the mouse with the active immunization directed against hamster zona pellucida (Gwatkin and Williams 1977, Gwatkin et al. 1977). Since these studies, numerous investigators have published reports detailing the effectiveness of using the development of antibodies against ZP proteins as an immunocontraceptive to induce infertility (as reviewed by Kirkpatrick et al. 2009).

The administration of a ZP-protein immunocontraceptive vaccine in wild populations as a humane alternative for population control has been demonstrated in several species such as feral horses (Bartholow 2007), white-tailed deer (Kirkpatrick et al. 1997), and dogs (Mahi-Brown et al. 1985). For wild animal immunocontraceptive vaccines, porcine zona pellucida (PZP) has become the antigen of choice due to the accessibility of porcine ovaries from abattoirs and the observation that antibodies developed against PZP prevents fertilization in a variety of species (Sacco 1977).

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

Studies performed in rabbits have implicated making antibodies against glycosylated ZP proteins in the pathogenesis of ovarian dysfunction associated with ZP antigen immunization,

and the authors of these research reports encourage the use of deglycosylated zona molecules in the continued efforts to develop a safe zona-based contraceptive vaccine (Keenan et al. 1991, Jones et al. 1992). However, using bacterially expressed recombinant ZP proteins, which are devoid of carbohydrates, do not induce an effective immune response (VandeVoort et al. 1995, Kaul et al. 1997). Also, their potential effectiveness might not be optimal because antibodies that were formed and bound at ZP glycosylation sites would more likely prevent the glycoprotein bases interaction between sperm and the ZP sperm receptor.

Summary

The ZP is a glycoprotein matrix that surrounds freshly ovulated oocytes. It functions as structural support for the oocyte and contains proteins that play a role in species specific sperm binding role during fertilization. In most species, the ZP coat consists of only 3 or 4 proteins called ZP proteins. However, the ZP proteins synthesized and utilized to make the ZP vary across vertebrate species. Once sperm are bound to ZP sperm receptors, the acrosome reaction is initiated which allows the sperm to penetrate the ZP and make contact with the plasma membrane of the oocyte for subsequent union of the male and female pronuclei.

CHAPTER 3

AVIAN ZONA PELLUCIDA PROTEINS

Identification of the Avian ZP Proteins

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

ZPC

The majority of the chicken's IPVL appears to be composed of two 42 kDa glycoproteins (ZPC and ZPD) and one 95 kDa glycoprotein (ZPB1) (Waclawek et al. 1998, Bausek et al. 2000, Okumura et al. 2004, Hughes 2007, Mann 2008). Based on sequence homology, one of the 42 kDa proteins was identified as being the chicken homologue of mammalian ZPC (Waclawek et al. 1998, Takeuchi et al. 1999). The mRNA transcript for chicken ZPC was detected by Northern Analysis in granulosa cells of the developing preovulatory follicles (Waclawek et al. 1998, Takeuchi et al. 1999). Western blot analysis with anti-chicken ZPC detected a 42 kDa protein in the IPVL isolated from large preovulatory follicles (Waclawek et al. 1998). In quail, both ZPC protein and RNA expression increases as the follicle matures towards ovulation (Pan et al. 2001). Interestingly, Western blot analysis of the IPVL isolated from freshly laid eggs revealed that chicken ZPC undergoes postovulatory modifications because anti-chicken ZPC antibodies only detected a single 34 kDa protein in these samples (Waclawek et al. 1998). This size reduction is independent of IPVL-sperm interaction since this smaller protein was detected in the IPVL of laid eggs from virgin hens (Waclawek et al. 1998). Pan et al. (2000) reported a similar size difference between quail ZPC detected in preovulatory follicles versus laid eggs. Both Pan et al. (2000) and Waclawek et al. (1998) suggested that the size modification is likely due to the presence of a protease secreted by the infundibulum. Pan et al. (2000), further suggested that, in addition to proteolytic processing involving the removal of the N-terminal amino acids, the molecular weight reduction of ZPC also resulted from N-linked and O-linked oligosaccharide chain modifications, however, Waclawek et al. (1998) reported that the size reduction was independent of oligosaccharide side chain modifications in the chicken.

ZPD

The mRNA for ZPD has been detected in preovulatory follicular granulosa cells of the chicken and quail (Okumura et al. 2004, Sato et al. 2009). ZPD, like chicken ZPC, is a 42 kDa protein (Okumura et al. 2004) and the two proteins co-migrate under reducing conditions. However, the two ZP proteins separate under non-reducing conditions due to a drastic and unexplained increase in ZPC mobility (Okumura et al. 2004).

ZPB1

A 97 kDa protein component of the IPVL was identified, isolated and then subsequently cloned from a liver cDNA library (Bausek et al. 2000). Based on the significant homology of the coding sequence of their isolated clone and mammalian ZPB1 sequences, the clone was classified as chicken ZPB1 (Bausek et al. 2000). Antisera to chicken ZPB1 bound a 97 kDa protein in immunoblot samples from the IPVL and liver but not from granulosa cell samples separated on SDS-PAGE gels under reducing conditions, indicating that chicken ZPB1 is synthesized in the liver and then transported to the ovary (Bausek et al. 2000). When IPVL and liver samples are separated on SDS-PAGE gels under non-reducing conditions and transferred to immunoblots, an additional 180 kDa band is detected (Bausek et al. 2000, Takeuchi et al. 2001). The detected 180 kDa peptide, which is approximately twice the mass of the 97 kDa peptide, was determined to be a dimer of the 97 kDa peptide formed through intermolecular disulfide bonds (Bausek et al. 2000, Takeuchi et al. 2001, Okumura et al. 2004). Similarly, ZPB1 in Japanese quail is transported to the ovary from the liver and has a high level of homology (87.8%) with chicken ZPB1 (Sasanami et al. 2003a). In fact, both ZPB1 and ZPC have been found to be sufficiently conserved across avian species to allow antibodies formed against the chicken

proteins to detect the orthologous proteins in other avian species including Galliformes, Anseriformes, Passeriformes and Columbiformes (Stewart et al. 2004).

ZPB2

In 1999, a cDNA sequence for ZPB was deposited in GenBank (Accession # AB025428) and subsequently renamed ZPB2 under the Spargo and Hope naming system (2003). Based on this sequence, Bausek et al. (2000) made a cDNA probe of ZPB2 for Northern analysis and detected expression of this message only in small stroma embedded follicles in the chicken ovary. Subsequently, by real time RT-PCR, Benson (2006) reported that ZPB2 mRNA expression was present in granulosa cells isolated from large preovulatory follicles of turkey hens and that the expression of ZPB2 was higher in granulosa cells isolated from the GD region than NGD regions of the F_1 and F_2 follicles.

ZPA and ZPX1

Smith et al. (2005) identified and subsequently mapped two additional ZP genes in the chicken genome: chicken ZPA and ZPX1. Although chicken ZPA and ZPX1 were classified as part of the chicken genome, expression analysis of these two ZP proteins in the chicken has yet to be characterized. In quail however, ZPA protein has been found to decrease dramatically during follicular development, with highest expression observed in the SWF (Kinoshita et al. 2010). In turkeys, ZPA and ZPX1 were both detected by real time RT-PCR in granulosa cells isolated from each of the hierarchical follicles (Benson 2006).

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 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 surrounding the developing oocyte. The chicken and quail, however, 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 also used in avian species for a vast majority of the yolk components that are deposited in the developing follicles (Lazier 1978, Nadin-Davis et al. 1980). Some species of teleosts such as the rainbow trout (Oppen-Burntsen et al. 1992),white flounder (Lyons et al. 1993) and medaka (Sugiyama et al. 1998) have also been found to express ZP proteins in the liver. However, these fish species rely exclusively on hepatic production of ZP proteins, while avian species produce only ZPB1 in the liver, and ZPA, ZPB2, ZPC, ZPD, and ZPX1 are produced by the granulosa cells of the developing preovulatory follicle (Waclawek et al. 1998, Bausek et al. 2000, Okumura et al. 2004, Benson 2006).

Hormonal Regulation of Avian ZP Proteins

Hormonal regulation of ZP proteins in avian species is not well characterized. Expression studies have revealed information on the transcriptional regulation of 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 results in a dramatic induction of ZPB1 production by the liver such that serum levels are elevated in these males to levels similar to those observed for an egg-producing laying hen (Bausek et al. 2000). Similar results were found in male quail after diethylstilbestrol treatment (Sasanami et al. 2003a). The induction of ZPB1 was so pronounced that Sasanami et al. (2003a) suggested that it could be used in males or immature females as a biological marker of environmental estrogen exposure.

In contrast to ZPB1, estrogen does not have an effect on ZPC expression in quail, (Pan et al. 2001). Quail treated with progesterone also have no significant increase in ZPC production (Pan et al. 2001). ZPC synthesis in cultured quail granulosa cells is stimulated by testosterone (Pan et al. 2001) and FSH (Pan et al. 2003). More recently, Benson (2006) found that ZPC and ZPD mRNA expression was up-regulated in chicken granulosa cells isolated from SYF and 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, while the mRNA expression of ZPD is higher in F_3 granulosa cells cultured with testosterone and estrogen (Benson 2006). In F_1 granulosa cells, LH lowered the mRNA expression of ZPC but not ZPD (Benson 2006). 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.

Secretion of ZP Proteins and Assembly of the IPVL

Avian ZPC is synthesized by the granulosa cells and is therefore an excellent model for studying the vectoral secretion of ZP proteins (Sasanami et al. 2003b). Avian granulosa cells in the hierarchical follicles are arranged on the surface of the oocyte as a single layer in mature follicles enabling several studies to investigate the intracellular trafficking and highly polarized secretion of avian ZPC. Waclawek et al. (1998) performed immunohistochemical staining on chicken granulosa cells and demonstrated the exclusive presence of chicken ZPC on the apical side of the granulosa cells. They proposed that hydrophobic domain near the carboxy-terminus of ZPC may serve as a glycosylphosphidylinositol (GPI) anchor which directs the newly synthesized ZPC to the apical side of the granulosa cells (Waclawek et al. 1998). Both quail and chicken ZPC have been reported to undergo post-translational proteolytic processing, which is a

common characteristic of GPI-anchored proteins (Jovine et al. 2005). For quail ZPC, this proteolytic processing of the C-terminal end is required for secretion and if this cleavage event is blocked, proZPC will accumulate in the endoplasmic reticulum (Sasanami et al. 2003b).

Once secreted, the avian ZP glycoproteins interact and assemble to form the three-dimensional network of fibers referred to as the IPVL or ZP in mammalian species. Assembly of the ZP matrix is best understood in the mouse where ZPC associates with ZPA and is cross-linked by ZPB1 to form filaments (Greve and Wassarman 1985, Wassarman 1988). Due to extensive variability in the ZP protein composition of the ZP across species, the mouse model of ZP assembly does not apply to all mammals or vertebrates. Jovine et al. (2004) proposed a regulatory mechanism of matrix assembly in the mouse that implemented two conserved ZP domains, the external hydrophobic patch (EHP) and the internal hydrophobic patch (IHP), with the two functioning as a control switch. The theory implicates EHP and IHP as binding partners and, when bound together, they prevent ZP assembly by folding the ZP domain of the pro-protein in half. Proteolytic processing cleaves the EHP, leading to the dissociation of the two domains and the mature ZP protein is then free to aggregate and form filaments (Jovine et al. 2004). This mechanism may also apply to chicken IPVL assembly as well. The high resolution crystal structure of chicken ZPC revealed the fold of the ZP domain that prevents premature incorporation of ZPC into the IPVL matrix (Han et al. 2010), supporting the IHP-EHP folding theory by Jovine et al. (2004). Even with this finding, little is known about the mechanism of IPVL assembly in birds. Ohtsuki et al. (2004) reported that granulosa cell cultures secrete ZPC in a soluble form, but ZPC becomes insoluble when IPVL homogenates are added to the cell culture media, implying that there are protein interactions leading to aggregations. In addition, chicken and quail ZPB1 and ZPC have been known to spontaneously form fibrous aggregates of

ZPB1-ZPC hetero-complexes *in vitro* through disulfide cross-linked bonds (Ohtsuki et al. 2004, Okumura et al. 2007b). As these ZPB1-ZPC complexes accumulate on the surface of the oocyte, they may act as scaffolding for subsequent matrix construction including ZPD association (Okumura et al. 2007a).

Sperm Binding to ZP Glycoproteins in Birds

In avian species, all three major ZP components of the egg envelope, ZPB1, ZPC, and ZPD, have been implicated in sperm-egg interaction (Bausek et al. 2004, Okumura et al. 2004). ZPC is the glycoprotein responsible for induction of the sperm acrosome reaction in the mouse, while ZPA acts as a secondary sperm receptor, maintaining the sperm/egg bond during early fertilization (Bleil and Wassarman 1980a, Bleil et al. 1988). Based on this model, researchers have investigated the role of ZPC in sperm binding to avian oocytes. Mori et al. (1998) and Pan et al. (1999) reported that antiserum against ZPC inhibits sperm binding and penetration of the IPVL in quail. Bausek et al. (2004) also presented evidence which suggested that ZPC may play a role in the sperm-egg interaction since chicken ZPC binds with far more affinity than ZPB1 to two isolated and purified chicken sperm proteins.

Both chicken ZPC and ZPB1 have an ability to bind individually to the acrosomal region of rooster sperm (Bausek et al. 2004). However, sperm binding to the IPVL leads to a proteolytic degradation of ZPB1 into discrete fragments, while chicken ZPC degradation is minimal (Bausek et al. 2004). The degradation of ZPB1 suggests that it may be degraded more during the acrosome reaction and therefore is more intimately involved in the sperm-IPVL interaction. Through these studies, Bausek et al. (2004) suggested that the binding of rooster sperm to the IPVL is mediated by both chicken ZPC and chicken ZPB1 with the initial adhesion occurring through ZPC. In addition, antiserum directed against ZPB1 significantly reduces the

number of sperm holes dissolved in the IPVL (Takeuchi et al. 2001, Bausek et al. 2004). Okumura et al. (2004) reported that dimeric ZPB1 was capable of stimulating the sperm acrosome reaction during an *in vitro* sperm activation assay. In experiments with two genetic lines of turkey hens that differ in female fertility, Benson et al. (2009) found higher levels of hepatic ZPB1 mRNA expression in the genetic line with higher fertility. These results imply that ZPB1 expression is correlated with better fertility in turkeys. However a role for ZPC was also implicated in the work by Benson et al. (2009) because hens from the genetic line with low fertility had higher levels of ZPC mRNA expression in NGD granulosa cells than GD granulosa cells. In contrast, the hens from the high fertility line had equal levels of ZPC mRNA expression between GD and NGD granulosa cells.

Benson et al. (2006) also examined ZPB2 expression in the two lines of turkey hens that differ in fertility. ZPB2 mRNA was higher in GD granulosa cells than NGD granulosa cells in both lines of 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, since sperm preferentially bind at the GD region (Howarth and Digby 1973, Bramwell and Howarth 1992, Birkhead et al. 1994, Wishart 1997).

One of the more recently discovered chicken IPVL glycoproteins, ZPD, was reported to play a role in the sperm egg-interaction immediately following its identification (Okumura et al. 2004). ZPD was found to bind loosely to the egg envelope matrix and stimulate *in vitro* sperm acrosome reactions more than ZPC and monomeric ZPB1 (Okumura et al. 2004). N-linked glycans have been shown to be vital in avian sperm-egg interactions (Horrocks et al. 2000, Robertson et al. 2000) and chicken ZPD has the most potential N-glycosylations sites of any of the known avian ZP glycoprotein. Benson et al. (2006, 2009), however, did not find a significant

difference in ZPD mRNA expression between the two genetic lines of turkey hens that differ in female fertility.

Oligosaccharides in Sperm Binding

Research on the carbohydrate moieties of ZP glycoproteins has consistently supported their necessity for successful sperm binding and penetration of the IPVL. Chicken ZPD, ZPB1, and ZPC proteins have four, three, and one potential N-glycosylation sites, respectfully (Okumura et al. 2004). In chicken, the removal of both N- and O-linked oligosaccharides from the IPVL results in a loss of sperm receptor activity (Howarth 1992). More specifically, Robertson et al. (2000) reported that the removal of N-linked, but not O-linked oligosaccharides led to an inhibition of avian sperm-IPVL interaction. In the well-studied murine model of sperm and egg binding, it is O-glycans that are important for sperm-egg interaction (Wassarman 1988). Horrocks et al. (2000) reported that the acrosomal reaction in chickens is induced by N-linked glycans which have a terminal N-acetyle-glucosamine residue. However, a more recent study with the high resolution crystal structure of chicken ZPC indicated that the elimination of a specific O-glycan led to a 80% decrease in sperm binding to ZPC (Han et al. 2010). Obviously, which ZP glycoprotein or proteins of the IPVL carries the carbohydrate moiety responsible for sperm-IPVL binding has yet to be determined.

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. There is evidence suggesting that ZPB1, ZPC, ZPB2 and ZPD may all play a role in sperm binding and induction of the

acrosome reaction. More research is needed to establish the exact ZP protein interactions involved in sperm binding and penetration of the IPVL in avian species.

CHAPTER 4

STATEMENT OF PURPOSE

When a follicle is ovulated in an avian species, it is surrounded by the IPVL. For successful fertilization, sperm must attach to and penetrate the IPVL. Sperm penetration of the IPVL is concentrated in the area around the GD of the follicle which contains the female pronucleus. Three of the protein constituents of the IPVL are ZPC, ZPB1 and ZPB2. ZPC and ZPB2 are synthesized and secreted by the granulosa cells of the developing follicle while ZPB1 is produced by the liver and transported to the developing follicle. In mammalian species, both ZPC and ZPB1 have been reported to possess sperm binding activity, and to assist with sperm penetration of the follicle.

Previously our laboratory conducted a series of studies collaborating with researchers at North Carolina State University to investigate the mRNA expression of ZPB1, ZPB2, and ZPC in two genetic lines of turkey hens selected for over forty generations for either increased egg production (E-line) or increased body weight (F-line). As a result of this genetic selection, hens from the E-line have a higher rate of fertility than hens from the F-line. Hepatic expression of the mRNA for ZPB1 was significantly greater in turkey hens from the E-line than the F-line (Benson 2006). The total mRNA expression of ZPC was equal between the two genetic lines of turkey hens. However, the distribution of the message within a follicle was not equal. Turkey hens from the F-line expressed a greater amount of ZPC in NGD regions than in the GD region. Turkey hens from the E-line had equal expression of ZPC between the GD and NGD regions of the preovulatory follicle (Benson 2006). The mRNA expression of ZPB2 was greater in GD

granulosa cells than NGD granulosa cells for both genetic lines. However, there was an indication that ZPB2 mRNA expression might decrease with follicular development (Benson 2006). These results suggested that the higher rates of fertility previously observed for eggs from the E-line versus the F-line of turkeys is related to differences in the expression of ZPB1 and ZPC. In addition, the preferential attachment of sperm to the GD region may be related to the higher expression of ZPB2 in this area. Finally the results also suggested that ZPB1 and ZPC mRNA expression could be used as genetic markers for selecting female lines of hens for greater fertility.

Although the turkey industry is an important component of the poultry industry, the broiler industry far exceeds turkey production, but shares the same problems with bird fertility. Therefore, given the results of our previous research the goals of the current research were (1) to determine if the mRNA expression of ZPB2 decreases with follicular maturity in broiler breeder hens, (2) to determine if the mRNA expression of ZPB1, ZPB2 and ZPC differ in four genetic lines of broiler breeder hens (3) to produce antibodies and verify their ability to detect ZPB1, ZPB2 and ZPC in turkey or chicken IPVL samples so that they could subsequently be used to determine if differences detected in the mRNA expression of these proteins were also present at the protein expression level.

CHAPTER 5

MATERIALS AND METHODS

Experiment 1: Characterization of ZPB2 mRNA Expression during Follicular Development in the Broiler Breeder Hen

Tissue Collection

The mRNA expression profile of ZPB2 was investigated in hierarchical and prehierarchical follicles in the broiler breeder hen. The broiler breeder hens were killed by cervical dislocation. The 4 largest follicles (F_1 to F_4), small yellow follicles (SYF, >5 to 12mm in diameter), and large white follicles (LWF, >2-5 mm in diameter) were removed from 8 Cobb 500 slow feathering hens at 45 week of age, 2 to 4 hours prior to ovulation. The granulosa cell layer was manually separated from the theca cell layers of each hierarchical follicle (Huang and Nalbandov 1979) and the theca and granulosa cells were enzymatically separated in the LWF and SYF as previously described (Davis et al. 2000). The granulosa and theca samples from 2 individual birds were pooled to generate 4 replicate samples of theca and granulosa tissue for each follicle size, except for the theca samples from the SYF and LWF which were not saved. The other theca samples were homogenized for 30 seconds with a PowerGen 700 tissue disrupter (Fisher Scientific, Pittsburgh, PA). The granulosa and theca samples were frozen and stored at -80°C in 1 mL of guanidinium isothiocyanate solution for subsequent RNA extraction. All experimental procedures for the broiler breeder hens were approved by the University of Georgia Animal Care and Use Committee.

Experiment 2: Characterization of ZPB2 mRNA in Theca and Granulosa Cells of Prehierarchical Follicles from Broiler Breeder Hens

Tissue Collection

This experiment followed a similar protocol as experiment 1, except that only the F_4 follicle, SYF and LWF were collected from broiler breeder hens at 47 week of age. The SYF were segregated based on size into the following 2 categories: >5 to 8 mm in diameter and >8 to 12 mm in diameter. Similarly the LWF were divided into size categories of <2 mm in diameter and >2 to 5 mm in diameter. The theca samples from each of the SYF and LWF size categories were saved and homogenized for RNA extraction. The granulosa and theca samples were frozen and stored at -80°C in 1 mL of guanidinium thiocyanate solution for subsequent RNA extraction.

Experiment 3: Expression of ZP Proteins in Four Genetic Lines of Broiler Breeder Hens

Animals

Pullets from 4 genetic strains of broiler breeder hens designated B, O, R, and G were obtained from a commercial hatchery and reared according to the recommended guidelines of the primary breeder for each genetic line using a 4/3 skip-a-day feed restriction program. At 21 weeks of age, the pullets were separated into 2 body weight categories: control (at or below primary breeder recommended target weight) and heavy (above primary breeder recommended target weight). The pullets in the heavy category were fed to maintain the 300 gram weight differential between the two weight categories for the rest of the experiment. One hundred pullets from each strain (50 heavy and 50 control) were placed into individual cages in random blocks of 10 cages (5 replicate blocks of cages for each weight category within each strain and 10 replicate blocks of cages per strain). Additional hens were housed in floor pens and were used to replace caged hens that died or ceased egg production during the trial that lasted until 65

weeks of age. At 21 weeks of age the pullets were photostimulated by providing 14 hours of light (14 light:10 dark) with 30 minute light increases given at 10 day intervals until 16 hours of light (16 light:8 dark) per day was reached. At 30 weeks of age all caged hens were artificially inseminated with 100 million sperm in a 50 µl volume from a pooled semen sample. Inseminations continued at 5 week intervals until 60 weeks of age. All eggs were recorded and collected daily to determine fertility by day post-insemination. All animal procedures were approved by the University of Arkansas Animal Care and Use Committee.

Tissue Collection

To determine if there were differences in the mRNA expression of ZPC, ZPB2, and ZPB1 in the four genetic lines of broiler breeder hens, granulosa and liver samples were collected from 18 hens at 65 weeks of age from each weight category from each genetic line. Hens were killed 2-4 hours prior to ovulation by CO_2 inhalation. For each hen, the F_1 follicle was removed and the connective tissue and theca cell layers were removed. Then, a one cm^2 section of the granulosa layer around the GD and an equivalent sized NGD area on the opposite side of the follicle to the GD region were collected. In addition, approximately 100 mg of tissue was removed from the left lobe of the liver from each hen. To obtain enough RNA for subsequent real time RT-PCR, granulosa and liver samples from 2 birds within each weight category for each genetic line were pooled into a single replicate tube (n = 9). All isolated granulosa cell samples and liver samples were stored in 1 mL of RNA*later* (Ambion, Austin, TX) for subsequent RNA extraction.

RNA Extraction and Two-Step RT-PCR

For Experiments 1-3, total RNA was extracted from each of the samples using the guanidinium isothiocyanate-phenol-chloroform method (Chomczynski and Sacchi 1987).

Taqman minor groove-binding probes and primers for detecting ZPB1, ZPB2, ZPC and GAPDH (endogenous control) were designed using Primer Express (Version 2.0, Applied Biosystems) based on published nucleotide sequences of these chicken genes (Table 1). All assays 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, 50, 100, and 150 ng of cDNA was used for each sample for the ZPC, ZPB1, and ZPB2 amplification, respectively. The GAPDH amplification utilized 50, 100, or 150 ng per sample based on the amount of cDNA utilized in the corresponding ZP protein amplification. The reactions were performed in a 25 µl volume of reaction buffer containing1x TaqMan Universal PCR Master Mix (Applied Biosystems) and 900 nM of either ZPB1, ZPB2, ZPC 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 Sequence Detection software (version 1.2.2, Applied Biosystems), and quantification was completed using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001). Briefly, the ZPB1, ZPB2 and ZPC C_Ts were determined for each sample and

Table 1. Oligonucleotide primer pairs and probes for real time PCR

Product	Primer	Oligonucleotide Primer and Probe Sequence	Product Size (Base Pairs)
ZPB1	Forward	5'-TTG GCA CCC GCT TCG A-3'	
	Reverse	5'-CAC CGT CCT CCC CAG TGT T-3'	69
	Probe	5'-ACT GCT CCA TCT GCA-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'-AAG GCA CAT GCT CTG-3'	
ZPC	Forward	5'-GTG ATG ACT GGA GCA CAG AGA GA-3'	
	Reverse	5'-GGC CTG GAT GTT GAG GAT GT-3'	68
	Probe	5'-CTT CAC CGG CTT CCA-3'	
GAPDH	Forward	5'-TTG GCA TTG TGG AGG GTC TT-3'	
	Reverse	5'-GGG CCA TCC ACC GTC TTC-3'	87
	Probe	5'-TGA CCA CTG TCC ATG CCA T-3'	

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). After all the ΔC_T values were obtained for an experimental replicate the ΔC_T values for each individual ZP protein were compared to the sample within the replicate that had the highest mRNA expression for that individual ZP protein using the $2^{-\Delta\Delta\ C_T}$ method. Thus, all data for each individual ZP protein is expressed as the fold-difference relative to sample with the highest expression.

Experiment 4: Cloning the Full Length Coding Sequences of ZPB1, ZPB2, and ZPC for Protein Expression and Subsequent Antibody Production

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Primers for RT-PCR were designed based on reported chicken cDNA sequences for ZPB2 (GenBank accession # AB025428), ZPB1 (GenBank accession # AJ289697) and ZPC (GenBank accession # AB031033). The forward primer was designed to contain a BamHI restriction site followed by the start codon of the appropriate ZP protein (Table 2). The reverse primer was designed to contain a XhoI restriction site followed by the stop codon of the appropriate ZP protein (Table 2). Each of the primer sets were synthesized by the University of Georgia Genomics Facility (Athens, GA). Reverse transcription reactions were performed as previously described (Davis and Johnson 1998) for ZPB2 and ZPC with total RNA extracted from granulosa tissue. For ZPB1, an aliquot of a purified preparation of pBlueScript plasmid containing a full length cDNA clone of chicken ZPB1 was used [generously provided by Dr. Nina Bausek from the University and Biocenter of Vienna (Bausek et al. 2000)].

Polymerase chain reactions (PCR) were conducted as previously described (Davis and Johnson 1998) with annealing temperatures of 45°C for ZPB2, 50°C for ZPB1 and ZPC. The annealing temperatures were lower than normal to allow for binding of the primers which were

Table 2. Oligonucleotide primer pairs for cloning

Product	Primer	Oligonucleotide Primer and Probe Sequence
ZPB1	Forward	5'-CGG'GATCCG**ATG**GGCCGCAGCCGCTCCCCTGCTG-3'
	Reverse	5'-TTAC'TCGAG**TTA**ACGCCTCCCTTCTGCAGGACC-3'
ZPB2	Forward	5'- CCG'GATCCG**ATG**GGTGTTGTAGGGCAGGCCATGGC-3'
	Reverse	5'-TAC'TCGAG**TCA**CTTCCTCCACCACAGCACAGCAGCAGCCACC-3'
ZPC	Forward	5'- CGG'GATCCG**ATG**CTGGGTGAGCTGGCAGCAGGC-3'
	Reverse	5'- TTAC'TCGAG**TCA**CACCGCAGTTGCGGTTCGGGTGCATC-3'

The BamHI (forward primer) and XhoI (reverse primer) restriction cut sites are denoted by '. Start and stop codons are shown in bold.

not complimentary at the 5' ends because of the extra bases added for the restriction sites. To produce sufficient quantity of each cDNA product for sequencing and further cloning activities, each PCR product was cloned into a pGEM-T vector (Promega, Madison, WI) and expressed in DH5-α cells according to manufacturer's instructions. The expected DNA sequences were verified by the University of Georgia, Georgia Genomics Facility (Athens, GA).

Protein Expression, Electrophoresis, and Immunoblotting

The cDNA clone for each ZP protein was then digested from the pGEM-T vector with BamHI and XhoI and inserted via the same restriction sites into the pET28b$^{(+)}$ protein expression vector (Novagen, Darmstadt, Germany) following the manufacturer's protocol. Recombinant ZPB1, ZPB2, and ZPC proteins were expressed in *Escherichia coli* BL21(DE3) cells (Novagen) following the pET28b$^{(+)}$ manufacturer's expression protocol. In brief, once the BL21 cells multiplied to have an optimal density of 0.6 at 600 nm, production of the ZP protein was induced with Isopropyl-β-D-thiogalactopyranoside (IPTG). One mL of media from the induced cells was collected at 0, 1, 2, 3, and 12 hours post induction. The media from each collection was centrifuged for 2 minutes at 2700 g at 4°C to pellet the cells. The supernatant was discarded and 200 µl of SDS-PAGE sample buffer (63 mM Tris HCl, 10% Glycerol, 2% SDS, 0.0025% Bromophenol Blue, pH 6.8) and 2-mercaptoethanol (0.5% BME) was added to the pellet. The resuspended pellet was sonicated for three, 4 second pulses for a total of 12 seconds. The sonicated sample was centrifuged at 20,000 g at 4°C for 5 minutes. The supernatant was saved and then heated to 100°C for 2 min before being electrophoretically separated using a 12.5% SDS-polyacrylamide gel (Laemmli 1978). For immunoblotting, the proteins from the gel were electroblotted onto a polyvinylidene fluoride (PVDF) membrane (Immobilon-P: Millipore, Bedford, MA). After blocking with 3% gelatin (Fisher Scientific), the membrane was incubated

with T7 Tag Antibody Alkaline Phosphotated Conjugate (Novagen), and visualized with chromogenic substrate by combining Alkaline Phosphatase Assay Buffer (1M Tris, pH 9.5, 5M NaCl, 1M $MgCl_2$, ddH_2O) with 0.0066% nitro-blue tetrazolium chloride (NBT, Fisher Scientfic) and 0.0034% 5-bromo-4-chloro-3'-indolphosphate ptoluidine salt (BCIP, Fisher Scientific).

Experiment 5: ZPB1, ZPB2, ZPC Protein Characterization in Two Genetic Lines of Turkey Hens

Animals

In order to obtain follicular and egg tissue samples for subsequent Western blot analyses of the ZP proteins, two lines of turkey hens that differ greatly in body weight and egg production were used. The egg line, or E-line, was selected by McCartney et al. (1968) from an established random bred control line (McCartney 1964), while the growth line, or F-line, was selected for increased 16 week body weight (Nestor 1977). Selection for increased body weight in the F-line resulted in reduced egg production in this genetic line due to a decrease in the intensity of egg laying as measured by average clutch size (Nestor et al. 1996, 2000). In contrast, selection for increased egg production in the E-line has greatly reduced broodiness while vastly increasing the intensity of egg laying in the hens of this genetic line (Anthony et al. 1991, Nestor et al. 1996). Eggs produced by the F-line hens also have lower rates of sperm penetration of the IPVL and fertility than the eggs produced by the E-line hens (Nestor and Noble 1995, Christensen et al. 2005).

In 2007, E-line and F-line poults were hatched at the Ohio Agriculture Research and Development Center and were soon thereafter shipped to the North Carolina State University Turkey Educational Unit. The turkeys were raised in floor pens and provided 10 hours of light per day until 25 weeks of age when the hours of light were reduced to 8. At 31 weeks of age, the

turkey hens were moved to breeding pens (6 birds per pen) and photo-stimulated for reproduction by providing them 14 hours of light per day. Each breeding pen was equipped with a nest box. The turkeys were provided with free access to appropriate commercial diets and water at all times through rearing and production. All animal procedures were approved by the North Carolina State University Animal Care and Use Committee.

Tissue Collection

Unfertilized eggs produced from the hens from each genetic line were collected over a two day period when the hens were 41, 43, and 44 weeks of age. Each egg was broken and the yolk was separated from the albumen. The yolk was placed into Krebs-Ringer bicarbonate buffer (pH 7.4). A one cm^2 section of the perivitelline layer around the GD and a NGD area, on the opposite side of the follicle to the GD area, was collected. To obtain enough protein for subsequent Western Blot analyses, the perivitelline layer samples from the GD and NGD regions were pooled from 3 birds of the same genetic line for each sample. All isolated perivitelline layer samples were frozen and stored at -80°C in 150 µL of lysis buffer containing protease inhibitors (Shirley et al. 2003).

Turkey hens (46 weeks of age) were killed by electrocution 2 to 4 hours before ovulation. The F_1 and F_2 follicles were removed from 20 E-line and 20 F-line hens for isolation of the granulosa cell layer. A one cm^2 section of the granulosa layer surrounding the GD area and a NGD area, on the opposite side of the follicle to the GD area, of the F_1 and F_2 follicles was collected. To obtain enough protein for subsequent Western Blot analysis, the granulosa samples from the GD and NGD regions were pooled from 2 birds for each follicle size. All isolated granulosa cell samples were frozen and stored at -80°C in 150 µL of lysis buffer containing protease inhibitors (Shirley et al. 2003).

Experiment 6: Synthesis of Synthetic Peptides for ZPB1, ZPB2, and ZPC and Antibody Production

Peptide fragments were selected for ZPB1, ZPB2, and ZPC which were 10-21 amino acids in length and did not contain potential glycosylation sites, potential phophorylation sites, or many hydrophobic amino acids. The potential protein fragments for each ZP protein of interest also had to have 100% sequence identity between chicken and turkey, so that antibodies raised against the protein fragment would potentially work in both species. The potential peptide fragments were also chosen in consultation with Bio-Synthesis, Inc. (Lewisville, TX). Once potential sequences were identified for each ZP protein, a BLAST homology (http://www.ncbi.nlm.nih.gov/) search of the selected peptides was performed against all proteins identified in the chicken genome in order to exclude future unwanted cross-reactions to other proteins with the antibodies produced for the specific ZP protein. The peptide sequence chosen for ZPB1 was PAGYEILRDEKVHGHQRPDRG-amidated which corresponds to amino acids 127-147 of the mature protein. The peptide sequence selected for ZPB2 was ATPSINPHQQTQWPVLVNG-amidated which corresponds to amino acids 378-396 of the mature protein. The peptide sequence picked for ZPC was acetylated-SWGAEAHSRAVAGSHPVAVQC which corresponds to amino acids 47-67 of the mature protein. The chosen ZPB1, ZPB2, and ZPC peptide fragments were synthesized by Bio-Synthesis, Inc. to a purity of >90%.

A portion of each of the synthesized protein fragments was then conjugated to Keyhole limpet hemocyanin (KLH) and then used by Bio-Synthesis, Inc to produce rabbit polyclonal antibodies. Conjugated synthetic ZP peptides were injected into rabbits with Freund's complete adjuvant at week 0, and Freund's incomplete adjuvant at weeks 2, 4, 6, and 8. Two New

Zealand White female rabbits at approximately 12 weeks of age were immunized for each ZP peptide (six rabbits total). Serum was collected at 0, 6, 8, and 10 weeks post initial injection and shipped to the University of Georgia. All rabbits were exsanguinated 12 weeks post initial injection and the serum was shipped to the University of Georgia.

ECL Western Blotting

SDS-PAGE gels containing 40 µg of turkey IPVL and/or granulosa cell samples were transferred to PVDF membrane (Immobilon-P) as previously described (Shirley et al. 2003). In order to determine the kilodalton (kDa) size of turkey ZPB1, ZPB2, ZPC protein, either MagicMark XP Western Protein Standard (Invitrogen, Carlsbad, CA) or Spectra Multicolor Broad Range Protein Ladder (Fermentas, Glen Bernie, MD) was electrophoresed in one lane of each gel. After blocking for 1 hour in Tris buffered saline, pH 7.6, containing 5% non-fat dried milk (LabScientific, Inc., Livingston, NJ), the protocol and reagents for the Amersham ECL (enhanced chemiluminescence) Plus Kit (Amersham Biosciences, Piscataway, NJ) along with goat anti-rabbit IgG-HRP conjugate (BioRad, Hercules, CA) were utilized to detect ZPB1, ZPB2, and ZPC proteins using the antibodies produced against the synthetic peptides for these proteins. Chemiluminescence was detected by using Super RX Fuji Medical X-Ray Film. Films were pan-developed using Kodak GBX developer and fixer/replenisher.

Statistics

Data in Experiments 1-3 were subjected to ANOVA according to the General Linear Model (GLM) with replicate and tissue as factors in Experiments 1 and 2 and with genetic lines, weight categories, and granulosa cell location as factors in Experiment 3. Single degree of freedom tests were used to determine significant differences in Experiment 3. Tukey's multiple comparison procedure (Neter et al. 1990) was used to detect significant differences among

genetic lines, hen weight categories, 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).

CHAPTER 6

RESULTS

Experiment 1

In the broiler breeder hen ZPB2 mRNA was not detected in theca cells isolated from the F_1, F_2, F_3 or F_4 follicles (Figure 1). Granulosa cell expression of ZPB2 was most abundant in the LWF followed by the F_4 follicle (Figure 1), but ZPB2 mRNA was not detected in the granulosa cells from the SYF (Figure 1).

Experiment 2

Theca cells isolated from LWF that were less than 2mm in diameter expressed the greatest amount of ZPB2 mRNA (Figure 2). Theca expression of the mRNA for ZPB2 decreased with follicular maturity and was not detected in the theca cells isolated from SYF that were greater than 8 mm in diameter (Figure 2). Similarly, the granulosa cell expression of the mRNA for ZPB2 decreased as LWF matured and it was not detected in SYF, but it was detected in granulosa cells from F_4 follicles (Figure 2).

Experiment 3

ZPC Expression

There were no differences in the overall expression of the mRNA for ZPC in F1 follicle granulosa cells between the 4 genetic lines of broiler breeder hens (Figure 3). Overall expression of ZPC mRNA was greater in NGD granulosa cells than the GD granulosa cells (Figure 4). However, in line O, the difference in the mRNA expression of ZPC between the GD and NGD granulosa cells was not present (Figure 5). There were no differences in the expression of ZPC

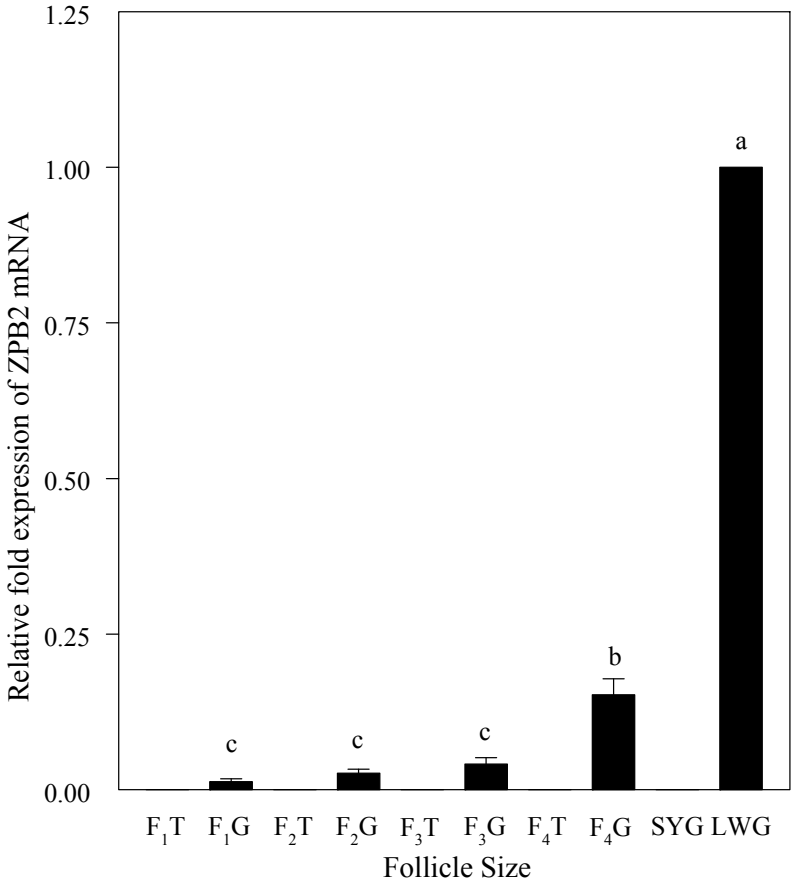

Figure 1. The relative expression of ZPB2 mRNA in granulosa (G) and theca (T) cells isolated from individual F_1 to F_4 follicles and in granulosa cells isolated from small yellow (SY) and large white (LW) follicles from broiler breeder hens. The mRNA expression data were normalized to glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference $\Delta\Delta C_T \pm$ SEM, n = 4. $^{a\text{-}c}$Means with different letters differ, $P < 0.05$. Expression of the mRNA for ZPB2 was undetectable in all the T samples and in the G sample from the SY follicles.

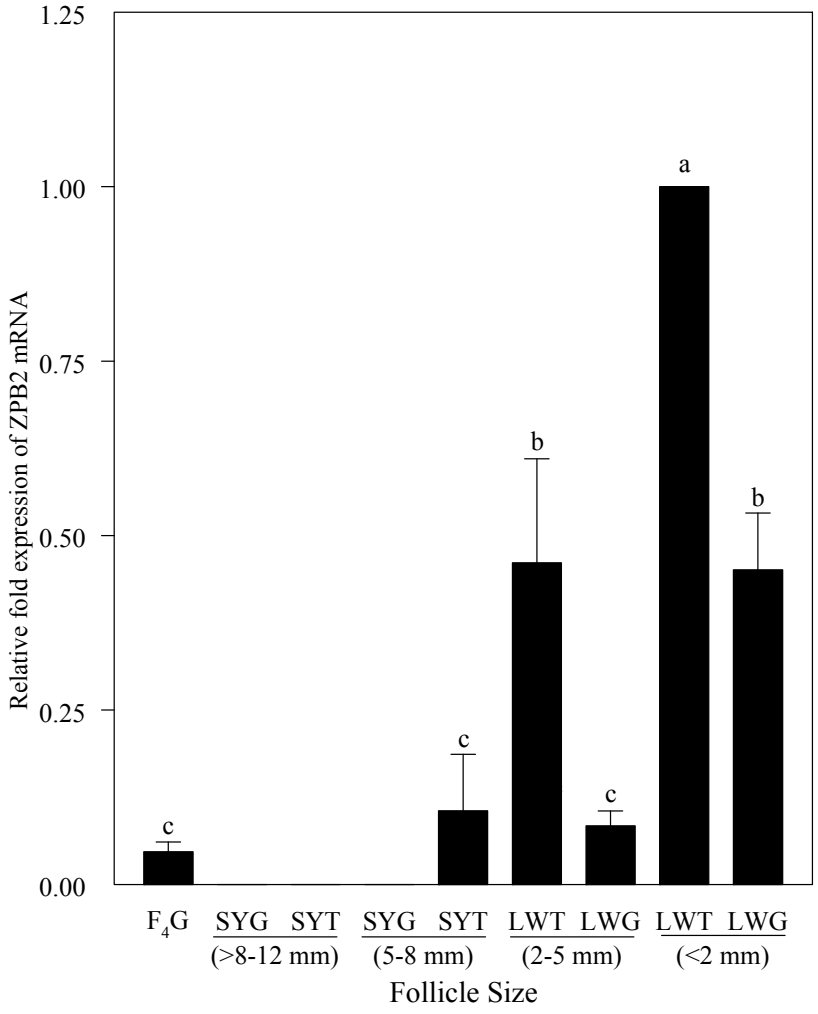

Figure 2. The relative expression of ZPB2 mRNA in granulosa (G) cells isolated from the F_4 follicle and in G and theca (T) cells isolated from small yellow (SY) and large white (LW) follicles from broiler breeder hens. The mRNA expression data were normalized to glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference $\Delta\Delta C_T \pm$ SEM, n = 4. $^{a\text{-}c}$Means with different letters differ, $P < 0.05$. Expression of the mRNA for ZPB2 was undetectable in T and G from SY follicles that were >8 to 12 mm in diameter, and in G from the SY follicles that were 5 to 8 mm in diameter.

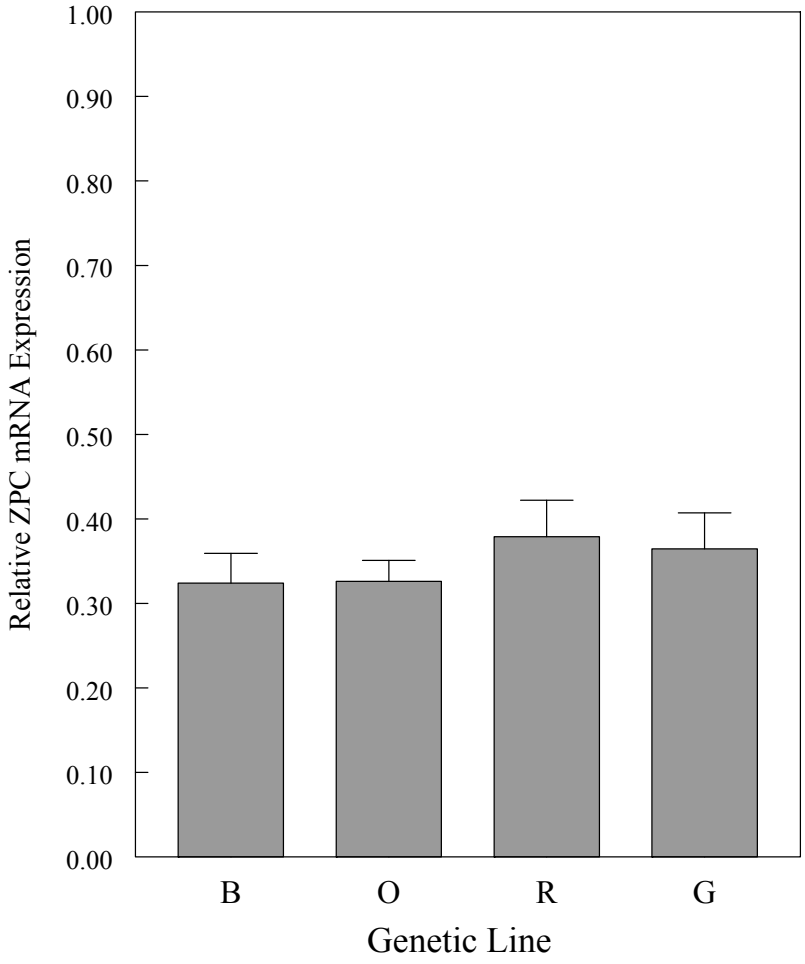

Figure 3. Overall relative expression (includes both GD and NGD) of the mRNA for ZPC in the granulosa cells of the F_1 follicle isolated from four genetic lines of broiler breeder hens maintained in two different body weight categories. Values are means ± SEM. There are 18 replicate GD and 18 replicate NGD samples (total n = 36 samples) for each genetic line.

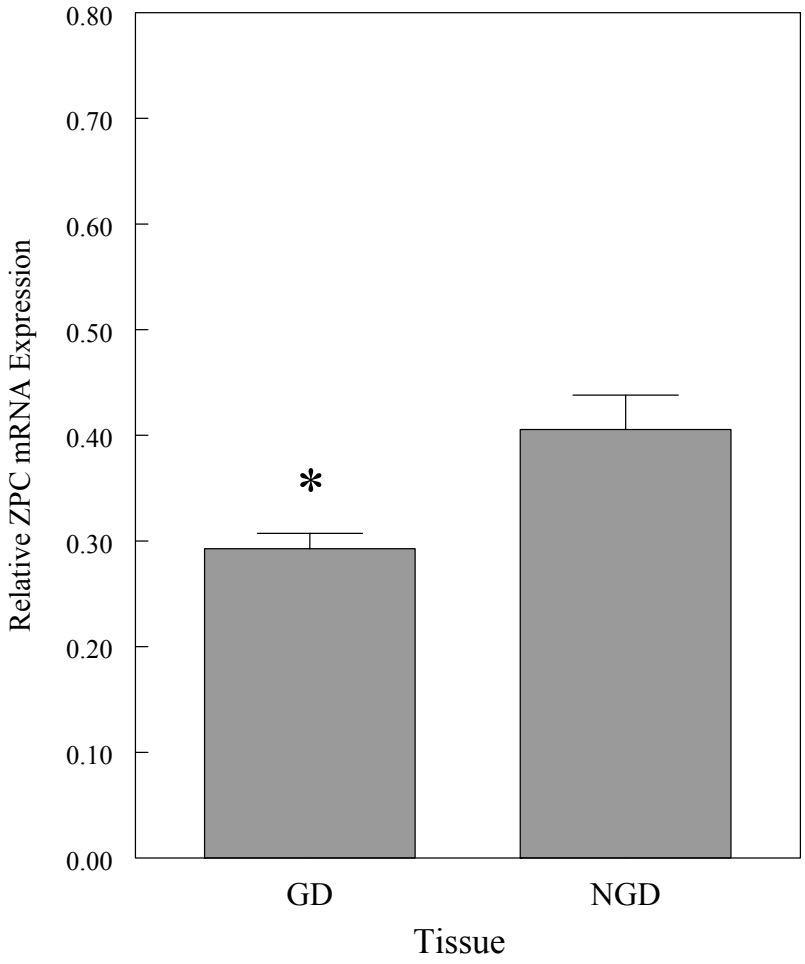

Figure 4. The relative expression of the mRNA for ZPC in the granulosa cells of the F_1 follicle isolated from four genetic lines of broiler breeder hens maintained in two different body weight categories. Values are means ± SEM. There are 72 replicate GD and 72 replicate NGD samples (18 samples from each genetic line) for each granulosa cell location. *Indicates significant difference $p < 0.05$.

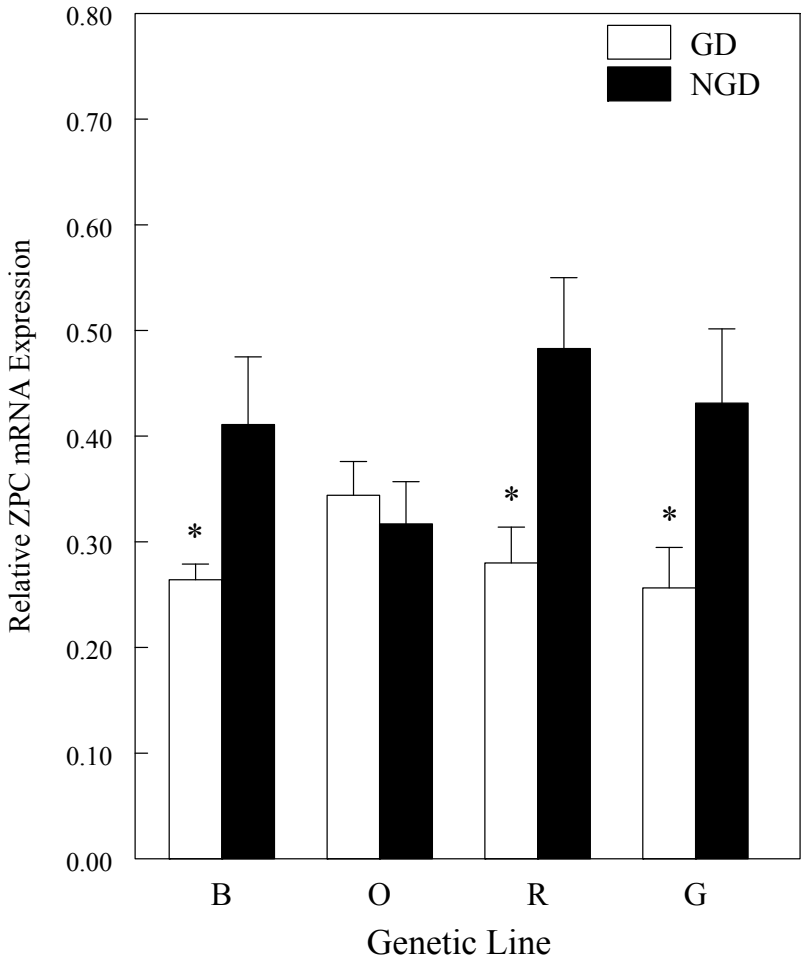

Figure 5. The relative expression of the mRNA for ZPC in the granulosa cells of the F_1 follicle isolated from four genetic lines of broiler breeder hens maintained in two different body weight categories. Values are means ± SEM. There are 18 replicate GD and 18 replicate NGD samples for each granulosa cell location for each strain. GD bars marked with an * indicates significant difference $p < 0.05$ from the corresponding NGD value within a genetic line.

mRNA in GD and NGD granulosa cells between control hens and heavy body weight hens (Figure 6).

ZPB2 Expression

There were no differences in overall expression of the mRNA for ZPB2 in F1 follicle granulosa cells between the 4 genetic lines of broiler breeder hens (Figure 7). Overall expression of ZPB2 mRNA was greater in GD granulosa cells than NGD granulosa cells (Figure 8), but this difference in GD and NGD expression was not significant for the smaller number of samples examined within each line (Figure 9). There were no differences in the expression of ZPB2 mRNA in GD and NGD granulosa cells between control and heavy hens (Figure 10).

ZPB1 Expression

Broiler breeder hens from line B had greater overall hepatic expression of ZPB1 than hens from line O (Figure 11). Control hens tended to have a higher expression of ZPB1 mRNA (Figures 12 and 13) than heavy hens with the exception of the hens from line G, which had the opposite expression trend for ZPB1 (Figure 13). If the expression data from line G is excluded, overall ZPB1 mRNA expression is higher for the control body weight hens compared to the heavy body weight hens (Figure 12).

Experiment 4

Based on sequence analysis, full length cDNA clones for ZPB1, ZPB2, and ZPC were successfully made with the desired restriction enzyme site placed before the start and stop codons (data not shown). These cDNA clones were then successfully subcloned into a protein expression vector and expressed. However, none of the three expressed ZP protein products were able to be readily solublized from the BL21 (DE3) cells that had been induced to express each ZP protein. A very small amount of soluble recombinant protein for each ZP protein was

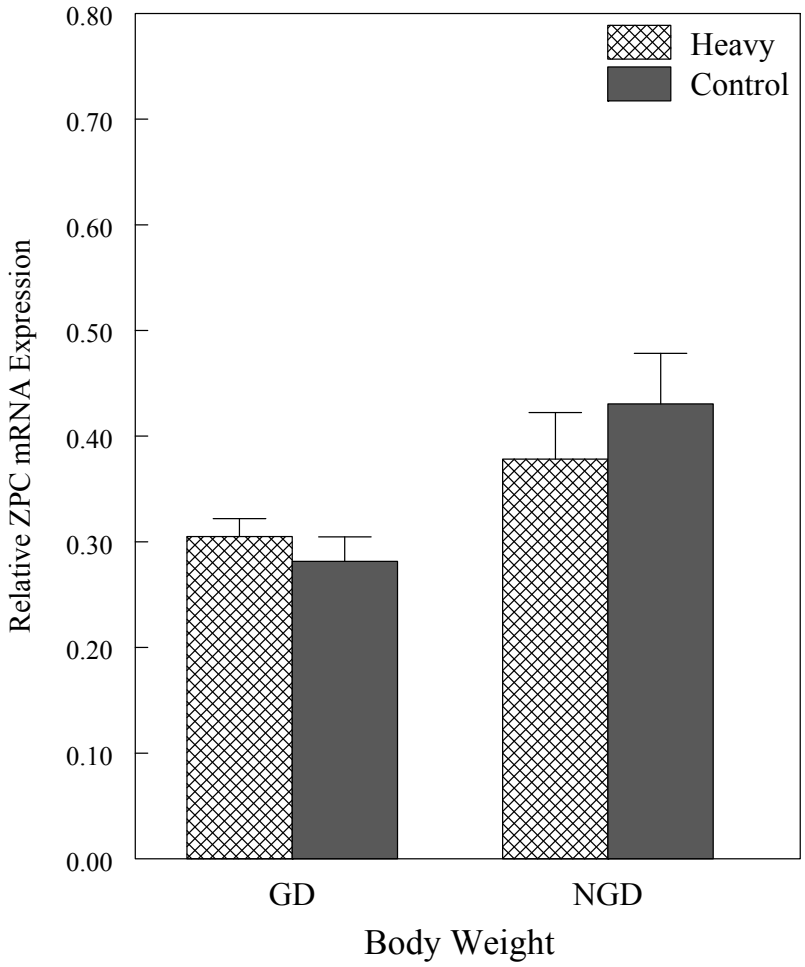

Figure 6. The relative expression of the mRNA for ZPC in the granulosa cells of the F_1 follicle isolated from four genetic lines of broiler breeder hens maintained in two different body weight categories. Values are means ± SEM. There are 36 replicate GD and 36 replicate NGD samples for each granulosa cell location for each body weight category (control and heavy).

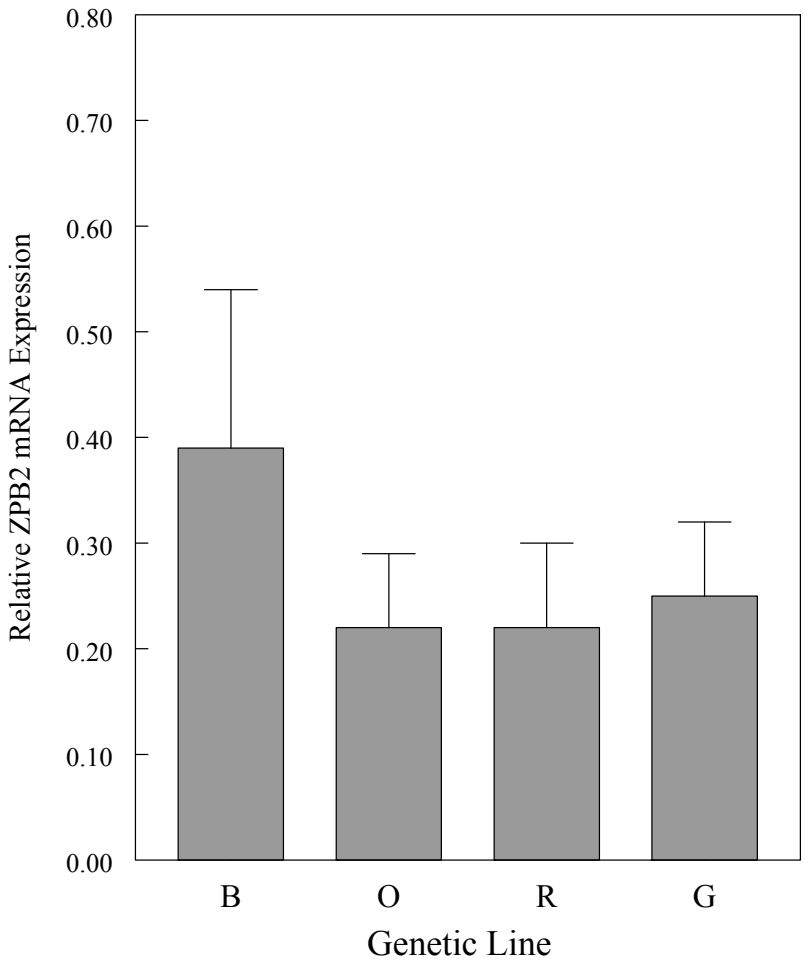

Figure 7. Overall relative expression (includes both GD and NGD) of the mRNA for ZPB2 in the granulosa cells of the F_1 follicle isolated from four genetic lines of broiler breeder hens maintained in two different body weight categories. Values are means ± SEM. There are 18 replicate GD and 18 replicate NGD samples (total n = 36 samples) for each genetic line.

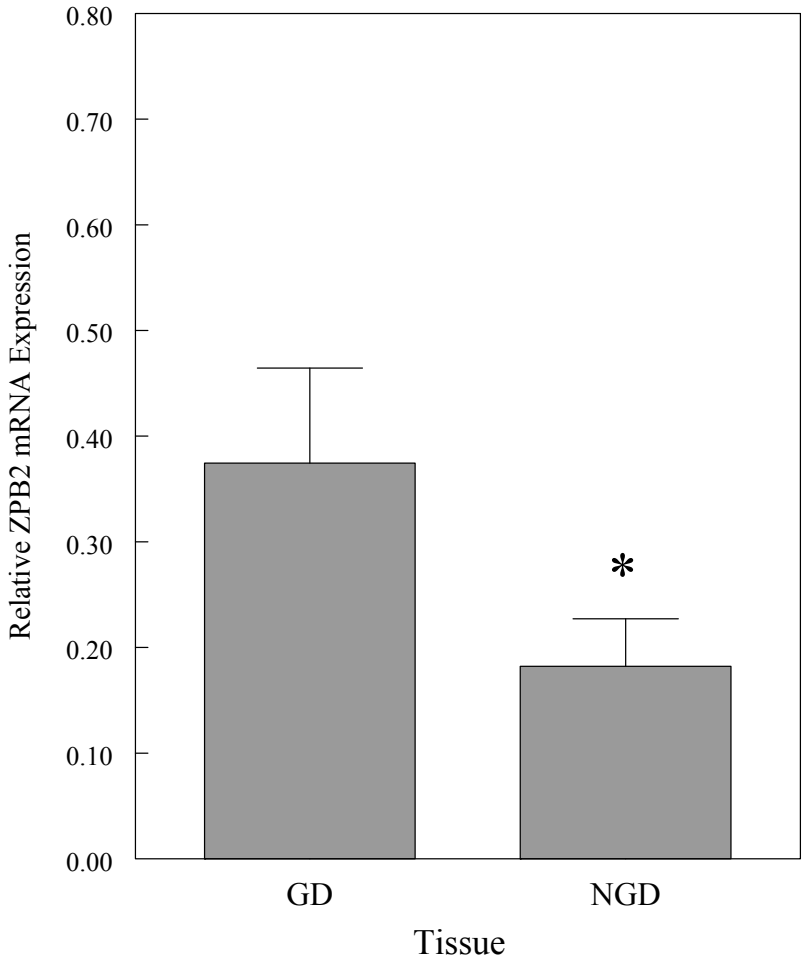

Figure 8. The relative expression of the mRNA for ZPB2 in the granulosa cells of the F_1 follicle isolated from four genetic lines of broiler breeder hens maintained in two different body weight categories. Values are means ± SEM. There are 72 replicate GD and 72 replicate NGD samples (18 samples from each genetic line) for each granulosa cell location. *Indicates significant difference $p < 0.05$.

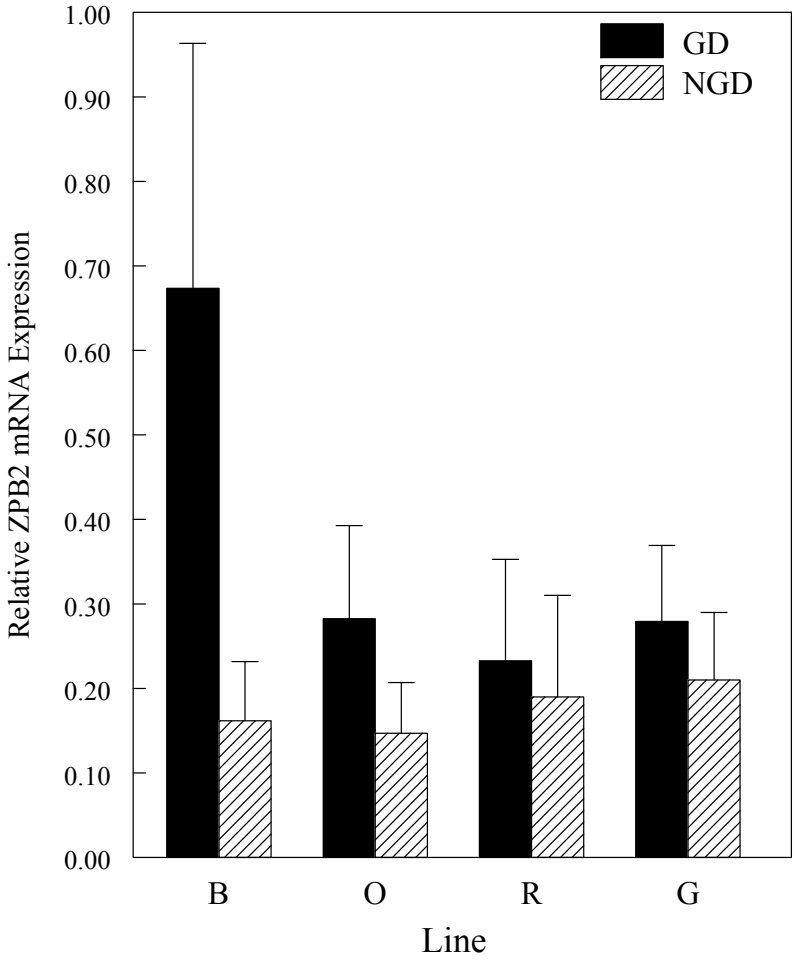

Figure 9. The relative expression of the mRNA for ZPB2 in the granulosa cells of the F_1 follicle isolated from four genetic lines of broiler breeder hens maintained in two different body weight categories. Values are means ± SEM. There are 18 replicate GD and 18 replicate NGD samples for each granulosa cell location for each strain.

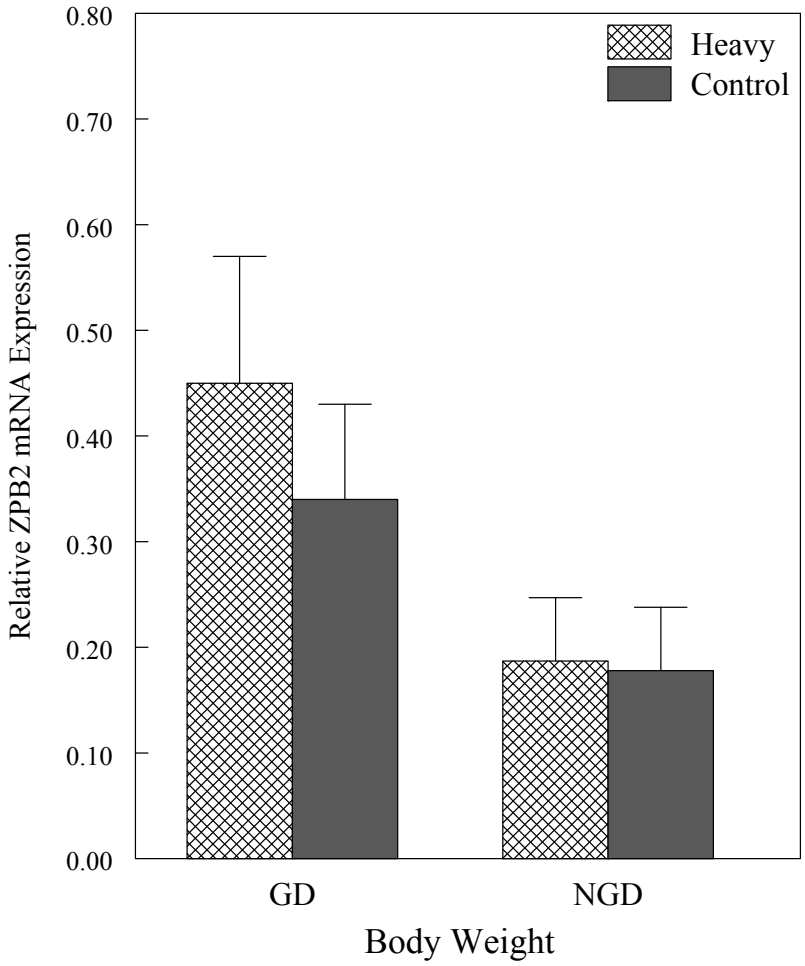

Figure 10. The relative expression of the mRNA for ZPB2 in the granulosa cells of the F_1 follicle isolated from four genetic lines of broiler breeder hens maintained in two different body weight categories. Values are means ± SEM. There are 36 replicate GD and 36 replicate NGD samples for each granulosa cell location for each body weight category.

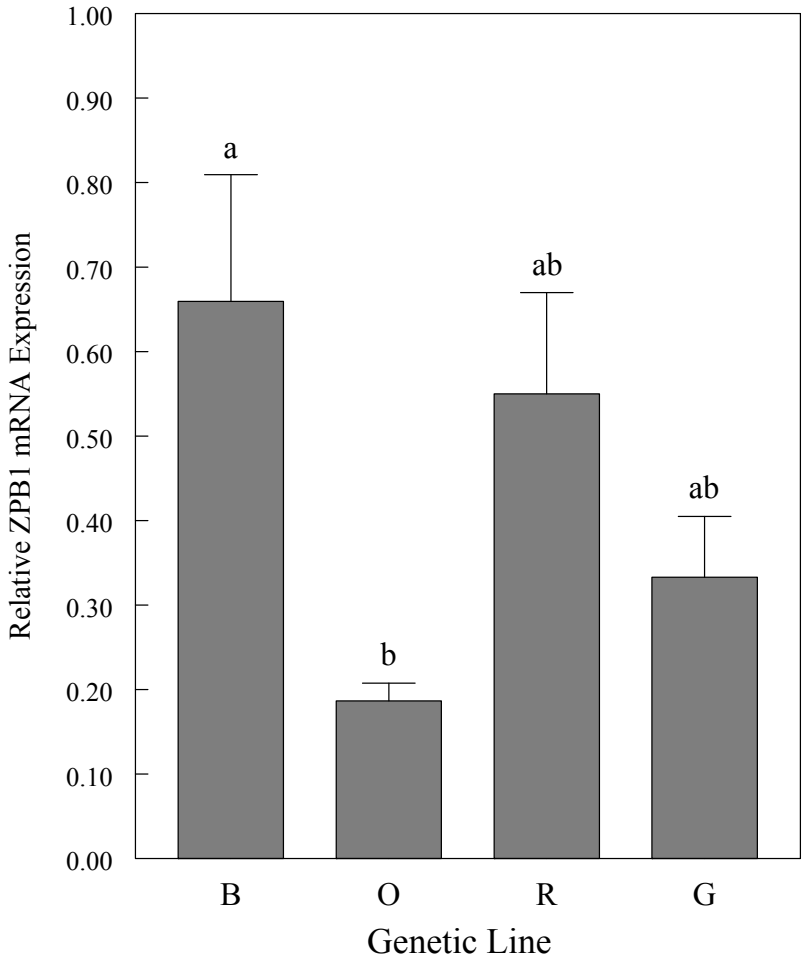

Figure 11. Relative expression of the mRNA for ZPB1 in hepatic tissue isolated from four genetic lines of broiler breeder hens maintained in two different body weight categories. Values are means ± SEM. There are 18 replicate samples (9 from the control and 9 from the heavy body weight category) for each genetic line. Bars without a common letter are significantly different from one another, $p < 0.05$. .

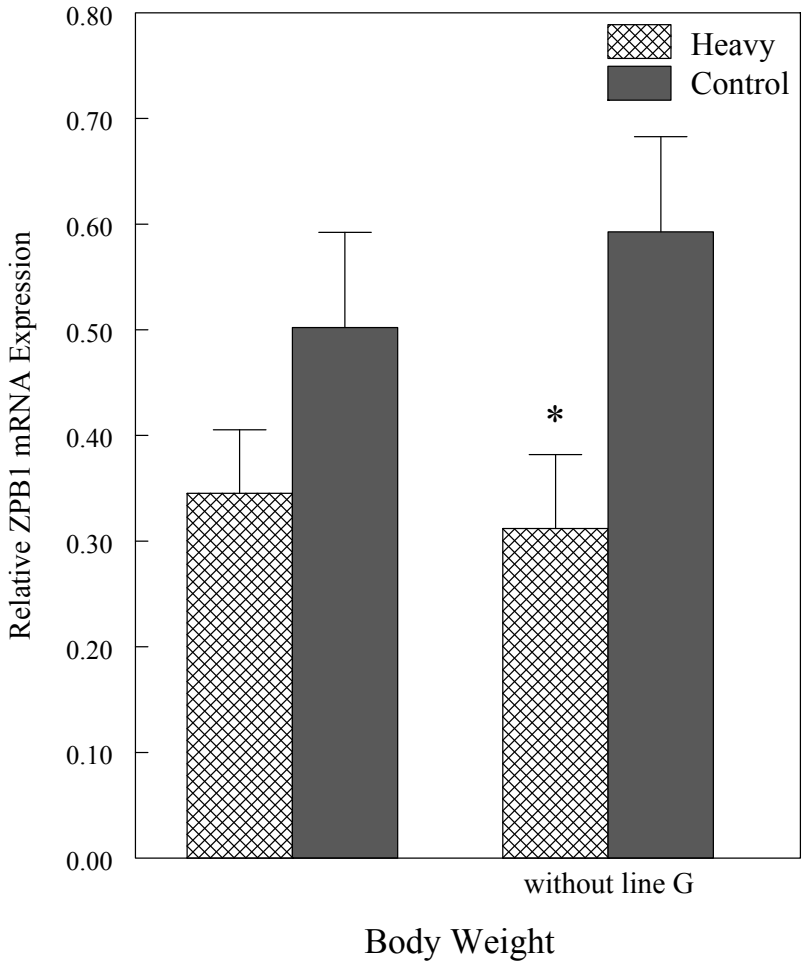

Figure 12. Relative expression of the mRNA for ZPB1 in hepatic tissue isolated from four genetic lines of broiler breeder hens maintained in two different body weight categories. There are 36 replicate samples for each weight category in the first 2 bars of the graph and 27 replicate samples for each weight category for the last 2 bars of the graph in which the results from line G are excluded. Values are means ± SEM. *Indicates significant difference $p < 0.05$.

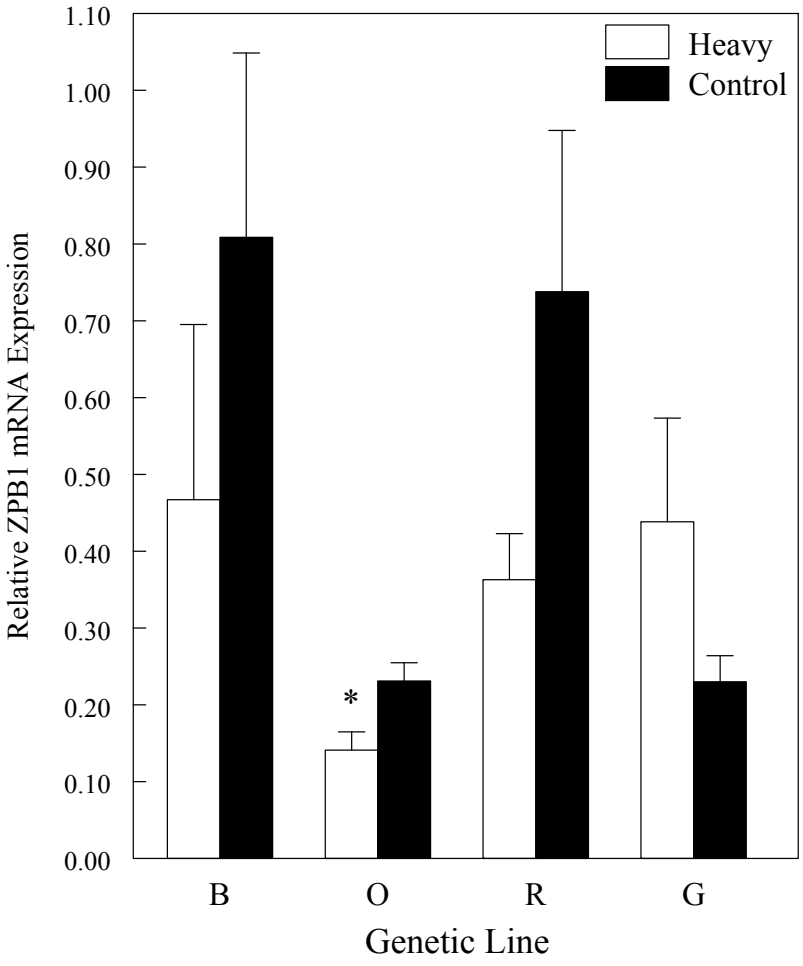

Figure 13. Relative expression of the mRNA for ZPB1 in hepatic tissue isolated from four genetic lines of broiler breeder hens maintained in two different body weight categories. Values are means ± SEM. There are 9 replicate samples for each weight category in each genetic line. *Indicates significant difference $p < 0.05$ from the corresponding weight category within a genetic line.

obtained based on Western blotting analyses of the soluble cell fraction obtained from a cell preparation for each protein (Figure 14). The T7 Tag Monoclonal antiboday used to detect the expressed proteins in the Western Blot analyses (Figure 14), bind specifically to an 11 amino acid leader peptide expressed by the pET vector in producing recombinant proteins. Use of this antibody in a Western Blotting application allows for the detection of less than 1 ng of recombinantly produced protein (Novagen) and the ZP protein bands detected by this antibody could not be seen by Commassie Blue staining techniques (data not shown).

Experiments 5 and 6

Western dot-blot analyses of each synthetic peptide using preimmune serum obtained from a rabbit prior to inoculation with chicken synthetic peptide ZPB1, ZPB2 or ZPC or using serum obtained 10 weeks post inoculation, indicated that specific antibodies for each ZP protein were successfully made (Figures 15-17). Although initial tests indicated that serum obtained 10 weeks post inoculation from both rabbits for each ZP protein had antibodies that detected chicken ZPB1, ZPB2 or ZPC, serum from only one of the rabbits for each ZP protein was used for subsequent Western blot analyses. Additional tests were conducted to determine that the optimum dilution of the rabbit serum for Western blot analyses were 1 to 5000 for ZPB1 and ZPB2 and 1 to 7000 for ZPC. The second antibody used for detection was diluted 1 to 3000 for ZPB1 and ZPB2 and 1 to 6000 for ZPC.

Western blot analysis of protein extracted from the IPVL surrounding the GD region of laid turkey eggs indicated a distinct putative ZPB2 protein band at about 60 kDa which was not detected with preimmune serum (Figure 15). For ZPB1, Western blot analysis of protein extracted from the IPVL surrounding the GD region of laid turkey eggs indicated bands at about 95 and 55 kDa that were not detected in the preimmune serum (Figure 16). For ZPC, Western

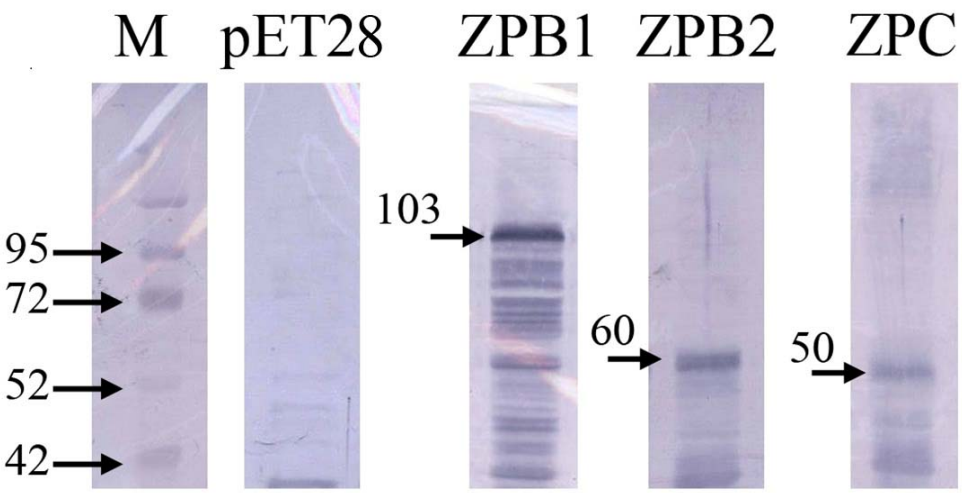

Figure 14. Western blot analyses of bacterial cell lysates using a T7 Tag Monoclonal antibody. Lane 1 contains protein markers at the indicated size (kDa). Lane 2-5 contain bacterial cell lysate from bacterial cells induced to express the pET vector with no insert (negative control, lane 2), or chicken ZPB1, ZPB2, and ZPC gene products, lanes 3, 4, and 5, respectfully. The approximate molecular weight of each ZP protein is indicated. The molecular weight values are higher than the expected 97, 59, and 42 kDa sizes for mature chicken ZPB1, ZPB2, and ZPC, respectfully, because the expressed protein has not undergone proteolytic processing and cleavage and has expression vector specific protein added to it. The native, mature protein size has a smaller molecular weight than the expressed proprotein.

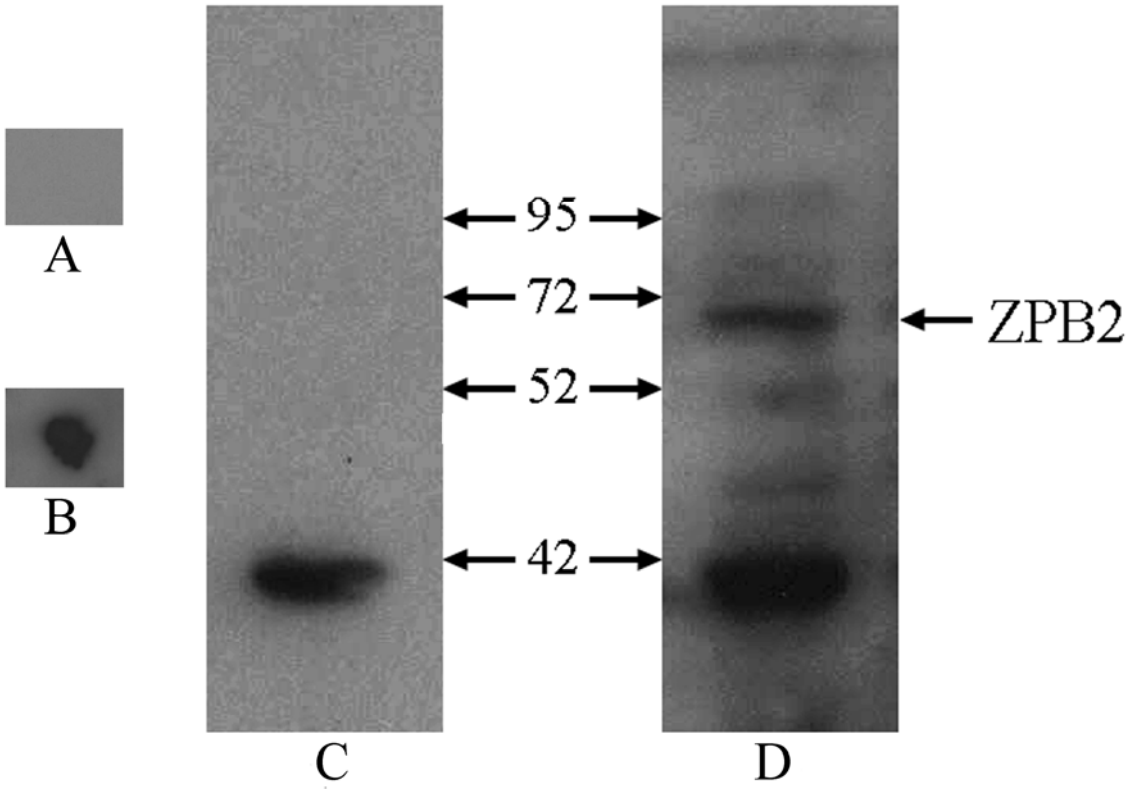

Figure 15. Autoradiograms from Western Analyses of ZPB2. Dot blots containing 10 ng of ZPB2 synthetic peptide analyzed with preimmune serum (A) and with immune serum containing chicken anti-ZPB2 antibodies (B). The provided kDa sized are based on Spectra Multicolor BroadRange Protein Ladder that was electrophoresed and transferred from a gel that also contained a duplicate 40 µg sample of total protein extracted from the IPVL of the GD region of turkey eggs. The PVDF membrane containing the samples was cut into two strips each containing a protein sample and then analyzed with preimmune serum (C) or with immune serum containing chicken anti-ZPB2 antibodies (D).

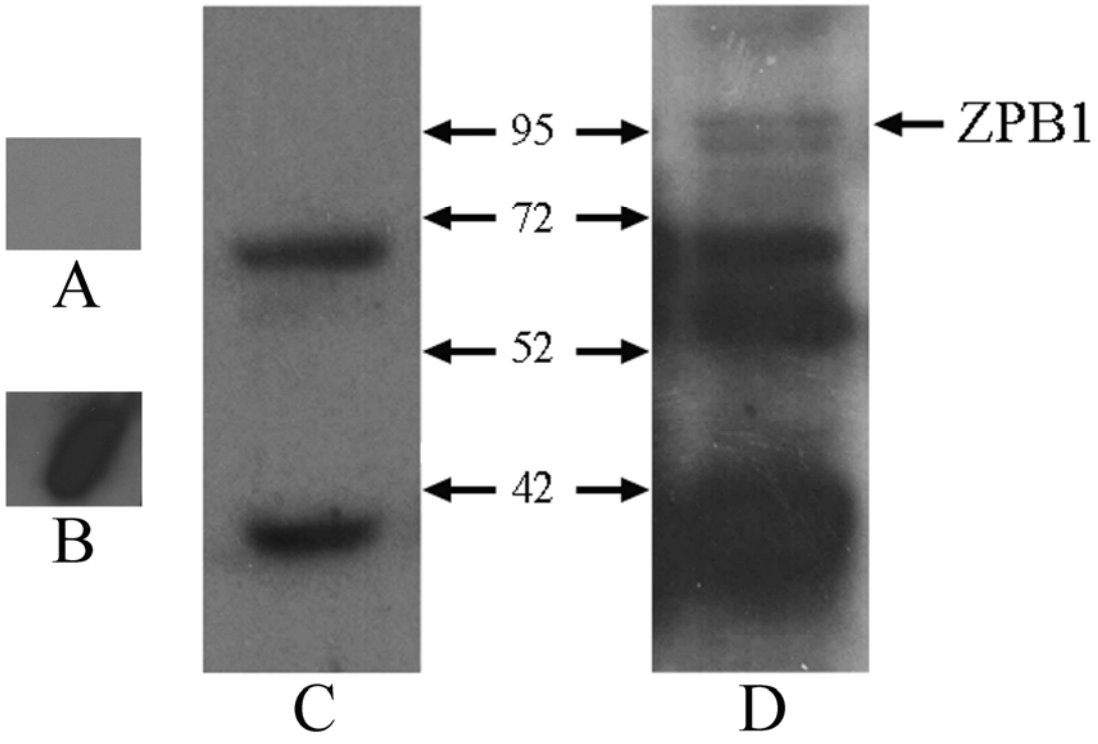

Figure 16. Autoradiograms from Western Analyses of ZPB1. Dot blots containing 10 ng of ZPB1 synthetic peptide analyzed with preimmune serum (A) and with immune serum containing chicken anti-ZPB1 antibodies (B). The provided kDa sized are based on Spectra Multicolor BroadRange Protein Ladder that was electrophoresed and transferred from a gel that also contained a duplicate 40 µg sample of total protein extracted from the IPVL of the GD region of turkey eggs. The PVDF membrane containing the samples was cut into two strips each containing a protein sample and then analyzed with preimmune serum (C) or with immune serum containing chicken anti-ZPB1 antibodies (D).

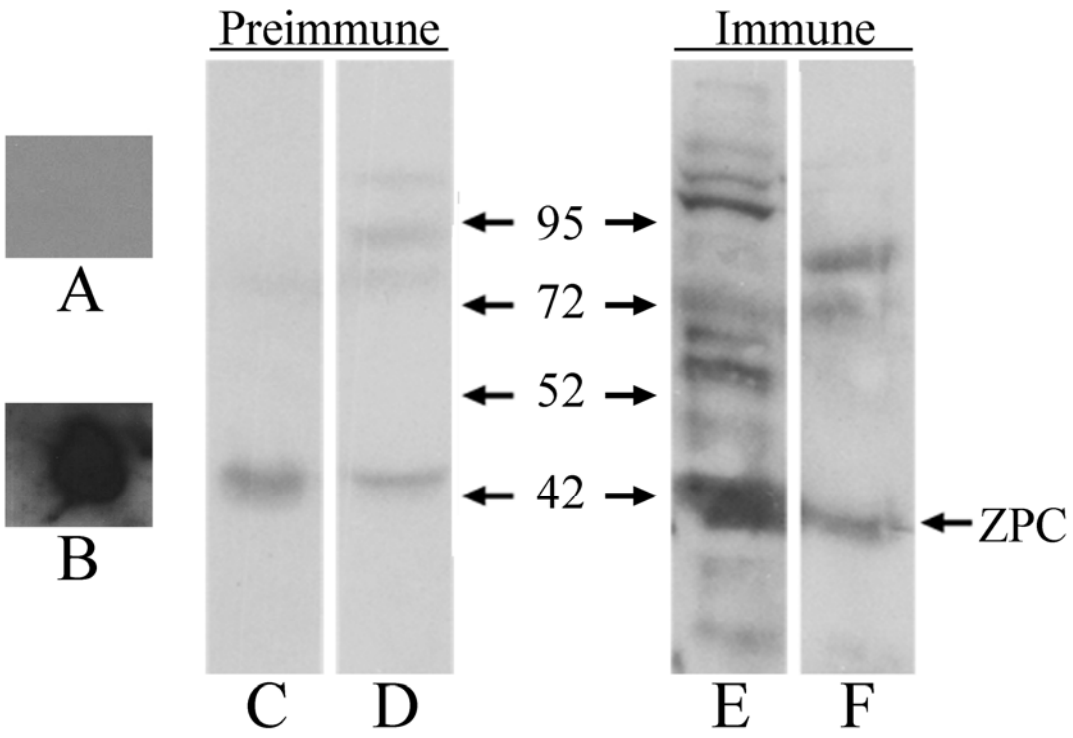

Figure 17. Autoradiograms from Western Analyses of ZPC. Dot blots containing 10 ng of ZPC synthetic peptide analyzed with preimmune serum (A) and with immune serum containing chicken anti-ZPC antibodies (B). The provided kDa sized are based on Spectra Multicolor BroadRange Protein Ladder that was electrophoresed and transferred from a gel that also contained duplicate 40 μg samples of total protein extracted from the GD region of granulosa cells (C and E) or from the IPVL of laid turkey eggs (D and E). The PVDF membrane containing the samples was cut into two strips each containing the two protein samples and then analyzed with preimmune serum (C and D) or with immune serum containing chicken anti-ZPC antibodies (E and F).

blot analyses were completed with preimmune and immune serum on protein extracted from either the IPVL or granulosa cells surrounding the GD, because differences in the size of ZPC are reported based on whether the sample is obtained near ovulation or after the egg is laid (Waclawek et al. 1998). Several distinct bands not detected with preimmune serum were detected in the granulosa sample, but fewer bands were detected in the IPVL sample (Figure 17). The only distinct protein band detected in both samples was at about 42 kDa (Figure 17).

CHAPTER 7

DISCUSSION

The current research expanded upon our previous research with the ZP proteins in two genetic lines of turkey hens by establishing that differences in ZP protein expression also exist between different genetic lines of broiler breeder hens, that the expression of the mRNA for ZPB2 is the most abundant in the smallest growing white follicles and in the granulosa cells surrounding the GD in the largest preovulatory follicle. The current research also yielded insight into producing chicken recombinant ZP proteins and antibodies against chicken ZP proteins.

In our previous research, of the five ZP proteins (ZPA, ZPB2, ZPC, ZPD and ZPX1) produced by the granulosa cells of the turkey hen hierarchical preovulatory follicles, only the mRNA expression of ZPB2 was significantly higher in the GD granulosa cells than in NGD granulosa cells (Benson 2006). ZPB2 mRNA expression was also greater in the GD granulosa cells of the F_1 follicle in the current research with broiler breeder hens. While the mRNA expression of ZPB2 was consistently higher for the GD granulosa cells than the NGD cells for each individual broiler breeder hen, the relative level of expression of ZPB2 in GD and NGD granulosa cells varied as much as 10 fold between hens within the same genetic line and weight category. This variability between birds was not seen in the previous research with turkey hens where the expression of ZPB2 mRNA was very consistent across hens and where the expression of ZPB2 mRNA was consistently 3 times greater in GD versus NGD granulosa cells from the F_1 follicle for all hens within both genetic lines. The turkey hens have been selected within their

lines for over 50 generations which may account for their lower variability in ZPB2 mRNA expression (McCartney 1964, McCartney et al. 1968, Nestor 1977).

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, Wishart 1997). 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. 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. Determining if the differences seen in the mRNA expression of this protein in GD and NGD granulosa cells are translated to the protein expression level will be accomplished by the use of antibodies specific for avian ZPB2.

ZPB2 and Early Follicular Development

Interestingly, the current research found expression of ZPB2 mRNA to be highest in the smallest prehierarchical follicles with the theca cells of these follicles expressing more ZPB2 mRNA than the granulosa cells. This expression pattern is very unusual compared to the mRNA expression of ZPC and ZPD. Both ZPC and ZPD expression increase with follicular maturity (Waclawek et al. 1998; Takeuchi et al. 1999; Pan et al. 2001; Okumura et al. 2004; Benson 2006) and only minimal theca mRNA expression of ZPC and ZPD can be detected in the F_1 follicle and none is detected in the other hierarchical or prehierarchical follicles (Benson 2006).

ZPB2 mRNA expression is not detected in the theca cells of the largest SYF or hierarchical follicles. ZPB2 mRNA was not detected in the granulosa cells isolated from the SYF, but is abundant in granulosa cells prior to the uptake of yellow yolk and is also detected in

granulosa cells from the F_4 follicle. This mRNA expression pattern implies that during the transition from the LWF stage of development to selection in the hierarchy, there is a factor that suppresses ZPB2 mRNA transcription. In addition, the granulosa cell ZPB2 mRNA expression results from experiments 1-3 collectively suggest that the ZPB2 may have a role in early follicular development that is not needed once follicles enter the SYF stage and that after selection into the follicle hierarchy, ZPB2 mRNA synthesis commences again for the production of ZPB2 protein to be localized at the GD region.

In retrospect, the idea that ZPB2 protein expression might play a role in early follicular development in avian species was first indicated when 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. Antibodies generated against ZP proteins have been successfully used as a contraceptive method with populations of wild horse, white-tailed deer, feral dogs, and laboratory animals as reviewed by Paterson et al. (2000) and Kirkpatrick et al. (2009). It is interesting to note that antibodies directed against the ZP glycoproteins can sometimes cause irreversible infertility in mammalian species via a harmful effect on ovarian function. 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 follicle viability and in the case of avian species it may be ZPB2 that plays this role. The production of recombinant chicken ZPB2 will allow for this hypothesis to be further tested by utilizing the protein in follicular tissue cell culture experiments and by immunizing hens against ZPB2.

Broiler Breeder Hen Genetic Lines

In broiler breeder hens, strains B, G, and R had higher ($P < 0.01$) ZPC expression in NGD granulosa cells than GD granulosa cells. However, in genetic strain O the expression of ZPC did not differ between the NGD and GD granulosa cells. In our previous turkey hen research, ZPC expression was equal between GD and NGD granulosa cells in the line with the best fertility while the genetic line with poor female fertility had higher expression of ZPC mRNA in NGD granulosa cells than GD granulosa cells (Benson et al. 2009). Thus, extrapolating from the turkey research it might be expected that the hens from strain O might have better fertility than the hens from the other three genetic strains. However, the hens from strain O had less hepatic ZPB1 mRNA than strain B while strains R and G had intermediate levels. In the previous work with the two genetic lines of turkey hens, the line with the highest level of hepatic ZPB1 mRNA had the best fertility (Benson et al. 2009). Therefore, based on this prior data it might be expected the hens in line O to have fertility levels similar to the other strains because the potential benefit in ZPC expression would be negated by the poor expression of ZPB1.

In actuality, the overall fertility of the hens in the four strains of broiler breeders was not different from 30 through 65 weeks of age (Bramwell et al. 2010a). There was also no difference in overall duration of fertility for any of the hen strains with all strains having a duration of fertility between 15.6 and 15.8 days following a single insemination every five weeks from 30 to 65 weeks of age (Bramwell et al. 2010a). The hens from the four strains also did not vary in the total number of sperm storage tubules located at the uterul/vaginal junction (Bakst et al. 2010).

The hens in the control body weight group from each strain had higher weekly egg production throughout the experiment compared to the hens that were heavy [above breeder guideline target weight (Bramwell et al., 2010b)]. For the entire experiment, the control hens had a 9% higher fertility rate and a 2 day increase in the duration of fertility when compared to the heavy hens (Bramwell et al. 2010b). While the expression of ZPC and ZPB2 did not vary between the control and heavy hens, the expression of ZPB1 mRNA tended to be reduced in the heavier hens compared to the control hens. As previously mentioned, elevated expression of hepatic ZPB1 mRNA is associated with higher fertility in turkey hens (Benson et al. 2009). Because ZPB1 is the only ZP protein produced by the liver and transported to the developing preovulatory follicle in poultry species, it may be the most likely ZP protein to be influenced by the metabolic state of the hen and thus provide an easy mechanism by which the metabolic state of the hen could affect fertility.

Expression of Chicken Recombinant ZP Proteins and Production of Antibodies Against Avian ZP Proteins

The results from the current research and our previous research (Benson 2006) underscores the need for producing recombinant chicken ZP proteins and antibodies that detect avian ZP proteins in order to further our understanding of ZP protein biology in poultry. The current research made some progress in these areas. A limiting factor for assessing the functions of individual ZP glycoproteins during fertilization is the formidable task of obtaining these proteins in reasonable quantities in highly pure form. To overcome this issue, several groups, have cloned and expressed ZP glycoproteins from various species using a variety of expression systems (Van Duin et al. 1994, Prasad et al. 1996, Gupta et al. 1997, Kaul et al. 1997, Harris et al. 1999, Sasanami et al. 2003, Okumura et al. 2007a). These expression systems include, but

are not limited to, utilizing a pcDNA3.1(+) vector with *E. coli* strain DH5α (Sasanami et al. 2003), a pCold TF DNA vector expressed in *E. coli*, strain BL21 cells (Sato et al. 2009), a pRSET-A vector expressed in BL21(DE3)pLysS *E. coli* cells (Chakravarty et al. 2005), a pET-32a$^{(+)}$ vector expressed in *E. coli* Origami B(DE3)pLysS cells and a pTargeT vector expressed in African green monkey kidney epithelial cells (Okumura et al. 2007a). In the current research, a pET28b$^{(+)}$ vector and BL21(DE3) cells were utilized to express full length cDNA clones of chicken ZPB1, ZPB2 and ZPC. However, only a very minute amount of the expressed recombinant ZPB1, ZPB2 and ZPC proteins were soluble (Figure 14) with the rest remaining bound to the lysed BL21 cells. Several solublization methods that have been used by other researchers that had solubility problems with their expressed proteins, were attempted and included the use of 1% Triton X-100 (Waclawek et al. 1998, Takeuchi et al. 1999; and pET System Manual, Novagen), 6M Guanidinium hydrochloride (Gupta et al. 1997), 4% Sarkosyl (Burgess 1996) and simply heating the lysed host cells to 95°C for 5 minutes in SDS buffer (Takeuchi et al. 2001, Okumura et al. 2007a). All of these methods proved unsuccessful.

The ZP proteins form the IPVL which is fairly insoluble. Ohtsuki et al. (2004) reported that Japanese quail granulosa cell cultures secrete ZPC in a soluble form, but ZPC becomes insoluble when IPVL homogenates are added to the cell culture media, implying that there are protein interactions leading to aggregations. In addition, chicken and quail ZPB1 and ZPC have been known to spontaneously form fibrous aggregates of ZPB1-ZPC hetero-complexes in vitro through disulfide cross-linked bonds (Ohtsuki et al. 2004, Okumura et al. 2007b). Thus the ZP proteins by nature want to aggregate and thus their solubility in expression systems could prove difficult. In future expression studies, replacing the pET28b$^{(+)}$ expression vector with the pCold TF DNA vector (Takara, Madison, WI) may be a productive choice. The pCold TF vector is

able to express proteins in a soluble form that are insoluble in conventional expression systems. This vector also works well with many *E. coli* host bacterial strains, including BL21, and Sato et al. (2009) reported that recombinant Japanese quail ZPD was readily soluble utilizing this vector with BL21 cells.

Western Blotting

The antibodies produced by rabbits against a synthetic peptide segment of ZPB1, ZPB2 and ZPC were able to detect specific proteins in protein samples extracted from turkey F_1 granulosa cells or IPVL membrane from laid turkey eggs. A 60 kDa protein was detected with immune serum from a rabbit injected with the synthetic fragment of ZPB2 and this protein was not detected with preimmune serum. This molecular size is in close agreement with an analysis of the chicken ZPB2 protein sequence which predicts a protein of 59.5 kDa (http://www.ncbi.nlm.nih.gov), Accession # AB025428). There has been no other research on ZPB2 in avian species beyond the research conducted in our laboratory and the initial report which indicated the mRNA for ZPB2 could only be detected by Northern analysis in stroma embedded chicken follicles (Bausek et al. 2000)

Western blot analysis of a protein sample extracted from laid turkey egg IPVL and reacted with anti-ZPB1 serum yielded protein bands of about 95 and 55 kDa that were not detected with preimmune serum. The molecular weight of 95 kDa is near the 95 to 97 kDa size reported for chicken ZPB1 (Bausek et al. 2000, Takeuchi et al. 2001, Okumura et al. 2007a). Further research will be needed to determine if the 55 kDa protein is the resulting protein fragment from further processing of the larger protein.

The immune serum produced against the ZPC peptide fragment was tested in turkey hen protein samples extracted from F_1 granulosa cells and from IPVL obtained from laid eggs. This

was done because differences in the molecular weight of ZPC have been reported based on whether the sample is obtained from preovulatory follicles or from laid eggs from chickens (Waclawek et al. 1998). ZPC in tissue isolate from preovulatory follicles has a molecular weight of 42 kDa and from laid eggs it is 34kDa (Waclawek et al. 1998). Susanami et al. (2003b) reported in Japanese quail that ZPC is synthesized by granulosa cells as a proZPC protein with a molecular weight of 43 kDa, and that after posttranslational modifications this protein is secreted to become part of the IPVL as a 35 kDa molecular weight protein. Takeuchi et al. (1999) reported a molecular weight of 42 kDa for ZPC based on Western blot analyses of follicular tissue.

In the current research, several protein bands were detected with anti-ZPC antibodies in the granulosa cell sample, that were not detected with preimmune serum, including one with a molecular weight near 42 kDa. This protein was also detected in the laid egg IPVL sample and was the only band that was detected in both samples. Further research will need to be completed to determine if turkey hens do not have further modifications in its molecular weight size after ovulation.

Importance of Research and Summary

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 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. In addition, the role of ZPC and ZPB1 expression as related to differences in fertility were suggested in the current research with broiler breeder hens as they had been with our previous work with turkey hens (Benson et al. 2009). Finally, producing recombinant chicken ZP proteins and antibodies that specifically detect avian ZP proteins will allow future research to identify the sperm receptor ZP protein or proteins in poultry species as well as whether ZPB2 plays a critical role in early follicular development and survival.

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APPENDIX

The Avian Biology Maymester in Costa Rica[1]

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[1]Mia N. Malloy and Adam J. Davis

To be submitted to the North American Colleges and Teachers of Agriculture journal

Abstract

Previously students enrolled in the College of Agricultural and Environmental Sciences (CAES) at the University of Georgia traditionally had not participated in study abroad programs at rate similar to the rest of the student body at the University. This lack of study abroad involvement by students in the College was related to the shortage of study abroad opportunities with content geared towards them. Therefore, an Avian Biology Study Abroad Program was created in 2008 to complement and enhance the avian biology major offered through the Department of Poultry Science within CAES. Because of its ecological diversity and large number of bird species, Costa Rica was chosen as the destination for the three-week, six-credit hour program. Student participation has reached the targeted goal of 15 students in 2008, 18 students in 2009, and 20 students in 2010 for a total of 53 students. Testing and survey results indicate that student learning objectives have been met and that students have been very satisfied with the overall experience of the program. The participating students have all been fulltime enrolled CAES students, except for 4 transient students enrolled through CAES and 1 student enrolled within another college at the University of Georgia. In addition, 19 of the participants were avian biology majors and 7 more became avian biology majors after participating in the program. Thus, the Avian Biology program has provided a capstone opportunity to avian biology majors and increased the study abroad participation of CAES students.

Introduction

With over 2,000 students of the entire combined undergraduate and graduate student enrollment participating in a study abroad program in a given year, the University of Georgia ranks 10th in the nation among higher education institutions for the number of students who study abroad each year (http://www.uga.edu/oie/office_archive_yb.htm, http://www.uga.edu/oie/sa_statistics.htm, Simmons 2011). About 30 percent of the total graduates from the University of Georgia each year have participated in a study abroad course during their academic program (http://www.uga.edu/oie/sa_statistics.htm). The University has faculty-led programs on every continent (http://www.uga.edu/oie/sa_statistics.htm).

Despite the University's overall stellar record in student involvement in study abroad experiences, students enrolled in the College of Agricultural and Environmental Sciences (CAES) within the University had traditionally participated in study abroad programs at a rate of less than 10 percent of each graduating cohort. Although the CAES had strived to incorporate various forms of international components into its curricula, weaving topics, sections, or a few lectures of internationally-related issues into curricula is not sufficient to provide students with understanding, compassion, and empathy for different global cultures (Crunkilton et al. 2003) and was not resulting in them seeking study abroad experience. McPherson (2001) argued that the time for debate as to whether colleges of agriculture and life sciences should incorporate international experiences into curricula had long passed and was now time to ask how agricultural colleges could participate in the internationalization of education through study abroad opportunities.

This lack of study abroad involvement by students in the CAES may have been related to the shortage of study abroad opportunities with content geared towards them and taught by

faculty within the College. Therefore, in 2008, in attempt to increase the study abroad participation of students enrolled within the CAES, the Department of Poultry Science created a 3 week, Maymester Avian Biology Study Abroad Program. This program was also developed to complement and enhance the Department's major in avian biology which it initiated in 2004.

Materials and Methods

Identifying the Country or Region of Study

One faculty member and one graduate student directed by this faculty member were given the responsibility to plan, design, and conduct the Avian Biology Study Abroad Program. Costa Rica was chosen as the destination for the program because it has many diverse, but distinct, ecosystems which allow over 850 species of birds to thrive in country that is slightly smaller in size than the state of West Virginia (Wainwright 2007, Henderson 2002). In addition, in 2001, the University of Georgia purchased a farm in the Monteverde region of Costa Rica and has since developed it to be a fully operational campus. To promote and recruit students to participate in study abroad programs at the Costa Rica campus, in the fall of 2005 the University of Georgia opened the Costa Rica Office at its main campus in Athens, GA. Therefore besides having a plethora of bird species, Costa Rica was chosen because the University already had a presence in the country and a support structure at the home campus to initiate a new study abroad program. Finally, the graduate student selected for planning the program was recruited based on her experience in Costa Rica which included a semester study abroad program in ecology and serving as a naturalist for seven months at a research facility in Costa Rica.

Planning the Program

Locations within different ecological zones of Costa Rica were chosen to allow the examination and analysis of the natural habitats and evolutionary adaptations of avian species in

each of these habitats and to provide the student participants the opportunity to see at least 300 species of birds, as well as a diverse array of mammals, insects and plants (Henderson 2002, Zuchowski 2007). The three week program was designed to utilize biological stations as well as ecological preserves and resorts to immerse students in the premontane cloud forest, lower montane rain forest, tropical dry forest, Caribbean lowlands, and Pacific coast lowland ecological zones (Henderson 2002). Lectures for the six credit hour course (POUL 4150, Field Studies in Avian Biology) were adapted for each environment in order to illustrate to students the incredible array of physiological and anatomical adaptations as well as nutritional strategies that the bird species of Costa Rica use to limit their competition with one another. The course has also designed to encompass field components which comprise about 80 percent of the course learning objectives and activities. The field learning components include guided bird and nature hikes that occur at least twice a day, and guided boat tours of mangroves and coastal estuaries on the Pacific and Caribbean coast locations.

Students are also able to gain an appreciation of the Costa Rican culture, conservation efforts, and sustainable and organic farming practices through discussions and visits to farming sites throughout the country. Recreational activities are also used to provide both direct and indirect cultural exposure. For example, one site visit is to a family farm near Monteverde that grows organic coffee that is sold to the local Co-Op Santa Elena, a fair-trade coffee cooperative that sells under the brand Café Monteverde. The small farm also produces sugar cane, beans, bananas, and other fruits and vegetables. The farmer and his family provide in depth knowledge about cooperative and organic farming practices as well as about their daily life. In addition, the students observe methods of harvesting sugar cane and then are able to extract the juice from the

cane by hand powering a century-old trapiche (Figure 1). The extracted juice is either consumed as a fresh beverage or concentrated by boiling.

To supplement the learning of the students, the same Costa Rican, bilingual travel guide with a degree in ecology has been hired each year for the duration of the trip. In addition, each location visited has staff guides which are utilized as necessary. The guides are very valuable in providing information about Costa Rica as well as the location of elusive flora and fauna.

Equipment and Group Projects

Due to the nature of the course and its focus on birds, specialized equipment was purchased to help observe, identify and record the avian fauna throughout the country. For bird identification, the course utilizes several copies of The Birds of Costa Rica by Richard Garrigues (2007) and many students opt to purchase and bring their own copy of this field guide with them. Other field guide references provided by the course include Wildlife of Costa Rica by Carrrol Henderson (2002), The Mammals of Costa Rica by Mark Wainwright (2007) and Tropical Plants of Costa Rica by Willow Zuchowski (2007). It is recommended that students bring their own waterproof binoculars, though the program has purchased several pairs for loan if needed. Additional equipment purchased for the course includes three digital SLR cameras with zoom lenses, two spotting scopes, three high definition video cameras with night recording capabilities and three laptop computers for student group projects.

Bird watching in the tropics is much more difficult than in the temperate zones due to the dense foliage and lack of sunlight under the canopy. At the beginning of the course, an introductory birding lecture is conducted that includes binocular and spotting scope use, birding etiquette, and field marks to look for on the birds. The students are also trained on the use of the photography equipment used to document what they have observed.

Using the observational techniques learned and the provided equipment, the students work in small groups to create photo and video documentaries depicting the immense array of flora and fauna seen, green initiatives observed and culture learned. The student projects are presented at the end of the term as a portion of their grade in the course. This activity combines several skills including observation, reflection, collaboration, and public speaking to reaffirm and test comprehension of the knowledge that the students have learned in the three-week duration of the program.

The students participated in pre and post testing to assess their gain in knowledge over the course. The same test covering bird biology and identification as well as Costa Rican culture, geography, and agriculture was given at both the start and end of the program. In addition, student surveys were conducted at the end of the program to determine overall student satisfaction and to provide an indication of things that could be changed to enhance the program. Students were asked questions to evaluate both the course and the overall program using a scale from 1-5 with 1 = poor, 3 = average, and 5= outstanding. Open-ended questions were also asked to encourage students to write their thoughts on the program experience.

Results and Discussion

Program popularity

The University of Georgia now has over 25 Costa Rica study abroad programs, yet the Avian Biology Maymester has met its targeted enrollment goals of 15, 18 and 20 students for the years 2008, 2009 and 2010, respectively. For 2011, the goal was to cap the course at 18 students because based on previous experience, this appears to have provided the best educational ratio of students to instructional staff. However, due to overwhelming interest, 20 students have been accepted to the program.

A total of 53 students have participated in the Avian Biology Study Abroad Program in Costa Rica. Of the total students that have participated, 49 have been from the University of Georgia and 48 of these students have been enrolled in the CAES. The remaining 4 students enrolled in the CAES as transient students to participate in the program. The Department of Poultry Science faculty advises the students enrolled in the animal health, avian biology, biological science, and poultry science majors within the CAES. Of the 48 CAES participants, 19 have been avian biology majors, 18 have been biological science majors, 5 have been animal health majors, 4 have been poultry science majors, 1 has been an animal science major and 1 has been an environmental economics and management major. The overwhelming participation in this program by CAES students supports our original hypothesis that providing a study abroad program with content geared towards these students and taught by faculty within the College would encourage their involvement in such a program.

The avian biology study abroad program was also created to enhance the relatively new avian biology major offered by the Department of Poultry Science. The created study abroad program has served this purpose by providing a capstone experience for many avian biology majors. In addition, the program has been an effective recruiting tool for the major. Seven students that participated in the program subsequently changed their major to avian biology.

Knowledge gained

Pre test results show a base knowledge average of 44% and the post-test yield 87%. This is a 43 point gain in knowledge attributable to the instruction and overall experience of the 3-week course. It has been readily evident by the end of the program each year, that the student participants are knowledgeable about basic avian science, can site-identify common Costa Rican birds, and are familiar with the natural history of dozens of species of birds. Each year, about

250 bird species, representing over 50 avian families, have been observed throughout the country. These birds include several near threatened, threatened, vulnerable, or endangered species such as the Resplendent Quetzal (near threatened), Black Guan (near threatened), Great Curassow (vulnerable), Bare-necked Umbrellabird (vulnerable), Three-wattled Bellbird (vulnerable), and Great Green Macaw (endangered) (BirdLife International IUCN Red List for birds 2011).

Student Satisfaction

The course designed for this study abroad program has been well received by the student participants from the first year (Table 1) and thus has not been altered for subsequent years except to provide new topics and creative angles for the assigned group photo and video documentaries. In contrast, aspects of the overall program logistics were changed based on student dissatisfaction from the first year of the program (Table 2). For the first year the staff of the University of Georgia Costa Rica Office handled the planning of the daily logistics of the program and conducted the orientation for the program. This seemed logical given their experience in doing this for the University's other Costa Rica study abroad programs. However, this did not work well because their experience was based on programs that stayed entirely at the University of Georgia Campus in Costa Rica or used the campus as their base for excursions to other locations in Costa Rica. Thus, the orientation the Costa Rica Office staff provided the students in our program and the arrangements made by them using several vendors were not suitable for our program that travelled Costa Rica coast to coast and only visited the University of Georgia Campus briefly.

After the initial year, the instructor and graduate student for the program started to handle all aspects of the program with the help of the tour company that employs our program guide.

This decision has allowed us to better prepare our students through orientations preceding our departure for the program (Table 2). In addition, the continuity provided by utilizing one tour company for all local arrangements has been invaluable. The addition of a constant tour bus driver throughout the program that is also highly knowledgeable about the wildlife and agricultural practices of his country has especially benefitted the program by allowing an increase in the number of student participants without a decrease in faculty and staff to student ratio.

Students' comments to the open-ended questions were very valuable as they summed up their thoughts about the overall experience. "Doing birding in the field and complementing that with lecture & activities allows the student to constantly reinforce what we learned daily. I will never forget certain lessons I have learned here," "I loved the Study Abroad Program. It opened my eyes to the world of birds and I will forever look for birds instead of just looking at the scenery around me," "I am happy that my first trip out of the country was one so deeply immersed in culture and nature," were most frequent on students' list of positive experiences. Students seemed pleased with the course format having a strong field component and one student wrote, "The experience I had here cannot even begin to compare with conventional classroom learning. I feel as though I have put to practice what others will never take out of the classroom." When asked of the value of the course in relation to cost, replies included, "Especially valuable for this length of a trip & the birds we saw," and "Best value ever! Would have paid twice as much!" The cultural component of the course also left a significant impact with comments including, "Writing the paper on conservation made me really admire the Costa Ricans and their way of life. It makes me want to make the United States more advanced in conservation," "Walking through the towns of each place we stayed, I feel had the greatest impact. It was so

different from home and it was interesting to see the livelihood, and "Seeing the culture & experiencing it helped me appreciate the people and their lifestyle."

Summary

In response to a need to increase the globalization of the students enrolled in the CAES of the University of Georgia, the Department of Poultry Science initiated an Avian Biology Study Abroad Program in Costa Rica. The three week summer program has been conducted successfully for the past 3 years and has significantly increased the number of CAES students that study abroad. It has also been an effective recruiting tool for the avian biology major and has provided a unique capstone experience for students enrolled in this major. Results from post-course surveys indicate that students were satisfied and perceived the experience to have had a very positive impact on their future academic endeavors, personal growth, and cultural understanding and awareness. This program will continue to be an indispensable component of the CAES undergraduate student experience and serve as a model for increasing the participation of students enrolled in colleges of agriculture in study abroad programs.

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Figure 1. Students observe how to harvest sugar cane and then are able to extract the juice from the cane by hand powering a century-old trapiche. The extracted juice is either consumed as a fresh beverage or concentrated by boiling.

Table 1: Summary of course perceptions of students participating in the Avian Biology Maymester in Costa Rica program from 2008 to 2010[1].					
		2008	2009	2010	Mean
1	Stimulation of interest in subject matter[2]	4.8	4.8	4.7	4.8
2	Instructor concern for students[3]	5.0	4.9	4.9	4.9
3	Academic quality and appropriateness of workload for the program environment	4.5	4.6	4.6	4.6
4	Course organization[4]	4.9	4.8	4.8	4.8
5	Methods of instructions[5]	5.0	4.9	4.9	4.9
6	Critical thinking[6]	4.9	4.8	4.7	4.8
7	Overall rating of the course[7]	5.0	5.0	4.9	5.0

[1]Values are the mean response for each question using a scale from 1-5 with 1 = poor, 3 = average, and 5= outstanding, of the 15, 18 and 20 students participating in year 2008, 2009 and 2010, respectively.

[2]Question continued: One measure of an instructor's success is the ability to convey enthusiasm for and generate interest in the subject matter. How well did your instructor(s) do so?

[3]Question continued: In a positive learning environment, an instructor shows respect and concern for the intellectural development of students. How well did your instructor(s) do so?

[4]Question continued: A well-organized course provides the student with a syllabus that outlines the course topics and assignments and clearly defines how the students' grades will be determined. How well was this course organized?

[5] Question continued: An instructor can use a variety of teaching and learning strategies to guide students toward an understanding of course material. How well did the instructional methods help you to understand course material?

[6] Question continued: A major goal of UGA Costa Rica's study abroad programs is to foster critical thinking. How well did this course improve your ability to think critically on your own about the subject matter?

[7] Question continued: Taking into account the many qualities that contribute to a good course, rate the overall quality of this course.

Table 2: Summary of program perceptions of students participating in the Avian Biology Maymester in Costa Rica program from 2008 to 2010[1].

		2008	2009	2010	Mean
1	Accuracy and usefulness of orientation programs and materials in preparing for the program	2.5	4.8	4.5	3.9
2	Safety of program locations, facilities, excursions, and transportation	4.1	4.6	4.1	4.3
3	Choice of locations for excursions, field trips and site visits	4.7	4.8	4.8	4.8
4	Overall effectiveness of program staff in managing program	3.2	4.9	4.8	4.3
5	Overall effectiveness of your program's professor and staff in dealing with students' academic and personal needs and concerns	5.0	4.9	5.0	5.0
6	Value of program in relation to the cost	4.8	4.8	4.9	4.8
7	Overall quality of the program in relation to your expectations	4.7	4.9	4.8	4.8

[1]Values are the mean response for each question using a scale from 1-5 with 1 = poor, 3 = average, and 5= outstanding, of the 15, 18 and 20 students participating in year 2008, 2009 and 2010, respectively.