Characterization of Pituitary Protein Expression Patterns During Stages in the Reproductive Cycle of Turkey Hens

by

Amy M. Spellerberg

Thesis submitted to the Faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

Master of Science

in

Animal and Poultry Sciences

APPROVED _____
Dr. Eric A. Wong, Chairman

_____ _____
Dr. C.L. Rutherford Dr. D.M. Denbow

April 11, 2004

Blacksburg, Virginia

Key words: turkey, protein expression, reproductive cycle, pituitary

Abstract

Characterization of
Pituitary Protein Expression Patterns During Stages in the
Reproductive Cycle of Turkey Hens

by

Amy M. Spellerberg

Animal and Poultry Sciences

Dr. Eric A. Wong

Improvement in turkey reproductive efficiency is a very desirable goal for the turkey industry. The ability to maintain turkey hens in the egg-laying (LAY) stage and produce one additional egg per hen a year is estimated to benefit the turkey industry approximately $1.5 million dollars per year. Overall protein expression patterns generated by tissues of the hypothalamic-hypophyseal complex, namely the anterior pituitary, of the mature turkey hen have a profound impact on reproductive cycling. One of the key physiological factors produced by the anterior pituitary and shown to play a significant role in the regulation of egg laying is the hormone prolactin (Prl). The objectives for this study are to examine the overall protein expression patterns from turkey hen pituitary tissue during the nonphotostimulated (NPS), photostimulated (PS), and egg laying (LAY) stages. Attempts to isolate transcription factors that regulate the expression of Prl using an affinity chromatography technique or southwestern screening of a bacteriophage expression library were not successful. A global analysis of protein expression, using two-dimensional polyacrylamide gels (2D gels), was conducted using whole cell, cytoplasmic and nuclear protein extracts from pituitary tissue collected during the NPS, PS and LAY reproductive

stages. Approximately 1,046 proteins ranging in pI from 4.6-8.2 and molecular weights between 100 kDa-6kDa were resolved. Protein expression patterns were replicated and verified using pituitaries harvested from NPS, PS and LAY stage turkey hens from another laboratory.

Proteins showing considerable changes (563 proteins increased in expression and 98 proteins decreased in expression from the NPS to the LAY stage) in their expression between the reproductive stages were grouped in analysis sets for future identification. These proteins may prove to be important to the reproductive cycling of the turkey hen and warrant future investigation. The results of this study contribute to the overall understanding of the role that the pituitary, as a critical part of the hypothalamic-hypophyseal complex, plays in turkey hen reproductive cycling.

Dedication

This thesis is dedicated to Dave, who began as my boyfriend and ended up as my husband. You have made this experience worth it all. I would also like to dedicate my work to the rest of my immediate family, Buster Brown, Mrs. Beau Jangles, Addie my fish and the bird. Thank you for your companionship and sacrifices. I'm looking forward to more quality time with you all. A special "Hello" to my daughter, Megan Emily…I love you and I hope the work I did here will help and benefit you in the future. I'm typing this seven days before your due date and I can't wait to meet you! Welcome to our family, little one! You and Daddy have been there with me for the hardest part…finishing it!! Thank you for being right with me to keep me focused on what matters. Thank you, Lord for your help

Acknowledgements

To my loving husband, Dave, thank you for supporting me in my decision to pursue graduate school and for all the trips you made down to Tech. We made it through together and I am so happy to have your love, encouragement and day to day support. I love you very much, Babe! Dr. Wong, thank you for taking me in and providing me with a wonderful masters degree experience. I know the process wasn't as smooth as expected, but I do appreciate your patience and constant help. Dr. Webb, thank you for taking me into your lab. I had a wonderful time working with you and I hope my work will help you all in the future. To Judy, my companion through it all, thank you, thank you, thank you. You have helped me tremendously. I am so happy we met each other and I know we will always be good friends and that has made the whole experience worth the effort. Julia, many thank you 's as well! I am so glad to have met you and I hope we always keep in touch. Matt, our companion in the lab; for all your help and for your friendship. Don, I am thrilled to have worked with you! You have been a great support to me and made my work very enjoyable. I hope retirement is great! Kris, it was wonderful to work with you. Thank you for all your help. I wish you the best for all your efforts in the future. Cindy and Ellie, thank you for being such a wonderful support! Alice, Elizabeth, Sarah, Xiao, and all the other graduate students and technicians at Tech. Thank you for being such wonderful friends. I had a great time working with you all. To my cousin, Anna, I had a wonderful time rooming with you and Rick. Thank you for all your support and for your excitement for life. To the very best Mom and Dad ever, thank you very much for all the love and support you have given me for my work and for all the efforts of my life. You have always given me the support and encouragement I have needed to press on and relentlessly finish what I have started. Thank you for your confidence in me, for preparing me so well, for enabling me to pursue my

dreams and make it this far. I love you both very much. To Mary and Pop, you are truly and by far the best parents-in-law that anyone could ever hope for. Thank you very much for all your patience, support and encouragement. I hope I will be able to return the favors in the future. To Grandma Spellerberg, Grandma Reese, Aunt Ruthie, Uncle Albert, and Grandpa Ambury, for all the love, prayers and encouragement you have given me as I worked on my masters, thank you very much. To Aunt Adeline, Grandma Ambury, Grandpa Spellerberg, Grandma Gee and everyone else that has been pulling for me up there. To my sisters and brothers, Charlene, Ken, Beth, Scott, Marie, Joben, Christie and Kyle, thank you very much for all the computer support, late nights, encouragement and stress management help you have all given me over the past two years. To my wonderful nieces, Andrea, Clara Rose, Madeline Marie and Mary Michelle, I can't wait to someday celebrate all of your graduations from graduate school someday!! Much love to you all. To my friends, Laura, Missy, Allen, I have been awfully hard to keep up with since I've been at school. I hope to change that soon! I know you have supported me long distance. Thank you very much for caring about me and always being there when I return. I miss you all a lot!

To Dr. Smith, for all the support you have given me for my masters work.. I enjoyed working on the turkey genome and I hope my little contribution was helpful. Good luck to you in all your pursuits. Dr. Siegel, it was an honor to meet you. I truly enjoyed all of our conversations and I will never forget you. To my committee members, Dr. Denbow and Dr. Rutherford, thank you for the time and consideration you have given me. To anyone I have missed, I am truly thankful for all your help and support. Despite the challenges, I enjoyed my time at Virginia Tech.

Table of Contents

Figures and Tables

I. Introduction

A. Rationale

The avian neuroendocrine system is a complex system of organs and glands including the hypothalamic-hypophyseal complex, gonads, pancreatic islets, the adrenal, thyroid, parathyroid, and ultimobranchial glands, as well as the endocrine cells of the gut. The function of this system is to express specific biochemical signaling proteins called hormones. The expression of a continuously changing complement of hormones enables the neuroendocrine system to regulate vital physiological processes in the bird. Cellular differentiation, metabolic and growth regulatory processes, as well as reproductive cycling are all regulated by hormones produced by the avian neuroendocrine system (Scanes, 2000).

The role of the hypothalamic-hypophyseal complex in regulating reproductive cycling in the turkey hen is particularly ideal for the study of complex protein expression and regulation patterns for many reasons. This complex is the primary system responsible for the expression of a full complement of hormones that affect numerous reproductive organs (Scanes, 2000). The anterior pituitary, which is part of the hypothalamic-hypophyseal complex, is a major contributor of the important hormones produced by this complex that specifically affect reproductive cycling. In addition, there are prominent behavioral characteristics associated with the different seasonal stages of the turkey hen's cycle that help to distinguish each easily for accurate collection of stage-specific pituitaries (El Halawani et al., 1988). Examining hormone gene expression patterns of the anterior pituitary during each of these stages is, therefore, an ideal way to study this cycle.

There are significant economic advantages for studying hormone gene expression patterns during the female turkey reproductive cycle. Developing the ability to regulate the expression of certain hormones during specific reproductive stages may enable turkey producers to maintain hens in the egg laying stage of their cycle, resulting in a significant increase in production efficiency (Crisostomo et al., 1998). Estimates indicate that eliminating broodiness in turkey hens and increasing egg laying production by one egg per hen per year would save the turkey industry approximately $1.5 million each year (Iowa State University Extension, 1997).

Finally, it is possible to obtain a significant amount of information about overall protein expression patterns of the anterior pituitary using available proteomic techniques. Two-dimensional gel electrophoresis and analysis permits the examination of each protein and its relative expression levels from cells isolated at different stages of development, under specific treatment conditions, or in this case, during various stages in cycling, to be detected and classified (reviewed by Rabilloud, 2000). Since a large number of proteins can be detected at one time, a study of overall changes in protein expression for *in vitro* as well as *in vivo* processes can be developed. Protein sequencing, bioinformatic tools and screening techniques can also be used to determine a protein's functional characteristics (Rabilloud, 2000). Information from all of these techniques can be combined to provide a comprehensive view of the anterior pituitary's role in turkey reproductive cycling.

B. Background

B-1: The Avian Hypothalamic-Hypophyseal Complex

The hypothalamic-hypophyseal complex is part of the neuroendocrine system that plays a central role in controlling the reproductive cycling of the turkey hen. This complex consists of the hypothalamus and pituitary gland and is found at the base of the brain. The structure of the hypothalamic- hypophyseal complex is shown in Figure I-1. The hypothalamus and the pituitary are intimately connected with the median eminence of the hypothalamus centrally located in the complex. The median eminence contains neurosecretory terminals from the Preoptic area (POA) and neurons of the supraoptic nucleus (SON). Neurons from the paraventricular nucleus (PVN) terminate in the posterior lobe of the pituitary and the median eminence of the hypothalamus. The posterior lobe is connected to the anterior lobe by hypophyseal portal blood vessels. These vessels enable neurosecretory molecules and hormones produced in the median eminence and posterior lobe of the pituitary to traverse to the anterior lobe of the pituitary (Scanes, 2000).

The anterior lobe of the pituitary consists of a caudal and a cephalic lobe. Each is characterized by prominent cell types and the secretion of certain hormones by each type. In avian species, including the chicken and the turkey, the caudal lobe consists mostly of gonadotrophs and somatotrophs that produce luteinizing hormone (LH)/ follicle-stimulating hormone (FSH) and growth hormone (GH), respectively. The cephalic lobe is characterized by gonadotrophs producing LH/FSH, corticotrophs producing adrenocorticotropic hormone (ACTH), and lactotrophs producing prolactin (Prl) (Hansen and Hansen, 1977, Jozsa et al., 1979, Mikami, 1986, Thommes et al., 1987, Naik et al., 1990 and Berghman et al., 1992). The same cellular distribution is also seen in

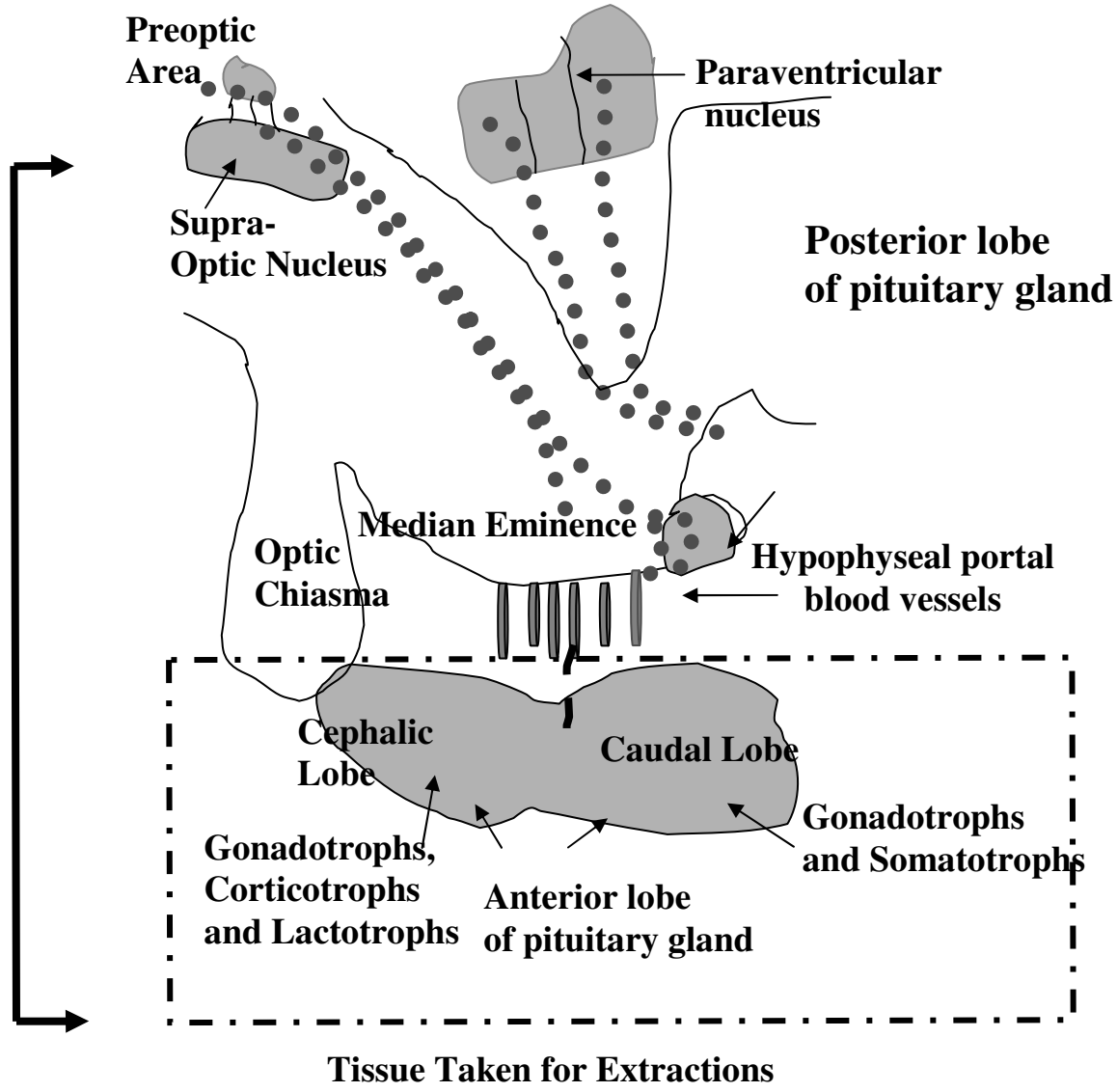


Figure I-1: Illustration of the Hypothalamic-Hypophyseal Complex. Illustration adopted from Sturkie et al, 2000 with permission from contributing author Dr. E.A. Wong.

mammalian species, namely the rat. (Watanabe and Daikoku, 1979, Chatelain et al., 1979, Hoeffler et al., 1985).

B-2: The Female Turkey Reproduction Cycle

The reproductive stages of the domestic turkey hen are controlled by cycles of light and dark photo periods and can be identified by behavioral and physiological characteristics (Scanes, 2000). Behaviorally, a reproductively quiescent turkey hen will begin egg laying after an increase in daylight exposure from 8 to 14 hr; a period known as photostimulation (PS) (reviewed by El Halawani et al., 1988). After a period of egg laying (LAY), turkey hens as well as those of other avian species will become photorefractory or non-responsive to light, cease to lay and begin to exhibit broody behavior (e.g., starlings; Dawson et al., 1985). Nesting and egg incubation activities are exhibited until the clutch hatches and hens return to a reproductively quiescent phase where the turkey hen is said to be photorefractory (Siopes, 2001; reviewed by El Halawani, 1988).

B-3: The Effect of Prolactin on Egg Laying

One of the most well characterized pathways of the hypothalamic-hypophyseal complex is the regulated expression of Prolactin (Prl) by lactotroph cells of the anterior pituitary (Burke and Papkoff, 1980; Mikami, 1980). Prl is an important hormone responsible for physiological responses promoting the onset of incubation and broody behavior in turkeys (reviewed by Rozenboim and El Halawani, 1993, Wong et al., 1991). Injecting mammalian Prl into laying turkey hens causes ovarian regression. As circulating levels of Prl increase, levels of circulating gonadotropins decrease. One hypothesis is that

Prl inhibits either gonadotropin synthesis or binding which would disrupt the positive feedback relationships of the hypothalamic-hypophyseal-gonadal axis (reviewed in El Halawani et al., 1988). There is a positive feedback loop between physical contact with eggs, Prl serum levels and nesting behavior (e.g., bantam hens; Sharp et al., 1988). The plasma levels of Prl correlate with the various stages of reproduction. Circulating Prl protein, and intracellular Prl mRNA levels increase from the nonphotostimulated (NPS) stage, to the photostimulated (PS) stage and again to the egg laying stage in a relatively linear manner (Karatzas et al., 1997). Prl levels sharply increase between the egg laying (LAY) stage and the incubation stage. This increased level of Prl is known as hyperprolactinemia and results in ovarian regression and loss of egg production (Ramesh et al., 1998). Prolactin levels abruptly fall from the incubation to the photorefractory stage (Karatzas et al., 1997; El Halawani et al., 1988; Tong et al., 1998).

B-4: Control of Extracellular Prolactin Protein Levels

Extracellular levels of Prl protein are dependent upon the extent of trans-differentiation of somatotrophs into lactotroph cells (Ramesh et al., 1998; Porter et al., 1991), the amount of lactotroph proliferation (Ramesh et al., 1995), and regulated expression by specific hormonal signaling pathways (Youngren et al., 1996; El Halawani et al., 1988; Schnell et al., 1999). Somatotroph and lactotroph cells of the anterior pituitary are anatomically separated. Lactotrophs are found in the cephalic lobe and somatotrophs predominantly in the caudal lobe during the NPS stage. As the hen cycles from the NPS to the LAY stage, the number of lactotroph cells in the caudal lobe of the anterior pituitary increases.

This increase is due to two factors. First, mammosomatotrophs are located in the caudal lobe of the anterior pituitary (Ramesh et al., 1998). Mammosomatotrophs are a transitional cell type that facilitate a bi-directional differentiation between somatotrophs and lactotrophs (Porter et al., 1991). In addition, the lactotroph cells of the cephalic and caudal lobes proliferate, causing an increase in the mass of the pituitary (Lopez et al., 1995). As somatotrophs become lactotrophs and lactotrophs proliferate, there is a 5- to 10-fold increase in the levels of circulating Prl during the onset of the incubation stage (Proudman et al., 1995).

Hypothalamic regulation of Prl gene expression is attributed to the primary Prl releasing factor, vasoactive intestinal peptide (VIP), which stimulates Prl secretion in a dose-dependent manner in birds (Xu et al., 1996; Chaiseha et al., 1999; Opel et al., 1988; Youngren et al., 1996, El-Halawani et al., 1988, Schnell et al., 1999). The release and expression of Prl are induced by VIP in the chicken (Talbot et al., 1991) and the turkey (Pitts et al., 1994). Both are reduced by active immunization with VIP in the turkey (Youngren et al, 1994). The ability of VIP to stimulate Prl production is inhibited by the biogenic amine dopamine (DA), produced by aminergic neurons terminating in the posterior lobe of the pituitary gland (Youngren et al., 1996). The binding of DA to receptors on the surface of cells of the hypothalamus prevents VIP secretion, and consequently, blocks Prl production (Youngren et al., 1999). Neurons that terminate in the median eminence of the hypothalamus produce VIP, which can traverse the hypophyseal portal blood vessels to the cephalic lobe of the anterior pituitary. VIP can then stimulate lactotroph cells to transcribe Prl through extracellular VIP receptors (Macnamee et al., 1989, El Halawani et al., 2001). VIP increases Prl mRNA abundance by increasing the rate

of Prl transcription and by enhancing the stability of Prl mRNA in lactotrophs (Chaiseha et al., 1998).

B-5: Intracellular Mechanisms Controlling Prolactin Expression

The intracellular mechanisms involved in VIP stimulation of Prl transcription and mRNA stability in turkey lactotrophs are not yet understood. Efforts to understand these mechanisms first began by defining the Prl gene itself and its regulatory units. The turkey prolactin-encoding gene and its upstream regulatory promoter have been cloned and sequenced (Kurima et al., 1995). Recent work in our laboratory has focused on characterizing the transcription factor(s) responsible for the expression of Prl in turkeys. Electrophoretic mobility shift assays (EMSA) have been performed covering the 5'-flanking region of the tPRL gene with turkey pituitary and liver nuclear extracts. The turkey prolactin promoter region (tprl-promoter) spans from nt –41 to –199 and contains three putative, high affinity transcription factor binding sites identified by electrophoretic mobility shift assay (EMSA) analysis (Gazzillo, 2000). Two regions of the promoter, one at –127 and one at –61, resemble the rat Pit1/GHF-1 transcription factor binding sites (Wong et al., 1991 for turkey Pit-1 and Nelson et al., 1988 for rat Pit-1). Shifted bands, indicating tprl-nuclear protein complex formation, were seen for promoter sequences –41 to –73 (tprl-1), -105 to –137 (tprl-2) and –175 to –199 (tprl-3). The sequence specificity of proteins that produced gel shifts, for the tprl-1, tprl-2 and tprl-3 regions, were determined by using unlabeled, binding site encoding oligonucleotides in a series of competition assays. Three shifted bands were observed in samples containing sequences for tprl-1 and tprl-2 binding sites. Competition assays indicated that these proteins had sequence specificity. Additional assays suggested that the same factors were binding to tprl-1 and tprl- 2. Supershift assays,

using an antibody specific to rat Pit-1, resulted in the loss of one of the bands. This result indicated that tPit-1, likely from somatotrophs, was one of the factors binding to these promoter regions (Gazzillo, 2000). The putative transcription factors responsible for the other band shifts have not yet been identified.

Pit-1 protein in rats has been identified as a VIP-responsive transcription factor that directly binds to promoter sequences in the mammalian prolactin gene (Mangalam et al., 1989). In chicken lactotroph cells in vitro, cPrl protein was produced in response to treatment with tVIP (Woods et al., 1998). The sequences for two cPit-1 cDNA's , cPit-1α and cPit-1γ, and changes in their mRNA expression during development have been characterized (Tanaka et al., 1999). Increasing cPit-1α and cPit-1γ mRNA levels parallel increases in the levels of cPrl, suggesting these transcription factors may be involved in cPrl gene expression. However, whether or not cVIP levels affect cPit-1α and/or cPit-1γ expression has not been established.

Just as Pit-1 has been shown to be a primary Prl regulatory protein in mammals (Mangalam et al., 1989), there is evidence to suggest Pit-1 plays a similar role in avian species. In cotransfection studies turkey Pit-1 was shown to activate turkey Prl, turkey GH and rat Prl promoters 3.8-, 3.7- and 12.5-fold respectively (Weatherly et al., 2001). However, dual-label immunofluorescence studies showed that tPit-1 was not detectable in Prl-containing cells, but was detectable in GH-containing cells. Therefore, although tPit-1 does function to activate the tPrl gene promoter in vitro, it does not appear to be involved in tPrl gene regulation *in vivo* (Weatherly et al., 2001).

In addition, previous work using DNase I footprinting and mutational analysis indicates that there may be several related, functionally similar, but tissue-specific

transcription factors that function in a combinatorial manner to activate rat Prl and rat GH genes. (Ingraham et al., 1988 and Mangalam et al., 1989). Although tPit-1 independently activates rPrl and tGH *in vitro*, the regulation of tPrl in vivo may also involve multiple factors or a tissue specific primary regulatory protein other than tPit-1. Previous studies also support the theory that second messenger systems may be responsible for intracellular regulation of tPrl expression (Tong et al., 1998). For example, results of previous studies have shown that there are significant increases in cAMP in cultured pituitary cells due to VIP treatment (Onali et al., 1983). In mammals, forskolin, a known stimulator of adenylyl cyclase, which produces intracellular cAMP, was used experimentally to confirm that the cAMP pathway is involved in signal transduction of VIP (Szabo et al., 1990). Therefore, cAMP as a second messenger is implicated in the regulation of Prl expression and protein release.

The combinatorial actions of multiple transcription factors may be responsible for the expression of Prl as well. The mechanisms for expressing genes involved in regulatory and/or developmental processes have often times consisted of multiple factors acting in concert (Mangalam et al., 1989, Chaiseha et al., 1998). Searching for a single protein product that binds to the Prl promoter does not take this into consideration, and therefore, may be too limited. A more general analysis of overall gene expression patterns during the reproductive stages of the turkey hen may be necessary to account for all possible scenarios.

II. Objectives

The first objective of this study is to isolate and characterize the putative transcription factors that were shown, by EMSA (Gazzillo, 2000), to bind to the tPrl promoter sequences, tprl-1 and tprl-2. The approaches to be used include affinity chromatography to isolate these putative transcription factors from nuclear protein extract from LAY stage turkey hen pituitaries. In addition, a λ-bacteriophage expression library from LAY stage turkey hen pituitary tissue will be screened for these putative transcription factors using a southwestern technique. This will provide information about specific proteins involved in the regulation of tPrl protein expression during reproductive cycling.

The second objective is to isolate and separate all expressed anterior pituitary proteins from two populations of turkey hens, obtained during the non-photostimulated (NPS), photostimulated (PS) and egg laying (LAY) stages, using two-dimensional gel analysis (2D gel analysis). These total protein separations will represent the protein expression patterns of the anterior pituitary gland of the hypothalamic-hypophyseal complex. Each separated protein will be classified by its unique molecular weight and isoelectric point and relative quantitative comparisons will be made for each of these proteins between each of the three reproductive stages. Based on these characteristics, individual proteins that show significant expression changes will be noted in analysis sets for future identification and analysis.

III. Experimental Methods

A. Animal Housing and Pituitary Collection

Nicholas Large White Turkeys (Nicholas Breeding Farms, Sonoma CA) were housed at the Virginia Tech Turkey Center and were raised on 8 hr of light: 16 hr of darkness. Turkey hens in this stage were considered nonphotostimulated (NPS). At 28-30 weeks of age, turkey hens were photostimulated (PS) with 16 hr of light: 8 hr of darkness to induce the onset of the LAY stage, which commenced with the laying of eggs. Pituitaries from hens that were in each of these three stages were dissected from electrocuted birds and immediately stored at –80°C. Liver was collected as a control tissue. Additional pituitaries were supplied by the laboratories of Dr. El Halawani (Department of Animal Science, University of Minnesota, St. Paul, Minnesota), from birds that were treated under similar conditions as described above. Pituitaries harvested at Virginia Tech are referred to as VT pituitaries and those harvested in Minnesota are referred to as MN pituitaries throughout the remaining sections. Pituitaries from turkey hens in the LAY stage were used for the preparation of nuclear protein extracts and the development of a cDNA library used in affinity chromatography and southwestern screening procedures respectively. Pituitaries from turkeys hens from both laboratories, collected in the NPS, PS and LAY stages, were used for 2D gel analysis of proteins expressed in each stage of the reproductive cycle. Turkey liver tissue was used for the preparation of a control extract for affinity chromatography.

B. Affinity Chromatography Procedure

B-1: Preparation of Turkey Pituitary Nuclear Extract, Probes and Columns

The procedure for preparing turkey pituitary nuclear extracts for affinity chromatography was adopted from Roy et al. (1991) and is also a modification of the procedure followed by Gazzillo, (2000). Nuclear extracts from turkey hen pituitary tissue and turkey liver tissue were prepared from approximately 0.4g of each tissue. The concentration of the pituitary and liver extract was determined using a commercially available protein assay (Bio-Rad Laboratories, Hercules, CA). The extract was diluted five-fold in 4°C, Buffer A (150 mM sucrose, 20 mM Tris-HCl, pH 7.9, 0.5 mM EDTA, and 2 µl/ml PMSF) and stored in aliquots at –80°C (adopted from Warner and Rutherford, 2000).

Oligonucleotides that included the putative transcription factor binding sites within the tPrl promoter region, identified by EMSA, were ordered from MWG Biotech, Inc. (Mendenhall Oaks, NC). Tprl-1 (5'-TCC ACA ACC TGC TGA ATG TAT GCA AAC TGG ACC -3'), tprl-2 (5'-AAG CAA GTA TTG AAT ATG AAT GTG GAA GAG AGG-3'), and tprl-3 (5'-TTT AAT GAA ATT CCC ACT CAC AGT A-3') oligonucleotide stocks were each annealed to antisense oligonucleotides to generate double-stranded sequences. Double-stranded oligonucleotides were phosphorylated and concatomerized using T4 Polynucleotide Kinase and DNA Ligase (New England Biolabs, Beverly, MA). The concatomers for each of the individual putative transcription factor binding regions were labeled at their 5'-ends with Biotin-16-ddUTP and DNA terminal transferase (Roche Molecular Biochemicals, Indianapolis, IN). Approximately 100 ng of the 300 ng of each labeled oligonucleotide obtained was resuspended in 25 µl Buffer A and incubated with 75

µl of a 100% slurry of agarose beads coated with immobilized streptavidin (Sigma-Aldrich, St. Louis, MO) for 2 hr, at 4°C with constant mixing.

A 1 cc syringe, containing a glass wool plug was washed with 100 µl of Buffer, followed by the application of 100 µl of the labeled oligonucleotide immobilized on streptavidin beads. The syringe was centrifuged at 4°C, 1800 rpm for 2 minutes to elute unbound oligonucleotides and pack the beads. The column was washed twice with 100 µl Buffer A and 100 µl eluted fractions were collected from each wash prior to the application of the extracts and designated fractions A and B. All washes consisted of a 5 min incubation period, on ice with 100 µl of buffer pipetted onto the column followed by centrifugation at 4°C, 1800 rpm for 30 seconds to elute each fraction.

B-2: Incubation Conditions

Approximately 500 µg of nuclear extract, diluted in 100 µl of Buffer A containing 2µg Poly I-C, was pipetted into the syringe column and washed. This procedure was repeated with the reapplication of 100 µl of the eluted extract to the column. The final eluted fraction was collected, stored at -80°C and designated fraction C. The column was then washed twice with 500µl of Buffer A, the eluted fractions were collected, stored at –80°C and designated D and E. The column was then washed twice with 500 µl Buffer B (150 mM sucrose, 20 mM Tris-HCl, pH 7.9, 0.5 mM EDTA, 2 µl/ml PMSF and 0.1 M KCl), Buffer C (Buffer A with 0.5 M KCl) and Buffer D (Buffer A with 0.7 M KCl). Eluted fractions from each were collected, stored at –80°C and designated in succession F-K (Kadonaga et al., 1986). A nuclear extract from liver tissue, prepared under the same

conditions was also applied to a new column and was used as a negative control. A column was also treated without extract to serve as a control as well.

B-3: Protein Detection

The 500 µl protein fractions, designated A-K were concentrated using Amicon Centricon-10 spin columns (Millipore Corp. Bedford, MA), following the manufacturer's instructions, to a volume of 50 µl. The protein concentration for each eluted fraction was determined using a commercially available kit (Bio-Rad Laboratories) based on a method by Bradford (1976). The full 50 µl volume of each fraction was separated on a standard 10% SDS-PAGE gel and proteins were visualized by silver staining (Bio-Rad Laboratories).

C. Development and Screening of a turkey pituitary cDNA library

C-1 RNA Isolation and cDNA Library Construction

Total RNA was isolated from 0.08g of layer turkey pituitaries and 0.08g of previously stored turkey liver tissue. The samples were homogenized in 800 µl of Trizol Reagent (Molecular Research Center, Inc., Cincinnati, OH), and RNA was isolated according to the manufacturer's instructions. The total RNA pellet was then air dried and resuspended in DEPC-treated, deionized water.

A cDNA library was constructed from total pituitary RNA using a SMART^{TM} cDNA Library Construction Kit from (BD Biosciences, Clontech, Palo Alto, CA). Briefly, first-strand cDNA was synthesized using Powerscript ^{TM} Reverse Transcriptase and lock-docking oligo(dT) primers to prevent excessive transcription of Poly A+ tails.

Asymmetrical S*fiI* restriction enzyme sites were also included in the primers' design for directionally cloning cDNA inserts into vectors downstream of a lac promoter sequence. First-strand cDNA was amplified by LD (long distance) PCR on a GeneAmp 2400 (PerkinElmer, Wellesley, MA) thermocycler. Three PCR reactions were prepared, each with a different concentration of total RNA. Each reaction was denatured at 95°C for 20 seconds and then cycled 21 times at 95°C for 5 sec and 68°C for 6 minutes, followed by a cooling to 4°C. Turkey liver full-length, double-stranded cDNA was also synthesized as a control. The turkey pituitary double-stranded cDNA was digested with *Sfi*I restriction enzyme and size fractionated using CHROMA SPIN^{TM} Columns (CLONTECH Laboratories, Inc.). A *λtrplEX* lambda vector, supplied in the SMART^{TM} cDNA Library Construction Kit, was also digested with S*fi*I and ligated with the digested double stranded cDNA fractions. The library was packaged into lambda phage using Gigapack Gold III Packaging Extract (Stratagene, La Jolla, CA) and titered on tetracycline plates supplemented with 10mM $MgSO_4$ and 0.2% maltose. The unamplified library was aliquoted and stored at –80°C with a titer of 9.85 X10^4 pfu/ml. The library was also amplified once to a titer of 4 X 10^9 pfu/ml.

C-2: Screening cDNA Expression Library

Approximately 500 pfu of the unamplified cDNA phage library were plated on twelve, 150 mm LB agar plates. For each plate, a 150mm nitrocellulose membrane was soaked in 10 mM IPTG solution for 2 minutes and gently overlayed on the surface of the plate. The membranes and the top agar were marked for orientation purposes. The plates were then incubated at 4°C for 8 hr, inverted and incubated at 37°C for 4 hr to initiate the

expression of protein from each recombinant phage. The nitrocellulose membranes were carefully removed and air-dried. To denature the proteins on the membranes and prepare them for exposure to the oligonucleotide probe, each membrane was washed for 5 minutes at 4°C in a series of guanidine hydrochloride buffer washes (Gordon et al., 1996 and Sarapura et al., 1997). The guanidine hydrochloride buffers (25 mM HEPES, pH 7.9, 3 mM $MgCl_2$, 40 mM KCl, 1 mM DTT) were identical except for a successively decreasing concentration of guanidine HCl (6M, 3M, 1.5 M, 0.75 M, 0.36 M and 0.19 M). The membranes were then blocked twice for 10 min, at 4°C, in Membrane Blocking Buffer (10 mM sodium phosphate, 5% nonfat carnation milk powder, 150 mM NaCl, 1% BSA, 2.5 % PVP-40, and 0.1% Trition X-100) for one hour, prior to probe application (Gordon et al., 1996).

A 300 µg sample of double-stranded trpl-1 oligonucleotide was phosphorylated using New England Nuclear, T4 polynucleotide kinase and 100 µCi of [gamma-^{32}P]-adenosine triphosphate with a specific activity of 4,500 Ci/mmol (New England Biolabs). Unlabeled oligonucleotides were removed by successively passing the sample through two 1 ml, Sephadex G-25 spin columns. T4 Ligase was added to the reaction mix and incubated overnight on ice. A 30 µl sample of this probe mixture was diluted 1:1000 in Binding Buffer (10 mM Tris-HCl, pH 7.5, 0.5% nonfat milk, 0.5% BSA, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 2 mM DTT, 0.1% Triton X-100, 5 % glycerol, 10µg/ml native salmon sperm DNA, and 10 µg/ml denatured salmon sperm DNA). The probe/ binding buffer mix was incubated with the blocked membranes overnight at room temperature with constant rotation. The membranes were then washed twice with 1X Binding Buffer and twice with Blocking Buffer for ten minutes each. The membranes were wrapped, marked

for orientation purposes, and exposed to Kodak XAR X-ray film for 30 hr or until spots were visible. Positive plaques were isolated using a wide-bore pipette tip and the bacteriophage were transferred to 0.5 ml LB broth containing 10 mM $MgSO_4$. These cultures were incubated overnight at 37°C, titered, replated and probed as described above for the primary screening. The southwestern screening procedure was repeated for 50 plates and approximately 25,000 pfu's were screened.

D. Proteomic analysis of turkey hen pituitary protein expression

D-1: Whole Cell Protein Extraction Preparation

The whole cell protein extraction procedure is a modification of the procedure described in "Protein Extraction Kit for Two-Dimensional Gel Electrophoresis" (Bio-Rad Laboratories, Inc). Approximately 0.025g of pituitary tissue from NPS, PS and LAY turkey hens was used for the preparation of whole cell protein extracts. Pituitaries from turkeys housed at Virginia Tech (VT pituitaries) as well as those from the University of Minnesota's Animal Science Department (MN pituitaries) were used. Tissue was kept at 4°C throughout the extraction procedure. The tissue was quickly minced in a chilled, plastic weigh boat, using forceps and a sterile razor blade in 0.5 ml of Protein Extraction Buffer (PEB) (8 M Urea, 2M Thiourea, 2mM TBP, 50 mM DTT, 5% CHAPS, 2% SB 3-10, 40 mM Tris Base, 0.2% Biolyte 3-10 range, and 10% Triton X-100). The formulation and content of the PEB buffer was based upon the extraction buffer from Bio-Rad's Protein Extraction Kit. The buffer-tissue mix was transferred to a Dounce homogenizer and 0.5 ml PEB buffer was used to collect all remaining tissue from the weighboat. The buffer-tissue mix was manually homogenized 40 strokes until it became a smooth, homogeneous

suspension. The sample was washed by transferring the contents of the homogenizer to a 1.5 ml microcentrifuge tube and centrifuged at 10,000xg for 10 min at 4°C. The supernatant was transferred to a clean, dry Dounce homogenizer and 50 µl of a 1U/µl stock of DNase I, and 1 µl of a 10mg/ml RNase A stock were added to the sample, homogenized with ten strokes and rewashed by transferring the contents of the homogenizer to a 1.5 ml microcentrifuge tube and centrifuged at 10,000xg for 10 min at 4°C. The supernatant was stored in aliquots at –80°C.

D-2: Nuclear and Cytoplasmic Protein Extract Preparation

Nuclear and cytoplasmic protein extracts were prepared from VT and MN pituitaries from the NPS, PS, and LAY stages. The procedure for preparing turkey pituitary nuclear and cytoplasmic extracts for proteomic analysis is a modification of the procedure for nuclear extract preparations from Roy et al. (1991) and the procedure followed by Gazzillo (2000). Two pituitaries weighing approximately 0.025g total from birds at each stage were quickly minced in a tray, on ice, in NE1 buffer. The buffer-tissue mix was transferred to a clean Dounce homogenizer and manually homogenized with 20 strokes. Remaining solid pieces of tissue were removed with a pipette tip and Triton X-100 was added to a final concentration of 0.1%. The sample was re-homogenized with 20 strokes. The homogenate was transferred to a 1.5ml tube and microcentrifuged at 4°C, 12,000xg for 5 minutes. The supernatant was transferred to a 1.5ml microcentrifuge tube and stored as a crude cytoplasmic protein extract. The crude cytoplasmic protein extract was diluted with 0.5 X volume of PEB buffer to improve the solubilization of proteins. An extract prepared in the same manner was also prepared and the supernatant was respun to remove any remaining

nuclei that may still be present. The supernatant from this second spin was considered the final cytoplasmic extract and was used for subsequent experiments. The crude and final cytoplasmic extracts were stored at –80°C and labeled appropriately.

The remaining pellet containing isolated nuclei was lysed and resuspended in PEB buffer. This nuclear extract was treated with 50 µl of a 50 U/µl DNase I stock, homogenized with 10 strokes, and centrifuged at 180,000 x g for 90 minutes at 4°C, in a Beckman TLA 100.3 rotor, to remove fine debris. The concentration of total protein was determined using an RC/DC Protein Assay Kit (Bio-Rad Laboratories, Inc.).

D-3: Western Blot Analysis of Extracts

Western blots of the crude and final cytoplasmic and nuclear protein extracts from VT pituitaries were performed to determine the presence and location of the nuclear protein tPit-1 in each preparation. Liver nuclear extract was used as a negative control. 20 µg per extract were mixed with sample loading dye (0.01% bromophenol blue, 1% SDS, 10% glycerol, 75 mM Tris buffer, pH 6.8) and separated on a standard 10% SDS-PAGE with a 4% stacking gel. Gels were run in 1 X Running Buffer (0.02 M Tris pH 8.3, 0.192 M glycine, 0.1% SDS) at 100 V until the dye front exited the stack, and 200 V for 3-4 hr for the remainder of the run. Gels were transferred for 2 hrs at 60 V, in Transfer Buffer (20 mM Tris, 144 mM glycine and 20% MeOH) to a 0.2 micron buffer equilibrated nitrocellulose membrane (Millipore, Inc.) using a Panther™ Semidry Electroblotter (Owl Separation Systems, Portsmouth, NH). The membrane was blocked for one hour in Blocking Buffer (1X PBS and 5% Carnation® milk powder). The blocked membrane was incubated with a 1:2,000 diluted solution of rabbit anti-rat Pit-1 polyclonal Ab (supplied by

Dr. Simon Rhodes, IUPUI, IN) in blocking buffer for 1 hour at room temperature. The membrane was washed three times for ten minutes, with Wash Buffer (1X PBS, 0.5% Carnation® powder milk and 0.1% Triton X-100). A 1:20,000 dilution of HRP-labeled goat anti-Rabbit IgG secondary antibody (Pierce, Rockford, Il) in Blocking Buffer was applied to the membrane for one hour at 25°C. The wash procedure was then repeated. tPit-1 was detected on Kodak RD X-Ray film using an HRP Chemiluminescent Detection Kit (Pierce) following the manufacturer's instructions.

D-4: Isoelectric Focusing, Equilibration and Separation of Cell Protein Extracts

Solubilized protein extracts were electrically focused to separate proteins according to their isoelectric points. Nuclear, cytoplasmic and whole cell extractions from VT and MN pituitaries from NPS, PS and LAY stage hens were all separated in this manner. For each protein extract, three Bio-Rad IEF gel strips (pH 3-10, 17 cm) were each soaked in 300 µl of a 1µg/µl preparation of the protein extract in IEF Running Buffer (IRB) (8 M Urea, 2 M Thiourea, 2 mM TBP, 4% CHAPS, 0.2% Biolyte 3-10, 50 mM DTT). Electrofocusing strips were allowed to actively rehydrate for 13 hr at 25°C, before focusing in the Bio-Rad IEF gel focusing apparatus. Strips were electrically focused, with 50 mA, 250V constant voltage for 15min and 20°C, followed by focusing from 250V to 10,000V over 5 hr at 20°C and then further electofocusing until a total of 60,000 Vhrs had accrued. Total focus time was usually 24 hr.

Twelve identical 8-14% polyacrylamide gels, 18cm X 20cm and 1mm thick, were simultaneously poured 24 hr before use. A Bio-Rad Dodeca gel casting unit with twelve gel plates, attached with silastic tubing to a peristaltic pump, a 50 ml syringe and a gel mixing

column was set up as shown in Figure III-1. 250 ml of an 8% acrylamide “Light” solution (8% of 29:1 acrylamide:bis acrylamide stock solution, 0.375 M Tris-Cl, pH 8.8, 2% SDS) and 250 ml of a 14% acrylamide “Heavy” solution (14% of 29:1 acrylamide: bis acrylamide stock solution, 0.375 M Tris-Cl, pH 8.8, 2% SDS, 10% Glycerol) were prepared to form the gradient gels. A separate, 50 ml solution of the “Light” acrylamide solution was prepared as well. All three solutions were mixed and degassed for 5 minutes at room temperature.

A final concentration of 0.05% ammonium persulfate (APS) and 0.025% TEMED were added to the 50 ml of Light solution and mixed. The 50 ml Light solution was drawn into the 50 ml syringe and the peristaltic pump was used to fill the gel casting chamber with 40 ml of the Light solution from the syringe. Figure III-1 illustrates the procedure for pouring these gradient gels. All twelve gels were filled simultaneously by capillary action in the casting chamber. The same final concentrations of APS and TEMED were added to the 250 ml Light and 250 ml Heavy solutions and they were simultaneously poured into their designated chambers of the gel mixing column. While constantly stirring the Light solution, the gel mixing column chambers were allowed to mix and the mixed contents were pumped to the gel casting chamber until the desired height of the gels was attained. dH2O was used to overlay each gel. The top of the gel casting unit was sealed with saran wrap to allow the gels to polymerize overnight. Three hours prior to IEF gel strip loading, 7 ml of a standard 4% polyacrylamide stacking gel solution was added to form a 4 cm stack on the top of each gel.

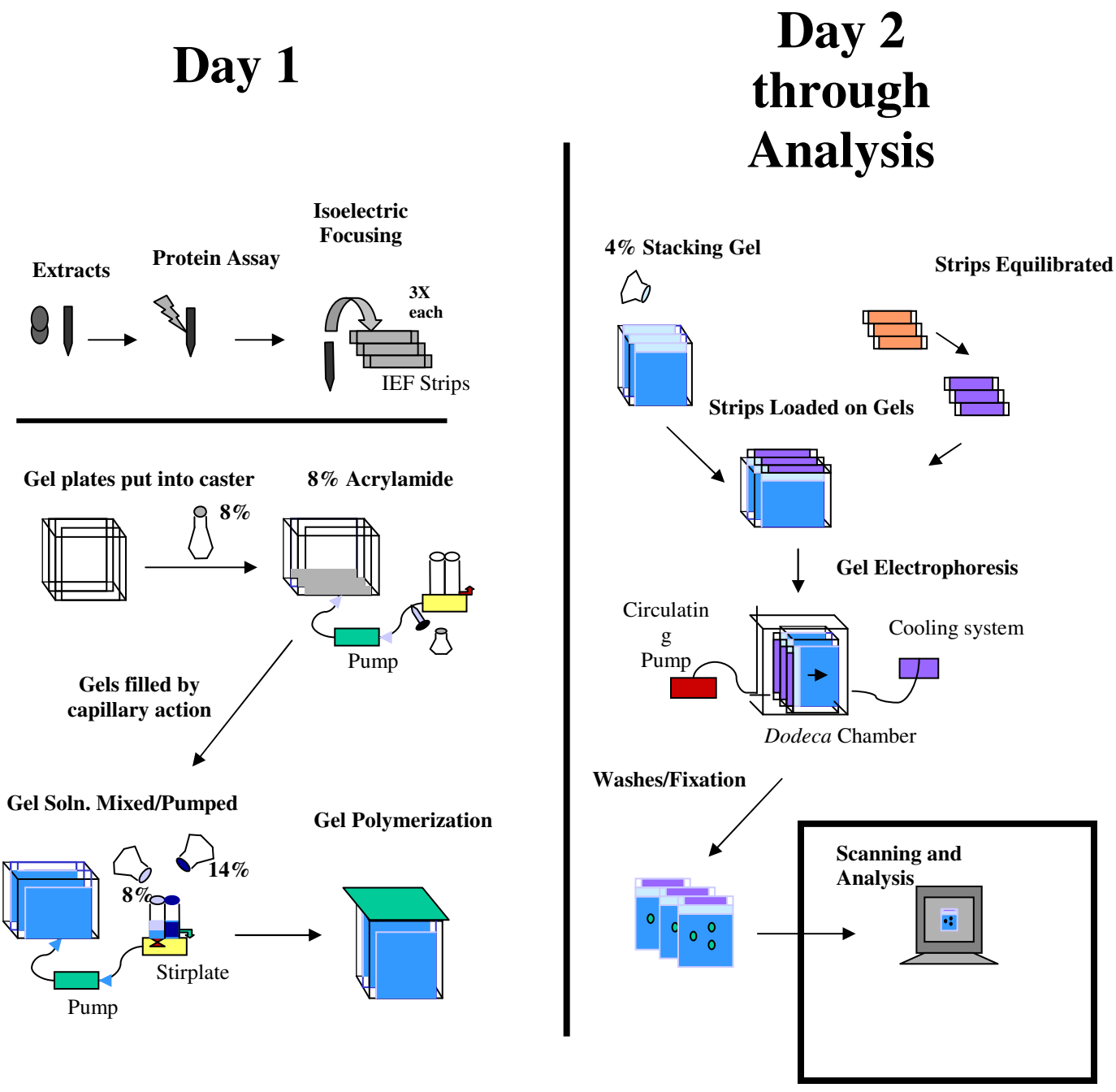


Figure III-1: Illustration of Two-Dimensional Electrophoresis Procedure. All procedures were optimized for turkey pituitary tissue and all steps are considered standard techniques for two-dimensional analysis except for the use of the peristaltic pump for the formation of our gradient gels.

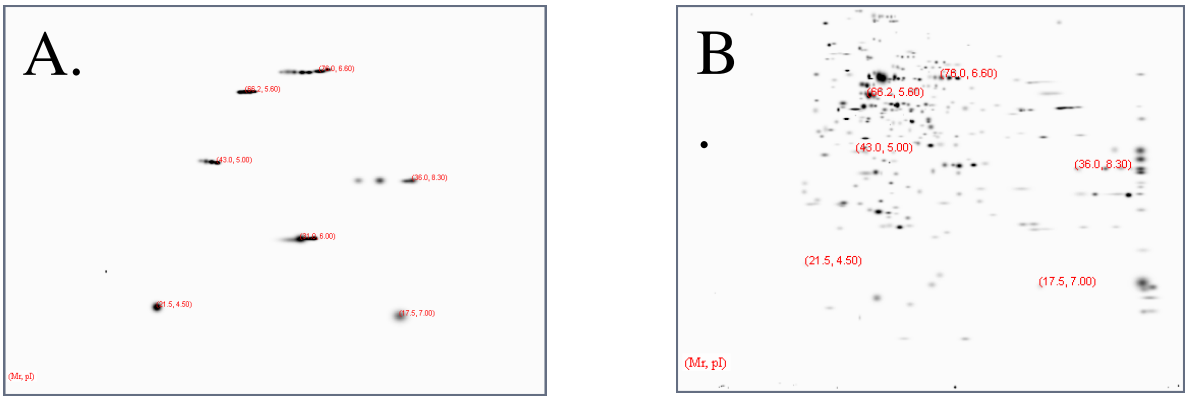
Focused IEF Strips were gently blotted on kimwipes and then equilibrated, with gentle rocking, for 30 minutes in EB1 (6 M urea, 4% SDS, 0.375 M Tris-HCl, pH 8.8 and 20% glycerol), EB2 (6 M urea, 4% SDS, 0.375 M Tris-HCl, pH 8.8 and 20% glycerol and 130 mM DTT), and EB3 (6 M urea, 4% SDS, 0.375 M Tris-HCl, pH 8.8 and 20% glycerol and 135 mM iodoacetamide). Focused and equilibrated strips were soaked in 1X TGS running buffer (25 mM Tris-Base, 192 mM glycine. 0.1% SDS pH 8.3) and sealed along the top edge of a gradient gel with 1% agarose in 1X TGS running buffer containing 0.01% bromophenol blue dye. Gradient gels were run for 8 hr at 200 V (constant voltage) in 1XTGS running buffer, which was constantly circulated and cooled to 22°C during the gel runs.

Gradient gels were fixed in a 10% methanol/10% acetic acid fixative solution for 30 minutes with gentle shaking. Gels were then stained and rocked overnight in Sypro Ruby staining solution (Bio-Rad Laboratories, Inc.). The stain solution was then removed and gels were washed 3 times, for one hour each, with fixative solution. Gels were placed upon the tray of a Molecular Imager FX Pro Plus MultiImager System (Bio-Rad Laboratories, Inc.) phosphorimaging unit and images of each gel were taken. Following the procedure described above, 2D gels were generated that offer protein expression information for each of the three stages in the reproductive cycle of the turkey hen and for the three different extractions performed on the pituitary tissue collected for each of these stages.

D-5: Establishing a Molecular Weight, Isoelectric Point Grid (MrpI grid)

Additional 2D gels were run, according to the procedure outlined above in order to characterize each separated protein by its molecular weight and isoelectric point. Figure III-

2 shows these gels and the resulting MrpI grid that was generated from them. A molecular weight and isoelectric point (Mr pI) standard grid was established by running 2D SDS PAGE internal protein standards (Bio-Rad Laboratories, Inc.) on 2D gels. The protein standards included seven, reduced and fully denatured proteins with known molecular weight and pI values. These proteins were: conalbumin (76 kDa/pI 6.6), albumin (66.2 kDa/pI 5.4), actin (43 kDa/ pI 4.7), GAPDH (36 kDa/pI 8.5), carbonic anhydrase (31 kDa/pI 6.0), trypsin inhibitor (22 kDa/pI 4.5) and myoglobin (17.5 kDa/pI 8.0). Triplicate 2D gels were run with just protein standards (Figure III-2A) and triplicate gels were run simultaneously with 15 µg of the internal protein standards (Figure III-2B). Triplicate gels containing 25 µg of the marker and 300 µg of protein extract from whole cell, LAY hen VT pituitaries were also run (Figure III-2C). The location of the marker proteins within the whole cell LAY hen pituitary extract were noted on the gels and their known molecular weights and pI's were entered with their SSP numbers. With these molecular weights and isoelectric points, the PDQuest software used for analysis was able to establish a MrpI grid across the entire surface area of the gel. With this grid, the molecular weights and isoelectric points for all proteins, designated as landmarks and/or matched protein spots in 2D gels generated using VT and MN pituitaries, could be determined. Proteins with a molecular weight between 21.5 kDa and 80.0 kDa, and an isoelectric point between 4.5 and 8.0 were resolved and characterized reliably on these 8-14% gradient gels with this MrpI grid.



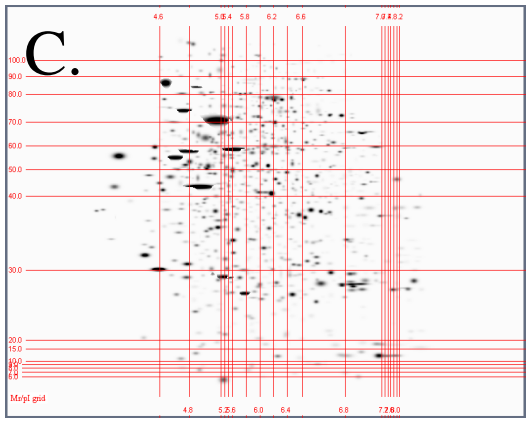


Figure III-2: Formation of MrpI Gradient. Illustration of (A) the separation of 15 µl internal standard proteins (BioRad Laboratories) in a 8-14% gradient 2D gel using an isoelectric focusing strip with a pH range of 3-10. Figure (B) depicts the gel used for the identification of the standard proteins in the 2D separation of whole cell extract proteins for LAY stage turkey hen pituitary cells. Figure (C) depicts the MrpI plot established by the internal standard proteins for all matched gels; represented here in the master gel for the VT matchset.

D-6: Establishing Matchsets

Two matchsets were established for analysis of protein expression patterns. One matchset was created containing the Raw, Filtered and Gaussian images for the triplicate gel separations of whole, cytoplasmic and nuclear extracts from VT pituitaries from NPS, PS and LAY hens. The second matchset was created from an identical set of gel images for MN pituitaries. For each matchset, PDQuest 7.0.1 gel image analysis software (BioRad Laboratories, Inc.) was used to generate a Raw, Filtered and Gaussian image of each gel and to conduct all subsequent 2D gel image analysis. Each scanned gel was cropped from the original image and the software's "Spot Detection Wizard" was used to determine background and standard spot parameters for the overall gel image. The background was established by setting sensitivity, smoothing and spot size scale parameters. A randomly selected "faint" spot was selected to determine the smallest/faintest spot that could be detected. Black marks less than or equal to 3X3 pixels were considered to be speckling due to dirt or other contaminants and were eliminated from analysis. A minimal intensity for spot identification was set for all gels. Smoothing the background was accomplished by suppressing the effects of "peppering", caused by undissolved gel staining reagent, and other background noise. To remove vertical and horizontal streaking, a randomly selected "large" spot was selected (33X12 pixels in size). Using standard settings set by the PDQuest software, vertical and horizontal streaks were removed that were lengths greater than 13 and 33 pixels respectively. Trends in signal intensity, indicating the presence of spots, from random intensity fluctuations, which were considered noise, were also resolved by the software.

Figure III-3A, B and C compares the visual representation of the Raw (A), Filtered (B) and Gaussian (C) images for the scanned gel with a nuclear protein separation from NPS stage turkey hens generated by the application of these parameters. The Raw image has not been manipulated. The Filtered image reflects the effects on the visualization of the gel by the application of the sensitivity, background smoothing and spot size scaling parameters and shows a clean, streak-free background. The Gaussian image reflects the software's adjustments made to normalize all the gels in the matchset and depict protein densities. The Gaussian image has the cleanest appearance, since it is a refined representation of protein spot densities. The Raw, Gaussian and Filtered image for each gel was examined throughout the manual matching procedure for each matchset. Consulting each of these three images for each gel ensured that protein spots were matched as accurately as possible in later stages when establishing matchsets. These same parameters for spot detection and background determination were applied to all gels used in each matchset.

Figure III-4 illustrates the gels grouped and analyzed together for the VT matchset Twenty-seven gels were grouped and analyzed in each matchset and a gel from the Whole cell extract, LAY hen pituitaries was used as the master gel for each matchset. All protein spots found in each gel were matched with those in the master gel, creating a single gel that depicts all spots present in every gel of the matchset. The "Automated Detection and Matching" option in PDQuest 7.0.1 was used initially to match spots in all the gel images. Spots clearly seen in all gels were marked as internal landmarks for orienting the gels. Manual matching was used to match every protein spot found in the master gel to the protein spots in other gels. Unmatched protein spots present in matchset gels that were not found in the master gel were added to the master and manually matched as well. The

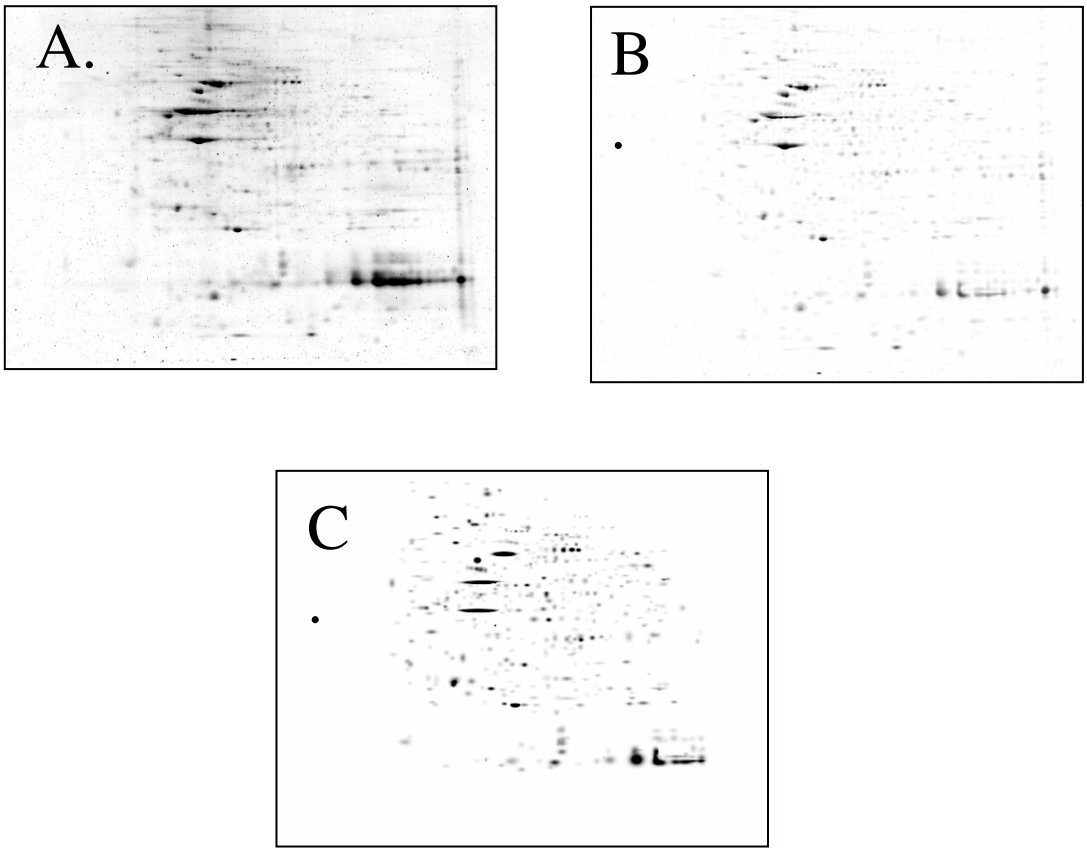


Figure III-3:Visual Representation of Image Types. Raw(A), Filtered(B), and Gausian (C) images used for matchset construction and for manually matching protein spots. All three images were generated from one gel in a triplicate group of nuclear extract proteins from NPS turkey hens.

matched gels were normalized and SSP numbers were assigned by the software system for every matched protein spot in the matchset. This number was used as a protein identification number throughout the analysis.

The molecular weight and isoelectric point (MrpI) values for the protein spots matched in each matchset were determined by visually comparing the spot pattern for each master gel with that of the MrpI gradient standard gels, which also used a whole cell, LAY hen pituitary extract. The MrpI values for proteins found on the MrpI standard gels that matched proteins in the matchsets' master gels were manually entered and a MrpI gradient for all the matchset gels was automatically generated by the software.

A higher level matchset was also designed to compare the VT matchset and MN matchset protein expression patterns by matching the master gels from each of the matchsets described above. Figure III-5 illustrates the steps taken to create the VT, MN and higher level matchset for protein expression pattern analysis. All represented protein spots in the master gels from the matchsets for VT and MN pituitaries were manually matched to one another. The same spot parameters were used to establish the higher level matchset and a MrpI gradient was established by referencing the locations of 2D SDS PAGE standard proteins in the master gels. The software assigned new SSP identification numbers for all matched protein spots.

D-7: Analysis of Protein Expression Patterns

The density, molecular weight and isoelectric point for every protein spot, identified by an assigned SSP number, in every gel in the VT, MN and higher level matchsets were determined by the PDQuest 7.0.1 image analysis software and exported to an Excel spreadsheet format.

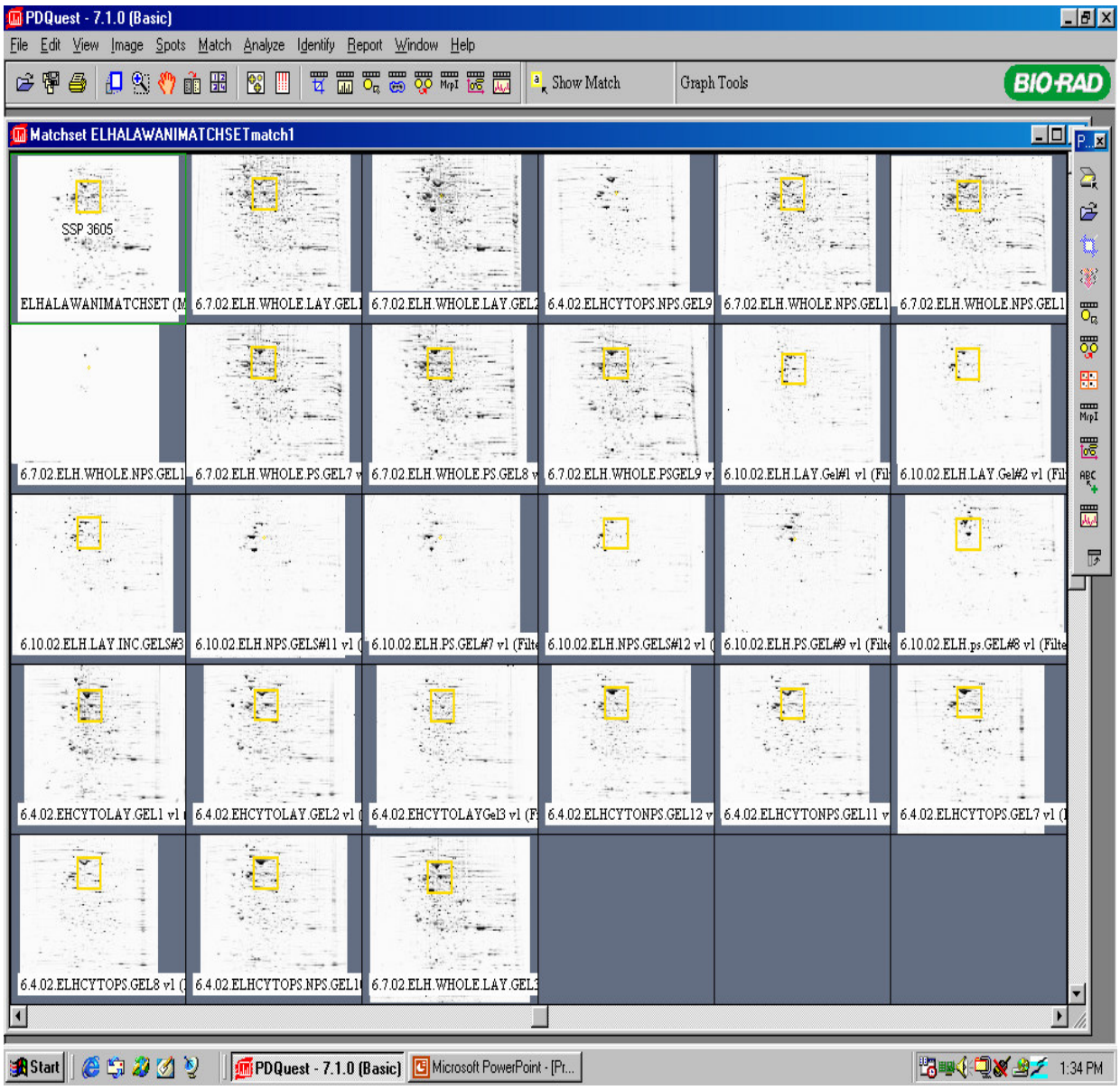


Figure III-4: Visualization of the MN Matchset. Representation is in PDQuest 7.1. The gel image in the top, lefthand corner is the master gel for this matchset. It represents a composite of all the protein spots detected in all the gels. Protein spot number 3605 is selected to show its matched representation in the gels of the matchset.

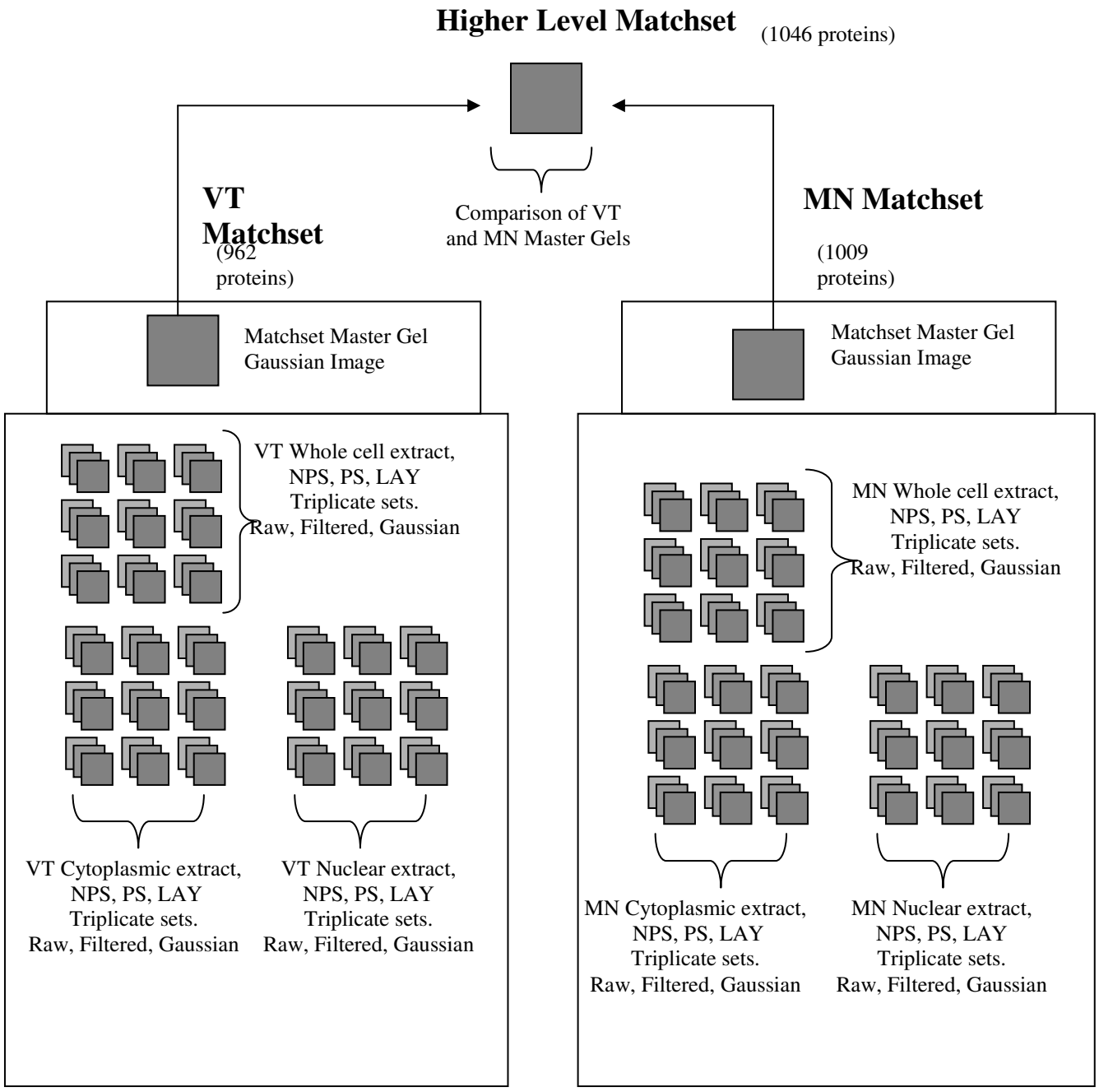


Figure III-5: Schema for Generating Matchsets for Analysis. Triplicate sets of gels for NPS, PS and LAY stage pituitary extracts in Nuclear, Cytoplasmic and Whole Cell extracts were run, scanned, and grouped, using the PDQuest software, as one matchset. A matchset with this same set of gels was generated for pituitaries from VT and MN turkeys. Once these two matchsets were formed, a Higher level matchset was generated using the master gels from the VT and MN matchsets. The master gel from each matchset displays all proteins expressed in every gel of the matchset and also retains the density values for all proteins expressed in these gels. These expression patterns and density values were compared between the two matchsets in the higher level matchset.

IV. Results

A. Isolation of specific putative binding proteins for the tPrl promoter region

An initial goal of this study was to isolate and characterize proteins, expressed by the anterior pituitary, that regulate reproductive cycling of turkey hens. The first objective was to specifically investigate the putative transcription factors that regulate tPrl gene expression using techniques that rely upon their specific interaction with the tPrl promoter for their isolation.

A-1: Affinity Chromatography

A procedure using affinity chromatography was designed to isolate putative nuclear transcription factors from LAY hens that bind to the tPrl promoter. Figure IV-1 depicts protein concentrations of fractions eluted from a representative affinity chromatography assay. The application of pituitary and liver nuclear extracts and an equivalent volume of Buffer A, as control lanes, are also depicted. Protein levels detected in the two pre-extract fractions (1 and 2), representing those columns receiving only Buffer A, were equivalent to background levels. Once the extracts were applied, 200 µg/µl of pituitary and 165 µg/µl liver protein were detected (fraction 3). The levels of protein eluted from each extract column dropped with further washes with Buffer A, demonstrated by fraction 4 in Figure IV-1. With the application of Buffers B, C and D, the amount of protein detected in fractions 5-10 were all below 20 µg/µl for all columns.

Figure IV-2 shows an image of a silver stained SDS PAGE gel with 60 µl of each eluted fraction from a column used to isolate putative transcription factors from LAY stage turkey pituitary tissue. Lane 1 contains a standard molecular weight marker. Lane 2 is

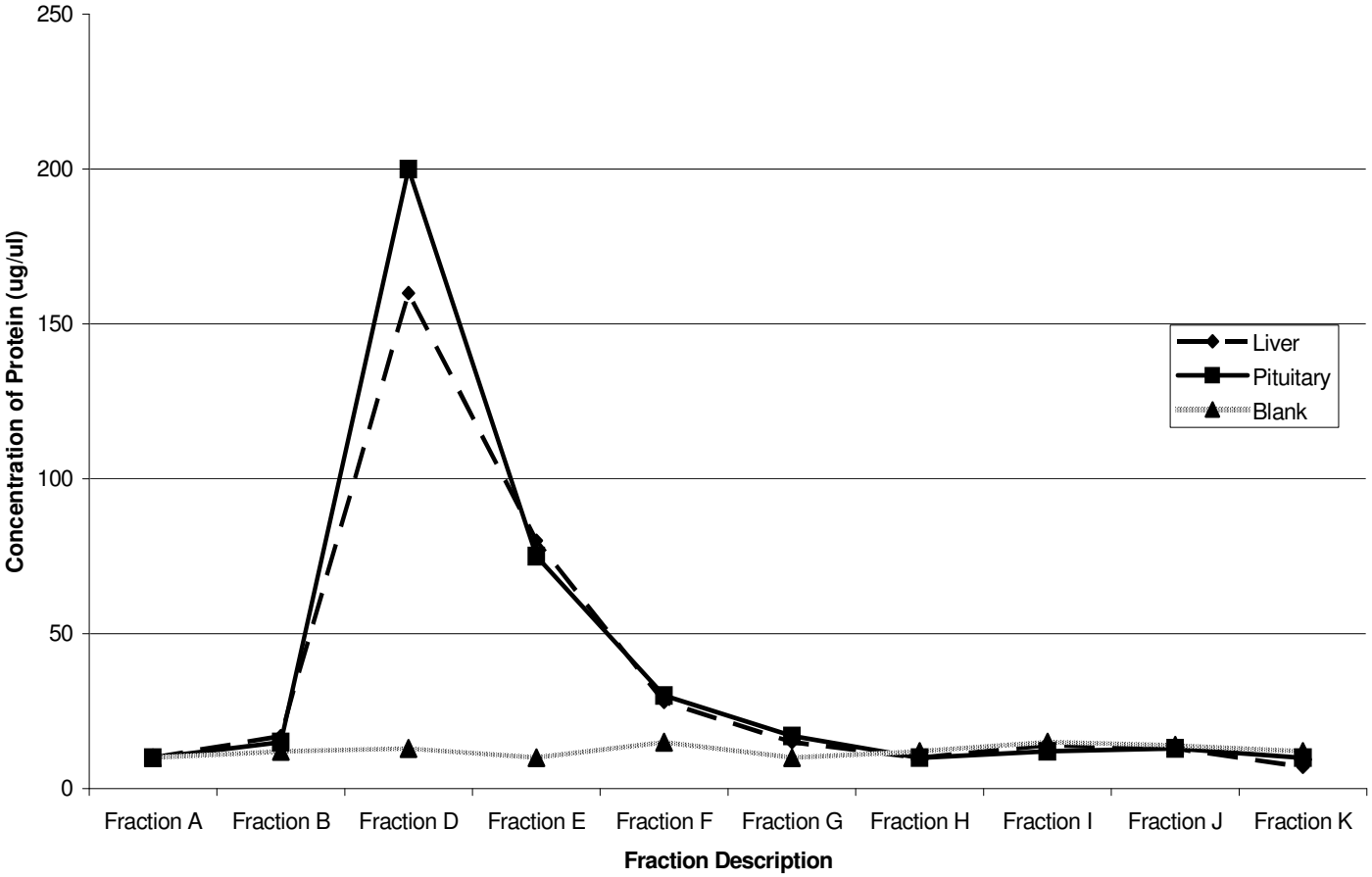


Figure IV-1: Protein Concentrations for Fractions Obtained from Affinity Chromatography Assay. Extract concentrations were measured in µg/µl. Fractions were collected from a column used for the separation of binding proteins from LAY stage turkey hen pituitary extract as well as from control columns for liver extract and buffer only. Fractions A and B were collected prior to the application of extract. Fractions D and E were collected after the addition of extracts to the columns and subsequent washes in Buffer A. Fractions F and G represent the last two washes with Buffer B, which had a slightly higher concentration of salt. Fractions H and I and collected after washing with Buffer C and Fractions J and K were collected after the last two washes with the highest concentration of salt in Buffer D.

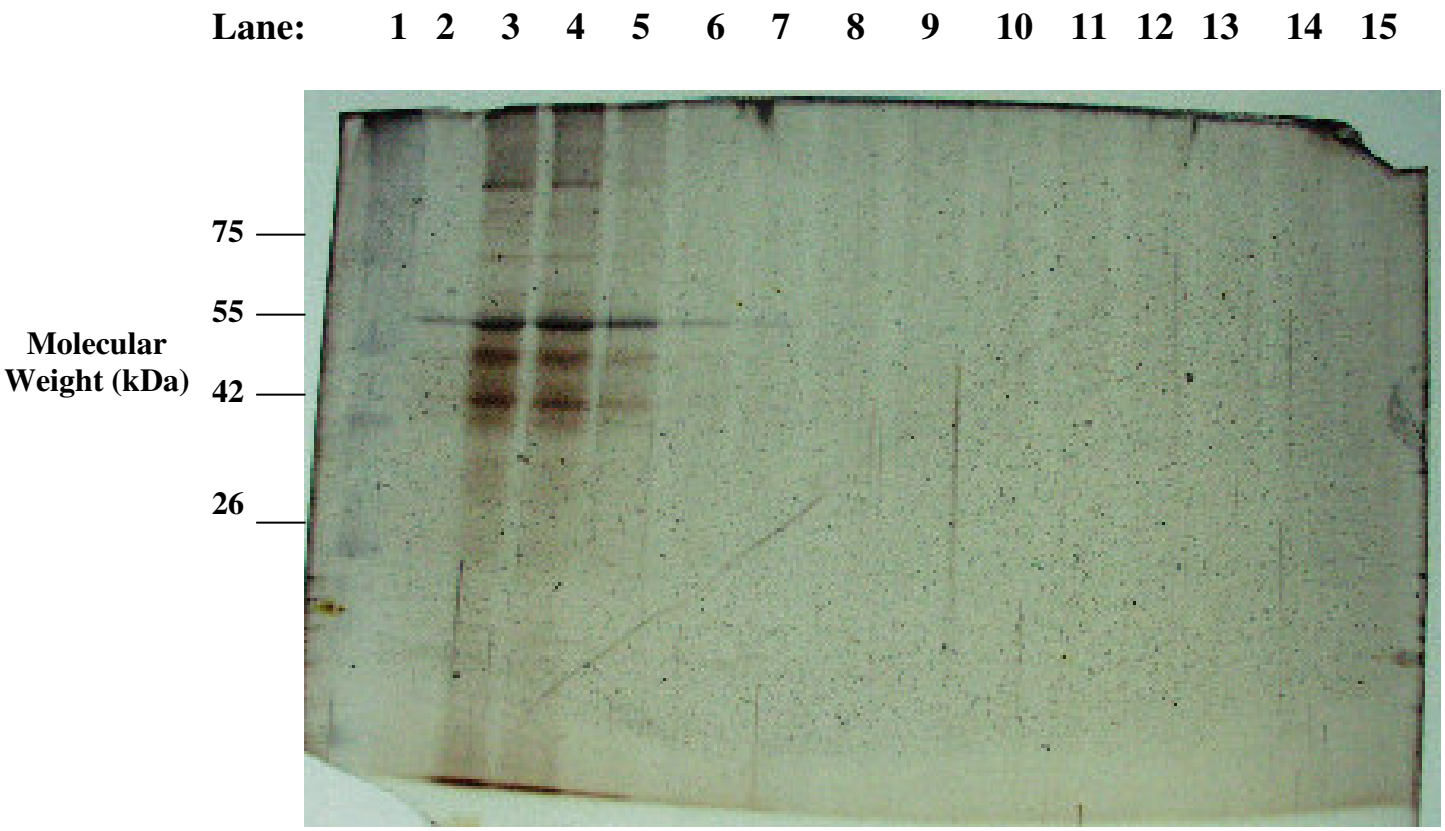


Figure IV-2: Silver Stained SDS PAGE Gel of Affinity Chromatography Fractions. Turkey pituitary protein fractions collected during affinity chromatography. Lane 1 shows a standard, molecular weight marker. Lane 2 represents Fraction B collected prior to the application of protein. Lanes 3, 4, and represent Fractions C, D and E, collected after applying nuclear extract and washing with Buffer A. Lane 6 represents the first wash with Buffer B. Lanes 7, 8 and 9 are Fractions G, I and K collected after the second washes with Buffer B, C and D respectively. Lane 10 through 15 represent fractions collected for the no protein control column and represent equivalent Fractions to the those in lanes 2 through 7.

Fraction B collected prior to the application of protein extract. Lanes 3, 4 and 5 represent Fractions C, D and E and show significant quantities of nuclear protein washed from the column with Buffer A. Lane 6 represents Fraction F, washed with Buffer B for the first time. Lanes 7, 8 and 9 represent Fractions G, I and K which were final washes with Buffers B, C and D respectively. No visible evidence of protein was detected in Fractions G, I or K from washes with increasing salt concentrations. Lanes 10-15 represent fractions from the no protein control column that were treated in the same manner as lanes 2-9.

The liver extract, acting as a control in the affinity chromatography assays, produced expected results. Since no putative transcription factors were present in this extract and, therefore would not bind specifically to the probe, protein was not expected to elute with the higher salt concentrations. However, since the pituitary extract does contain putative transcription factors with affinity for the probe, protein was expected to elute after an increase in the salt concentration. The higher salt concentrations should elute any putative transcription factors binding specifically to the probe from the column. Figure IV-1 and IV-2 do not show detectable levels of protein in any of the fractions from the washes with higher salt concentration buffers.

A-2: Southwestern Screening of a cDNA Expression Library

A second attempt was made to isolate these putative transcription factors by screening a cDNA λ phage expression library made from LAY stage pituitary RNA. A southwestern screening technique, modified from a procedure by Gordon et al. (1996), was used to identify clones expressing protein products that specifically bound to the tPrl promoter binding site. An unamplified pituitary cDNA λ phage library was constructed and

each cDNA insert was properly oriented into the *λtrplEX* lambda vector using *Sfi*I sites. Restriction enzyme digestion of twenty random clones using *Sfi*I revealed variable length inserts, indicating that the library successfully represented an array of cDNA sequences expressed by the LAY turkey hen pituitary cells (data not shown). However any positive plaques after the primary screening of approximately 25,000 pfu's, were not confirmed to be positive in the secondary screening procedure (data not shown). Therefore, the radioactively labeled probe was not specifically bound to any of the protein expressed by individual plaques.

In summary, these approaches to the study of specific proteins involved in turkey Prl gene expression were unsuccessful. The affinity chromatography and southwestern techniques were unable to isolate putative transcription factors that have been shown previously to bind to the tPrl promoter by EMSA assays. A pituitary cDNA λ phage library, however, was successfully constructed that may be used for future research efforts.

B. 2-D Gel Analysis of Pituitary Protein Expression

The second major objective of this study was to isolate and separate on 2D gels all expressed proteins from turkey hen pituitary tissue, from various reproductive stages. This approach to the study of overall protein expression leads to the isolation and characterization of many proteins that may be involved in turkey hen reproductive cycling. Although the more specific approach used in the first objective was unsuccessful, this second, more general approach may also result in the isolation and characterization of the same putative transcription factors involved in tPrl gene expression. In this way, results from this more generalized approach not only expand upon specific proteins that have already been

pursued, but they also offer avenues for the characterization of new proteins that regulate turkey hen reproductive cycling.

In addition the possibility of individual protein discoveries, the experimental methods designed for separating solubilized proteins from turkey pituitary tissue established a baseline for future investigations using 2D gel analysis. The quality of the protein extracts, the effectiveness of the 2D gel separation techniques, and the ability to replicate protein separations in triplicate gel sets were important components for meeting this objective. Therefore, particular attention was given to evaluating these procedures.

B-1: Evaluation of Tissue Extracts

Previous research efforts have suggested that the quality of protein extraction procedures are often times tissue specific (Molloy, 2000; Rabilloud, 2000). For example, Molloy (2000) noted that membrane proteins are more difficult to solubilize and separate, due to their chemical character and membrane compartmentalization. In general, the protein extraction buffers and procedures must be optimized given the unique solubilization characteristics of the tissue type used. They must enable the separation of compartmentalized proteins, i.e. nuclear from cytoplasmic proteins or mitochondrial from membrane bound proteins. In this study, the nuclear and cytoplasmic protein extractions were prepared from the same starting material. Table IV-1 lists the amount of tissue used for the preparation of each extract from VT and MN pituitaries. Quantities ranged from 21 mg to 17 mg, averaging 19.6 mg. Fluctuations were due primarily to variations in whole pituitary weights. LAY pituitaries were approximately two times larger than NPS and PS pituitaries which is consistent with previous reports that lactotroph cells proliferate during the laying

Extract Type and Tissue Source	Total Protein Yield (ug)	Tissue Weight (mg)	Concentration of Extract (ug/ul)
Nuclear NPS/ Virginia Tech	1121.66	19.00	1.50
Nuclear NPS/Minnesota	1604.70	18.00	2.14
Nuclear PS/Virginia Tech	1826.48	20.00	2.44
Nuclear PS/Minnesota	2051.70	20.00	2.74
Nuclear L/Virginia Tech	1017.07	21.00	1.36
Nuclear LAY/Minnesota	1464.23	20.00	1.95
Cytoplasm NPS/Virginia Tech	1351.61	19.00	1.80
Cytoplasm NPS/Minnesota	1605.15	19.00	2.14
Cytoplasm PS/Virginia Tech	789.54	20.00	1.05
Cytoplasm PS/Minnesota	1907.25	21.00	2.54
Cytoplasm L/Virginia Tech	1275.20	21.00	1.70
Cytoplasm L/Minnesota	1470.24	22.00	1.96
Whole NPS/Virginia Tech	1389.20	17.00	1.85
Whole NPS/Minnesota	1415.48	19.00	1.89
Whole PS/Virginia Tech	1298.44	18.00	1.73
Whole PS/Minnesota	1089.75	19.00	1.45
Whole L/Virginia Tech	2747.51	19.00	3.66
Whole LAY/Minnesota	1925.25	20.00	2.57
Average Yields/Virginia Tech	1495.60	19.53	1.99
Average Yields/Minnesota	1542.87	19.59	2.06

Table IV-1: Protein Extract Concentration and Total Protein Yields. Tissue weights, extract concentrations and protein yields for whole, cytoplasimic and nuclear extracts of NPS, PS and LAY stage pituitaries. Extracts were resuspended in 750 µl of buffer.

stage of the turkey's cycle (Lopez et al., 1995). Multiple pituitaries were used from the NPS and PS stages to obtain the same amount of tissue for the extractions. Table IV-1 also lists the total protein yields obtained in each extraction, for VT and MN pituitaries. Following the procedures described above for whole cell, cytoplasmic and nuclear protein extracts, on average, 19.6 mg of pituitary tissue generated 1.5 mg of total solubilized protein in 750 µl of buffer. The average concentration of the extracts was 2.0 µg/µl. The pituitaries collected from each of the two locations produced consistent quantities of protein from the same amount of tissue. Since 2D gel analysis requires 300 µg of total protein from each extract for each gel, on average, each extraction produced enough protein to run three to four gels. This quantity was sufficient for the completion of the study.

To verify that these preparations successfully separated nuclear proteins from cytoplasmic proteins, each of these extracts were separated and compared by western blot analysis, shown in Figure IV-3. Using an anti-rat Pit-1 antibody, which has been shown to detect the nuclear transcription factor tPit-1 (Weatherly et al., 2001), western blot analysis indicates a high quantity of tPit-1 protein is present in the nuclear extracts. The quantity of tPit-1 decreases drastically in extracts taken after one wash of the nuclear pellet. Turkey Pit-1 levels were even lower in extracts after a second wash step and tPit-1 protein is not detected in turkey liver nuclear extracts, which is expected, since tPit-1 is not expressed by this cell type. Turkey Pit-1 is exclusively a nuclear protein, found in some pituitary cells (e.g., somatotrophs, probably also present in thyrotrophs) of the anterior pituitary. Since our turkey pituitaries have somatotroph cells present, tPit-1 protein should be present in turkey pituitary extracts. However, tPit-1 should not be present in cytoplasmic extractions since it

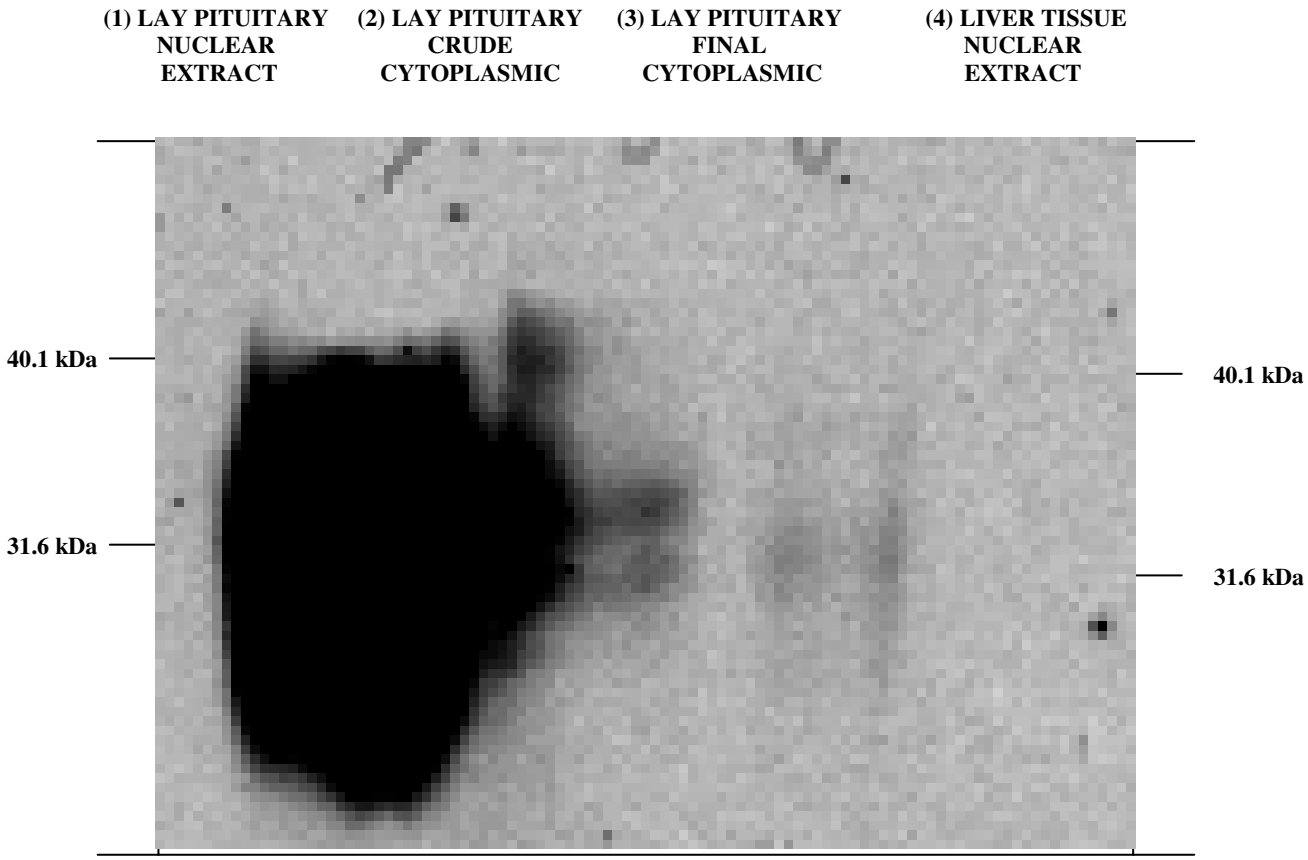


Figure IV-3: Illustration of Western Blot Analysis Results. Western blotting was used to evaluate the relative levels of tPit-1 in layer hen cytoplasmic and nuclear extracts (4). Equal quantities of extract (20 μg) were loaded into each lane of a 10% SDS-PAGE gel. Samples of the LAY stage nuclear extract (1), the crude cytoplasmic extract (2) and the final cytoplasmic extract (3). Liver nuclear extract (4) was used as a negative control. Conditions for the detection of tPit-1 using an anti-rPit-1 antibody are described in the experimental methods.

is primarily located in the nuclei. This result demonstrates that the extraction procedure successfully separates nuclear proteins from cytoplasmic proteins.

B-2: Evaluation of 2-D Gel Separations

Since the goal was to analyze the total protein expression patterns of the pituitary tissue during the NPS, PS and LAY stages in the turkey hen reproductive cycle, the 2D gel system was optimally designed to separate all solublized proteins by molecular weight and pI. Figure IV-4 shows three representative 2D gels with nuclear (A), cytoplasmic (B) and whole cell protein extracts (C) from VT harvested, LAY stage pituitaries. These three gels are representative of the protein expression pattern for each of the three extractions. Usually the nuclear protein separations did not have as many visible protein spots as in the cytoplasmic and whole cell separations. Figure IV-5 shows 2D gel separations of NPS (A), PS (B) and LAY (C) stage VT harvested pituitaries from whole cell extracts. The NPS stage protein separations appeared to have fewer protein spots than the LAY and PS stage separations. Each 2D gel shows a thorough, quality separation of solubilized proteins with major streaking limited to the outer edges of the gels. These gels show that the experimental methods for 2D gel separations did successfully separate proteins in each type of extract and from each stage in the reproductive cycle that was included in the study.

B-3: Determination of Triplicate Set Quality

Three 2D gels were produced for each extract in order to obtain a more accurate account of the total protein expression for each condition. Figure IV-6 shows a representative triplicate set from VT harvested, LAY stage, whole cell extracted pituitary

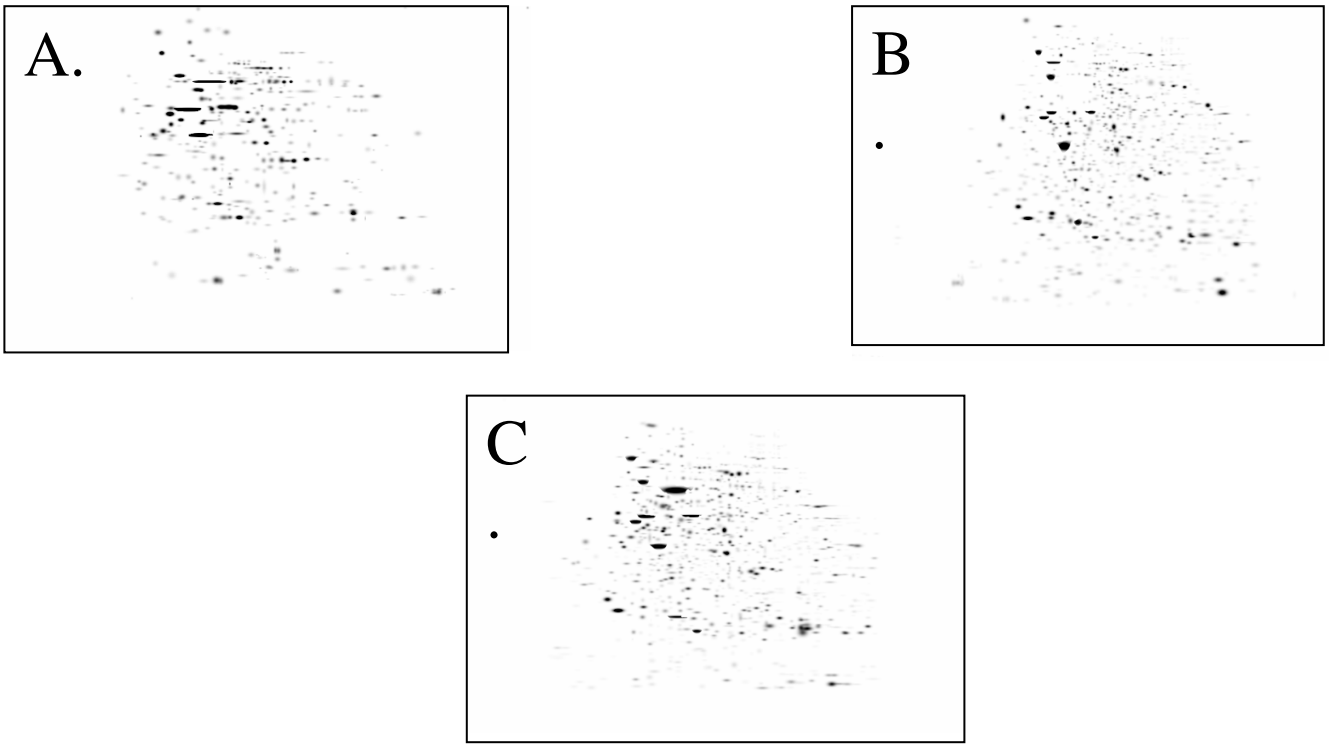


Figure IV-4: Gausian Images of 2D gels from LAY Stage Hens. Images representing proteins expressed in (A) nuclear, (B) cytoplasmic and (C) whole cell extracts. Extracts were from turkey pituitaries harvested at VT.

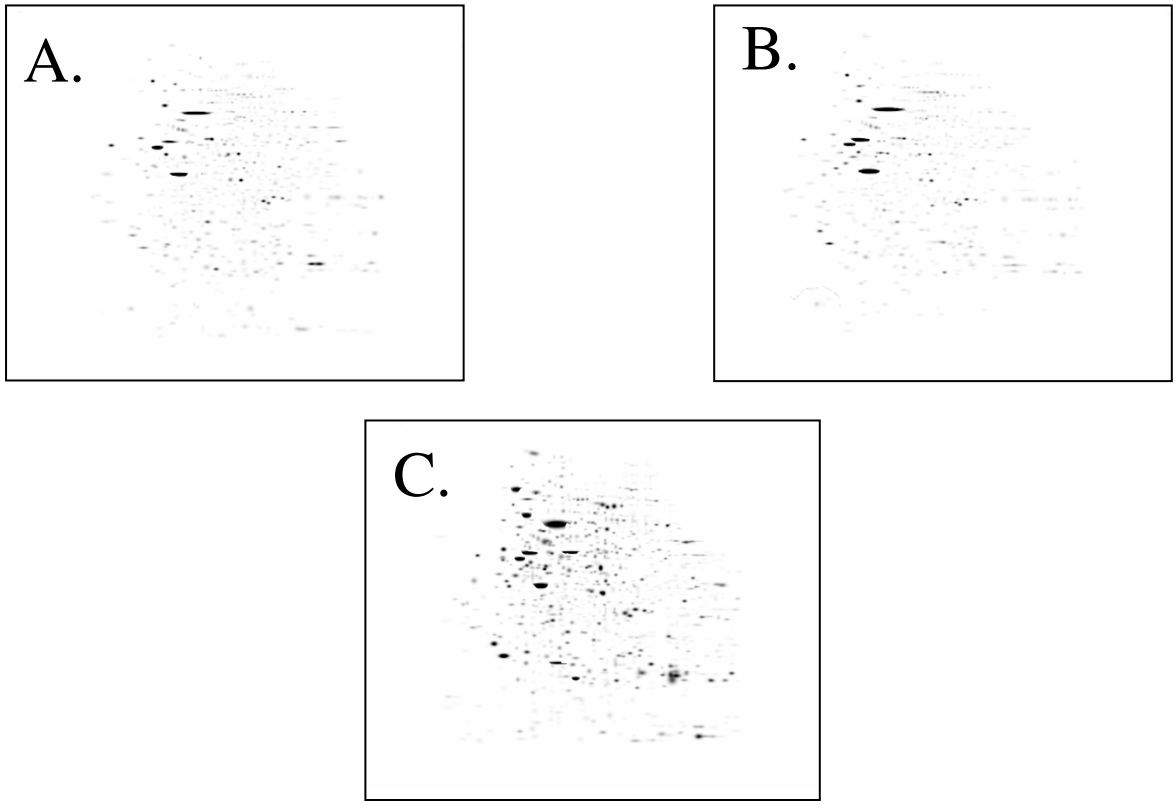


Figure IV-5: Gausian Images of 2D Gels from Whole Cell Extracts. Images representing proteins expressed in (A) NPS, (B) PS and (C) LAY stage turkey hens. Extracts were from turkey pituitaries harvested at VT.

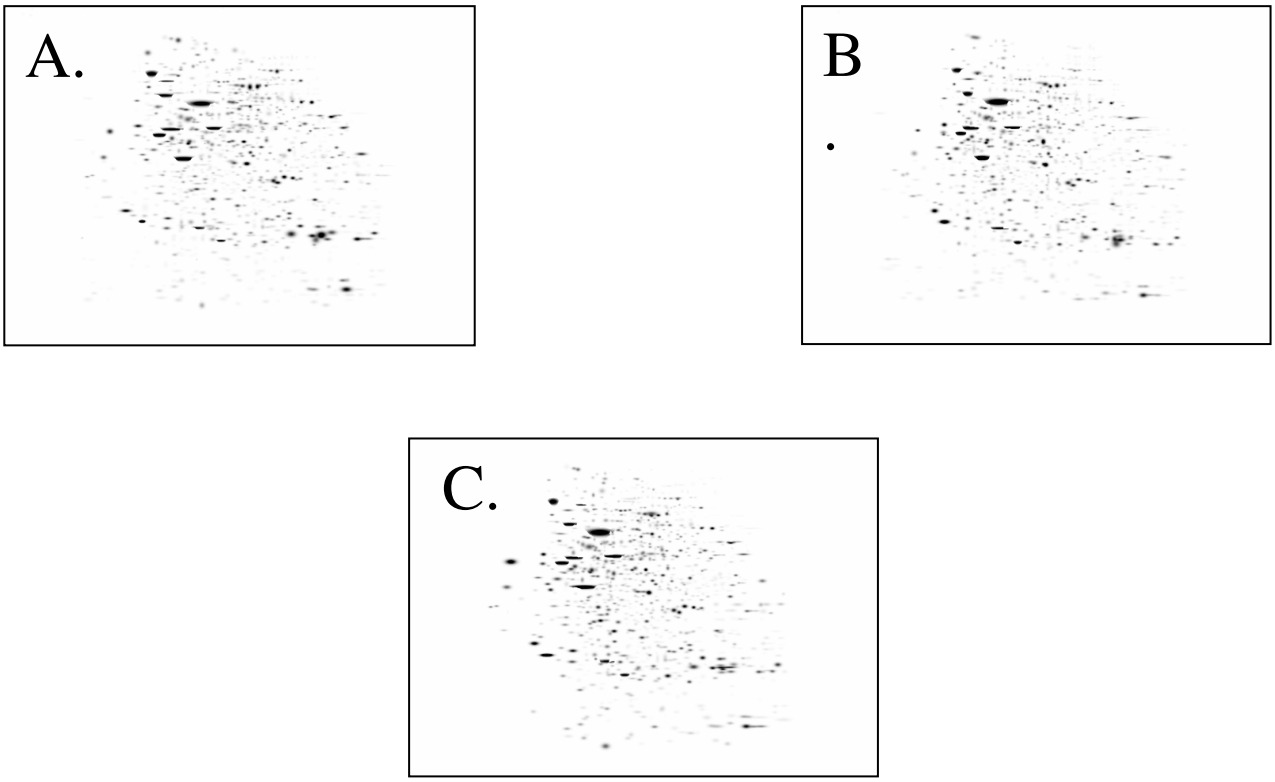


Figure IV-6: Gausian Images of a Triplicate Set. Images of three 2D gels belonging to the same triplicate set from whole cell extracts of pituitary tissue harvested at VT from LAY stage hens.

protein. Tables IV-2A, IV-2B and IV-2C show the total number of proteins identified on each 2D gel in each triplicate set of the VT matchset. Each of these tables includes the number of protein spots matched for each gel in a triplicate group. A correlation coefficient is also listed for each gel, which reflects the degree to which each gel's protein pattern matched the protein pattern of the master gel for the matchset. Averages for each replicate group's total protein counts, the number matched, overall matching rate and correlation coefficients are also listed.

A number of the triplicate sets have a consistent number of proteins detected on each gel. For example, gels from cytoplasmic extraction of PS pituitaries have 812, 919 and 942 proteins detected on each gel. NPS, whole cell extracted proteins have 638, 681 and 763 proteins in each gel of the triplicate set, which is a relatively consistent set of totals as well. In comparison, the NPS nuclear extracts have 228, 371 and 462 proteins and the PS nuclear extracts have 668, 385 and 285 proteins per gel, which were relatively inconsistent. Between 58% and 80% of all the proteins detected in each triplicate were matched within the VT matchset, either within the triplicate set or with other gels of the matchset.

B-4: Evaluation of Matchset Replication Quality and the Total Number of Proteins Detected

The MN pituitaries of turkeys were used to replicate the results obtained from the 2D gel analysis of protein expression from VT pituitaries and are represented in the MN matchset. The MN turkey pituitary extracts were made. Subsequent protein pattern comparisons were done to verify the protein expression patterns for pituitary cells during the NPS, PS and LAY stages. A higher level matchset was generated to compare the two matchsets more closely. This matchset matches and aligns the protein spots represented in

the two master gels from each matchset. A molecular weight, Isoelectric point (MrpI) plot was also generated for this higher level matchset. The VT matchset's master gel was designated as the master gel for the higher level matchset. The proteins shown in Tables list the SSP identification number, molecular weight and isoelectric point for each protein spot detected in the higher level matchset. A total of 1048 individual proteins were detected in the higher level matchset. MN matchset contained a total of 1009 individual protein spots and in the VT matchset, 962 protein spots were detected. Out of the 1009 protein spots in the MN matchset, 82% matched the pattern on the higher level matchset's master gel. There were 642 manually matched protein spots between the two matchset masters, indicating identical molecular weight and isoelectric point coordinates for these proteins. Approximately the same number of proteins were detected in the MN and VT matchsets and 82% of the proteins were matched between the two matchsets. This information indicates that the protein separations on the 2D gels can be replicated following the described experimental methods.

B-5: Trends in Protein Expression between Reproductive Stages and Protein Extractions

Significant trends in overall protein expression levels from the NPS to LAY stage of the turkey hen reproductive cycle in the anterior pituitary can be determined from the data obtained from the VT and MN matchsets. The VT matchset data represented in Tables IV-2A, IV-2B and IV-2C indicate there is a general increase in the number of detectable proteins from the NPS to the LAY (B) stage in all three types of extractions. For

Table IV-2A: Spot Count and Matching Analysis for VT Matchset Gels with LAY Stage Proteins

Gels from LAY CYTOPLASMIC Extracts

Stage	Gel #	CYTO Extract # SPOTS	CYTO #MATCHED	CYTO MATCH RATE	CYTO CORR COEF
LAY	1	854	505	59%	0.663
LAY	2	907	531	58%	0.688
LAY	3	677	478	70%	0.624
LAY AVE.		**812**	**505**	**62%**	**0.658**

Gels from LAY WHOLE CELL Extracts

Stage	Gel #	WHOLE Extract #	WHOLE #MATCHED	WHOLE MATCH RATE	WHOLE CORR COEF
LAY	1	992	700	70%	0.820
LAY	2	965	692	71%	0.845
LAY	3	930	914	98%	1.000
LAY AVE.		**962**	**769**	**80%**	**0.888**

Gels from LAY NUCLEAR Extracts

Stage	Gel #	NUCL Extract # SPOTS	NUCL #MATCHED	NUCL MATCH RATE	NUCL CORR COEF
LAY	1	465	358	76%	0.576
LAY	2	367	242	65%	0.473
LAY	3	585	420	71%	0.524
LAY AVE.		**472**	**340**	**71%**	**0.524**

Table IV-2B: Spot Count and Matching Analysis for VT Matchset Gels with PS Stage Proteins

Gels from PS CYTO Extracts

Stage	Gel #	CYTO Extract # SPOTS	CYTO #MATCHED	CYTO MATCH RATE	CYTO CORR COEF
PS	1	942	516	54%	0.572
PS	2	919	548	59%	0.653
PS	3	812	544	66%	0.626
PS AVE.		**891**	**536**	**58%**	**0.617**

Gels from PS WHOLE CELL Extracts

Stage	Gel #	WHOLE Extract # SPOTS	WHOLE #MATCHED	WHOLE MATCH RATE	WHOLE CORR COEF
PS	1	532	421	79%	0.615
PS	2	385	315	81%	0.638
PS	3	580	462	79%	0.644
PS AVE.		**499**	**399**	**80%**	**0.632**

Gels from PS NUCLEAR Extracts

Stage	Gel #	NUCL Extract # SPOTS	NUCL #MATCHED	NUCL MATCH RATE	NUCL CORR COEF
PS	1	668	422	63%	0.485
PS	2	385	257	66%	0.432
PS	3	285	181	63%	0.485
PS AVE.		**446**	**287**	**64%**	**0.467**

Table IV-2C: Spot Count and Matching Analysis for VT Matchset Gels with NPS Stage Proteins

Gels from NPS CYTO Extracts

Stage	Gel #	CYTO Extract # SPOTS	CYTO #MATCHED	CYTO MATCH RATE	CYTO CORR COEF
NPS	1	563	426	75%	0.681
NPS	2	928	519	55%	0.586
NPS	3	615	436	70%	0.673
NPS AVE.		**702**	**460**	**67%**	**0.647**

Gels from NPS WHOLE CELL Extracts

Stage	Gel #	WHOLE Extract # SPOTS	WHOLE #MATCHED	WHOLE MATCH RATE	WHOLE CORR COEF
NPS	1	763	530	69%	0.658
NPS	2	681	527	77%	0.683
NPS	3	638	528	82%	0.686
NPS AVE.		**694**	**528**	**76%**	**0.676**

Gels from NPS NUCLEAR Extracts

Stage	Gel #	NUCL Extract # SPOTS	NUCL #MATCHED	NUCL MATCH RATE	NUCL CORR COEF
NPS	1	228	112	49%	0.613
NPS	2	462	292	63%	0.391
NPS	3	371	285	76%	0.433
NPS AVE.		**354**	**230**	**63%**	**0.479**

cytoplasmic extracts, the number of proteins detected increased from 702 in the NPS stage to 812 in the LAY stage. Whole cell extracts increased from 694 to 962. Nuclear extracts increased as well from 354 to 472. In addition, comparing Tables IV-2A, IV-2B and IV-2C indicate the nuclear extracts have approximately 42% fewer proteins detectable overall than the cytoplasmic extract and 52% fewer proteins detectable than whole cell extracts. This is expected, since the nuclear proteins are only a portion of the proteins present in each cell.

However, there was a relatively large discrepancy with the number of proteins in the whole cell extracts between stages. For the NPS stage, there was an average of 694 proteins detected. In the PS stage, 499 were detected and then 962 were detected in the LAY stage. One concern is that the inconsistency with these numbers may be due to the fact that the comparison of the 2D gels from whole cell extracts and those from nuclear extracts do not consider relative concentrations of individual proteins. Nuclear proteins may actually be at much lower concentrations in the cell than proteins found in the cytoplasm. Each protein present in nuclear extracts is loaded at a much higher concentration in the gels than each of the cytoplasmic proteins since the same quantity of protein extract is loaded on each gel (300 μg), and there are fewer proteins in the nucleus than in the cytoplasm overall.

The number of proteins separated from cytoplasmic extracts increased from 702 to 891 between the NPS and LAY stages. These totals do not indicate that combining the number of nuclear extract proteins with the cytoplasmic proteins yields a quantity of proteins equivalent to those in the whole cell extracts. Given the results depicted in Figure IV-3,which indicates that the nuclear protein Pit-1 is successfully isolated in the nuclear extract, the discrepancy in these protein numbers may mean that unlike Pit-1, other nuclear proteins may not have been confined to the nucleus of the cell. Therefore, there may also be

a considerable number of proteins that are naturally present in the cytoplasmic and nuclear portions of cells that would then show up in both separations and inflate the total number of proteins detected.

Tables IV-3A, IV-3B and IV-3C show the protein spots detected in each gel of the MN matchset. These totals reflect the same overall trend as the VT matchset. On average, there were more proteins present in the whole cell extracts for LAY, PS and NPS stages (between 405-1142) than in the cytoplasmic (418-516) and nuclear extracts (88-208). In all three extracts, there was an increase in the number of proteins present from the NPS to LAY stage as well. For example, there was an average of 405 proteins in the NPS/whole cell extract gels and 942 in the LAY/whole cell extract gels. However, similar to the VT matchset gels, there was no significant increase in the number of proteins detected from the PS to LAY stage. For example, there were on average 418 proteins in the PS stage, cytoplasmic extract and 516 proteins in the LAY stage cytoplasmic extract. The number of proteins detected on each gel of the MN matchset did not closely match the number of proteins on the comparable gel from the VT matchset in most cases. These observations indicate that there is a general trend increase in the overall number of proteins expressed from the NPS to LAY stage in the reproductive cycle, although there may be individual proteins that decrease as well. The 2D gels separations showed fewer proteins in the nuclear extracts than in the cytoplasmic or whole cell extracts as well.

Table IV-3A: Spot Count and Matching Analysis for MN Matchset Gels with LAY Stage Proteins

Gels from LAY CYTO Extracts

Stage	Gel #	CYTO Extract # SPOTS	CYTO #MATCHED	CYTO MATCH RATE	CYTO CORR COEF
LAY	1	724	486	67%	.570
LAY	2	503	373	74%	.468
LAY	3	321	266	82%	.626
LAYER AVE.		**516**	**375**	**74%**	**.555**

Gels from LAY WHOLE CELL Extracts

Stage	Gel #	WHOLE Extract #	WHOLE #MATCHED	WHOLE MATCH RATE	WHOLE CORR COEF
LAY	1	544	424	77%	.641
LAY	2	1058	671	63%	.691
LAY	3	1223	990	80%	1.00
LAY AVE.		**942**	**695**	**73%**	**.777**

Gels from LAY NUCLEAR Extracts

Stage	Gel #	NUCL Extract # SPOTS	NUCL #MATCHED	NUCL MATCH RATE	NUCL CORR COEF
LAY	1	210	172	81%	.180
LAY	2	190	158	83%	.439
LAY	3	223	183	82%	.402
LAY AVE.		**208**	**171**	**82%**	**.340**

Table IV-3B: Spot Count and Matching Analysis for MN Matchset Gels with PS Stage Proteins

Gels from PS CYTO Extracts

Stage	Gel #	CYTO Extract # SPOTS	CYTO #MATCHED	CYTO MATCH RATE	CYTO CORR COEF
PS	1	294	244	82%	.569
PS	2	666	468	70%	.543
PS	3	294	249	84%	.677
PS AVE.		**418**	**320**	**79%**	**.596**

Gels from PS WHOLE CELL Extracts

Stage	Gel #	WHOLE Extract # SPOTS	WHOLE #MATCHED	WHOLE MATCH RATE	WHOLE CORR COEF
PS	1	771	539	69%	.669
PS	2	1440	753	52%	.675
PS	3	1216	691	56%	.705
PS AVE.		**1142**	**661**	**59**	**.683**

Gels from PS NUCLEAR Extracts

Stage	Gel #	NUCL Extract # SPOTS	NUCL #MATCHED	NUCL MATCH RATE	NUCL CORR COEF
PS	1	109	90	82%	.503
PS	2	46	32	69%	.336
PS	3	109	49	44%	.586
PS AVE.		**88**	**57**	**65%**	**.475**

Table IV-3C: Spot Count and Matching Analysis for MN Matchset Gels with NPS Stage Proteins

Gels from NPS CYTO Extracts

Stage	Gel #	CYTO Extract # SPOTS	CYTO #MATCHED	CYTO MATCH RATE	CYTO CORR COEF
NPS	1	637	383	71%	.600
NPS	2	545	277	70%	.600
NPS	3	316	244	87%	.522
NPS AVE.		**499**	**301**	**76%**	**.584**

Gels from NPS WHOLE CELL Extracts

Stage	Gel #	WHOLE Extract # SPOTS	WHOLE #MATCHED	WHOLE MATCH RATE	WHOLE CORR COEF
NPS	1	503	427	84%	.621
NPS	2	667	494	74%	.590
NPS	3	46	9	19%	.331
NPS AVE.		**405**	**310**	**59%**	**.541**

Gels from NPS NUCLEAR Extracts

Stage	Gel #	NUCL Extract # SPOTS	NUCL #MATCHED	NUCL MATCH RATE	NUCL CORR COEF
NPS	1	111	91	81%	.327
NPS	2	113	89	78%	.393
NPS	3	120	90	75%	.349
NPS AVE.		**157**	**90**	**78%**	**.356**

B-6: Individual Protein Expression Patterns between the Reproductive Stages

The expression patterns for each of the 962 detected spots from the VT matchset and the 1009 protein spots detected in the MN matchset were determined by comparing the densities for each spot between each of the reproductive stages. In addition to each protein's higher level match set SSP number, molecular weight and isoelectric point, Tables IV-4 to IV-7 list average density values for proteins detected in each triplicate gel in the VT matchset. Proteins with the largest decreases and increases in expression from the NPS to PS and the NPS to LAY stages in VT whole cell pituitary extracts with molecular weights between 46.2 and 21.6 kDa and isoelectric points between 4.5 and 8.0 are shown in each individual table. Ranges for molecular weight and isoelectric points were used to eliminate proteins that may not have been accurately separated or matched in the periphery of the gels.

Using Microsoft Excel, the average density values for whole cell, NPS triplicate set proteins were all normalized to 1.00. The average change in expression for matched protein spots in each of the VT triplicate sets are reported in each table representing the relative change in the expression level of each protein from the NPS to PS and NPS to LAY stages. Although relative increases and decreases for all proteins in the matchset were determined, the Excel tables were sorted so that only those proteins that demonstrated the greatest increases or decreases (the top 40 proteins) in their expression in the whole cell, NPS triplicate set are shown in each table.

Tables IV-8 to IV-11 list the relative density values for matched protein spots detected in the MN matched set that indicate the largest decreases and increases in their expression as well. Average density values are reported in the same manner as those

SSP	Mr	pI	VT WC NPS	VT WC PS	VT WC LAY
7113.0	28.0	6.9	1.0	0.0	1.5
6109.0	25.7	6.7	1.0	0.0	5.9
7308.0	35.3	6.9	1.0	0.0	1.8
5218.0	29.3	6.4	1.0	0.0	4.2
5307.0	35.3	6.2	1.0	0.0	1.6
5409.0	42.6	6.4	1.0	0.0	3.0
5201.0	31.3	6.2	1.0	0.0	1.7
5117.0	26.1	6.6	1.0	0.0	1.5
4221.0	30.4	6.1	1.0	0.0	0.7
6425.0	40.5	6.7	1.0	0.0	2.0
5111.0	27.8	6.5	1.0	0.0	4.4
6409.0	41.3	6.7	1.0	0.0	1.2
2119.0	27.5	4.9	1.0	0.0	0.8
3303.0	35.8	5.2	1.0	0.0	8.3
7402.0	41.1	6.8	1.0	0.0	0.2
7419.0	42.0	6.9	1.0	0.0	1.6
6107.0	24.0	6.6	1.0	0.0	5.4
5213.0	31.3	6.4	1.0	0.0	2.9
6304.0	34.8	6.6	1.0	0.0	0.5
5418.0	40.7	6.5	1.0	0.0	0.8
4405.0	44.0	5.8	1.0	0.0	7.3
2416.0	42.9	5.0	1.0	0.0	2.4
5311.0	34.2	6.3	1.0	0.0	1.7
4413.0	39.1	6.0	1.0	0.0	4.4
6222.0	33.4	6.7	1.0	0.0	3.2
7220.0	31.2	7.0	1.0	0.0	1.7
2207.0	33.4	4.9	1.0	0.0	0.6
8103.0	28.1	8.0	1.0	0.0	5.5
4217.0	31.3	6.1	1.0	0.0	8.1
4302.0	34.9	5.8	1.0	0.0	5.1
2218.0	30.2	4.9	1.0	0.0	50.1
1319.0	33.7	4.8	1.0	0.0	8.7
6417.0	46.0	6.7	1.0	0.0	6.3
3205.0	32.0	5.3	1.0	0.0	1.6
2303.0	35.1	4.8	1.0	0.0	6.0
7120.0	28.3	7.1	1.0	0.0	16.0
3307.0	35.4	5.3	1.0	0.0	39.3
3412.0	38.0	5.5	1.0	0.0	4.4
5410.0	45.8	6.4	1.0	0.0	8.1

Table IV-4: Proteins with Largest Decrease in Expression from the NPS to PS Stage in VT Whole Cell Pituitary Extracts. The average for whole cell (WC), nonphotostimulated (NPS) extract triplicate set protein expression values were normalized to 1.00. The average relative change in expression of matched protein spots from each of the triplicate sets in the Higher Level matchset from VT pituitaries are shown. Rows with highlighting are proteins that show a similar change in expression in both the VT and MN matchsets. A density of 0.0 indicates a relative density between 0.00 and 0.05.

SSP	Mr	pI	VT WC NPS	VT WC PS	VT WC LAY
7313	37.2	7.0	1.0	0.8	0.2
7402	41.1	6.8	1.0	0.0	0.2
2104	28.5	4.8	1.0	1.7	0.2
7422	45.9	6.9	1.0	1.1	0.5
7107	28.4	6.8	1.0	0.0	0.5
6304	34.8	6.6	1.0	0.0	0.5
2207	33.4	4.9	1.0	0.0	0.6
6422	43.3	6.7	1.0	0.6	0.6
5227	32.5	6.6	1.0	0.2	0.6
4221	30.4	6.1	1.0	0.0	0.7
2119	27.5	4.9	1.0	0.0	0.8
5418	40.7	6.5	1.0	0.0	0.8
6209	29.6	6.7	1.0	1.1	0.8
7302	36.0	6.8	1.0	0.4	0.9
5110	22.0	6.4	1.0	0.1	0.9
4308	36.2	5.9	1.0	0.4	0.9
6426	44.5	6.7	1.0	0.5	0.9
7215	31.3	6.9	1.0	0.5	0.9
1118	26.3	4.8	1.0	0.4	0.9
7209	32.4	6.9	1.0	0.7	1.0
2201	31.8	4.8	1.0	0.0	1.1
6409	41.3	6.7	1.0	0.0	1.2
113	21.5	4.5	1.0	0.3	1.3
415	42.2	4.5	1.0	1.7	1.3
5413	43.1	6.5	1.0	0.4	1.3
2106	24.8	4.9	1.0	0.4	1.4
5323	34.6	6.6	1.0	0.4	1.5
5117	**26.1**	**6.6**	**1.0**	**0.0**	**1.5**
5206	30.5	6.3	1.0	0.1	1.5
7404	39.3	6.8	1.0	0.6	1.5
3204	33.1	5.3	1.0	0.7	1.5
4415	37.9	6.0	1.0	0.9	1.5
7113	28.0	6.9	1.0	0.0	1.5
7110	27.7	6.9	1.0	0.5	1.5
6216	33.0	6.7	1.0	0.9	1.6
5307	35.3	6.2	1.0	0.0	1.6
3205	32.0	5.3	1.0	0.0	1.6
7403	44.0	6.8	1.0	0.3	1.6
7419	42.0	6.9	1.0	0.0	1.6
6416	39.5	6.7	1.0	0.4	1.7

Table IV-5: Proteins with Largest Decrease in Expression from the NPS to LAY Stage in VT Whole Cell Pituitary Extracts. The average for whole cell (WC), nonphotostimulated (NPS) extract triplicate set protein expression values were normalized to 1.00. The average relative change in expression of matched protein spots from each of the triplicate sets in the Higher Level matchset from VT pituitaries are shown. Rows with highlighting are proteins that show a similar change in expression in both the VT and MN matchsets. A density of 0.0 indicates a relative density between 0.00 and 0.05.

SSP	Mr	pI	VT WC NPS	VT WC PS	VT WC LAY
5302	**35.0**	**6.2**	**1.0**	**18.4**	**8.9**
320	34.8	4.5	1.0	9.5	14.6
3302	34.2	5.2	1.0	4.8	16.5
4315	35.0	6.0	1.0	4.2	7.1
1401	**44.9**	**4.6**	**1.0**	**4.1**	**9.8**
5216	31.4	6.4	1.0	4.0	2.5
6110	28.6	6.7	1.0	3.5	16.5
4408	42.3	5.9	1.0	3.4	25.5
7118	28.5	7.0	1.0	3.3	4.1
4104	23.7	5.9	1.0	3.2	3.1
5210	31.5	6.3	1.0	3.0	6.0
6116	22.3	6.7	1.0	3.0	11.9
2310	36.4	4.9	1.0	2.8	5.1
5313	35.4	6.4	1.0	2.7	21.7
6302	**34.5**	**6.6**	**1.0**	**2.6**	**3.9**
4414	44.5	6.0	1.0	2.4	6.1
5308	35.5	6.2	1.0	2.3	2.1
3101	22.1	5.0	1.0	2.2	12.2
6301	35.7	6.6	1.0	2.2	2.9
4102	24.0	5.8	1.0	2.2	10.5
6204	31.2	6.6	1.0	2.2	9.8
5229	29.4	6.6	1.0	2.1	3.8
3117	28.9	5.7	1.0	2.0	8.1
4204	32.9	5.8	1.0	2.0	2.4
7211	**29.5**	**6.9**	**1.0**	**2.0**	**12.5**
1306	**35.8**	**4.7**	**1.0**	**1.9**	**4.5**
3421	44.4	5.8	1.0	1.9	5.1
1107	**26.6**	**4.7**	**1.0**	**1.8**	**6.4**
2104	28.5	4.8	1.0	1.7	0.2
7112	27.3	6.9	1.0	1.7	7.3
415	42.2	4.5	1.0	1.7	1.3
8302	35.9	7.9	1.0	1.7	4.7
5113	26.6	6.5	1.0	1.7	7.2
4404	41.6	5.8	1.0	1.6	3.8
217	32.4	4.6	1.0	1.5	3.6
325	33.8	4.6	1.0	1.5	2.6
6306	35.8	6.6	1.0	1.5	4.7
8101	27.3	7.7	1.0	1.3	11.1
3208	32.8	5.4	1.0	1.3	8.2
7417	45.3	6.9	1.0	1.3	2.8

Table IV-6: Proteins with Largest Increase in Expression from the NPS to PS Stage in VT Whole Cell Pituitary Extracts. The average for whole cell (WC), nonphotostimulated (NPS) extract triplicate set protein expression values were normalized to 1.00. The average relative change in expression of matched protein spots from each of the triplicate sets in the Higher Level matchset from VT pituitaries are shown. Rows with highlighting are proteins that show a similar change in expression in both the VT and MN matchsets.

SSP	Mr	pI	VT WC NPS	VT WC PS	VT WC LAY
2218	**30.2**	**4.9**	**1.0**	**0.0**	**50.1**
7315	35.2	7.0	1.0	1.2	47.1
3307	35.4	5.3	1.0	0.0	39.3
3215	29.1	5.2	1.0	0.3	32.9
4408	42.3	5.9	1.0	3.4	25.5
7104	28.0	6.8	1.0	1.1	23.4
2101	25.9	4.8	1.0	0.0	23.3
2120	28.6	5.0	1.0	1.3	22.7
5313	35.4	6.4	1.0	2.7	21.7
2211	32.9	4.9	1.0	1.0	19.8
5420	46.2	6.5	1.0	1.2	18.5
5109	21.8	6.4	1.0	1.3	17.4
5108	24.7	6.4	1.0	0.0	17.3
7108	27.2	6.8	1.0	0.4	16.7
3302	34.2	5.2	1.0	4.8	16.5
6110	**28.6**	**6.7**	**1.0**	**3.5**	**16.5**
7120	28.3	7.1	1.0	0.0	16.0
6118	21.8	6.7	1.0	0.0	15.3
320	34.8	4.5	1.0	9.5	14.6
7406	44.5	6.8	1.0	0.0	12.9
7211	29.5	6.9	1.0	2.0	12.5
1402	42.5	4.6	1.0	1.0	12.4
3101	22.1	5.0	1.0	2.2	12.2
7202	32.6	6.8	1.0	0.0	12.2
2315	34.1	4.9	1.0	1.1	12.0
5101	24.2	6.2	1.0	0.0	12.0
1311	35.9	4.8	1.0	0.2	11.9
6116	22.3	6.7	1.0	3.0	11.9
8101	27.3	7.7	1.0	1.3	11.1
4102	24.0	5.8	1.0	2.2	10.5
7217	31.7	6.9	1.0	0.5	10.4
7420	39.3	6.9	1.0	0.6	10.2
1401	**44.9**	**4.6**	**1.0**	**4.1**	**9.8**
6204	31.2	6.6	1.0	2.2	9.8
4207	33.1	5.9	1.0	0.2	9.3
2313	33.8	4.9	1.0	0.7	9.2
5302	35.0	6.2	1.0	18.4	8.9
1319	33.7	4.8	1.0	0.0	8.7
3409	41.9	5.4	1.0	0.5	8.7
2212	29.3	4.9	1.0	1.3	8.6

Table IV-7: Proteins with Largest Increase in Expression from the NPS to LAY Stage in VT Whole Cell Pituitary Extracts. The average for whole cell (WC), nonphotostimulated (NPS) extract triplicate set protein expression values were normalized to 1.00. The average relative change in expression of matched protein spots from each of the triplicate sets in the Higher Level matchset from VT pituitaries are shown. Rows with highlighting are proteins that show a similar change in expression in both the VT and MN matchsets. A density of 0.0 indicates a relative density between 0.00 and 0.05.

for the VT matchset described above. The SSP numbers, molecular weights and isoelectric points for all protein spots are based upon values assigned in the higher level matchset. Therefore, any SSP number listed in the tables from the VT matchset that is also found in the tables from the MN matchsets would indicate the same protein spot was detected in the various gels of both matchsets and that protein spot would demonstrate a large increase or decrease in expression in both matchsets.

It is important to note that the densities for many of the proteins were assigned an extrapolated value by the software if that protein was relatively light, was not detected, or if an even density could not be determined for the entire surface of the spot image. For example, the densities for many of the proteins in a given gel were assigned the same, low density value by the PDQuest software. This was considered to be due to fluctuations in the the level of background for the gel and, in order to normalize the densities for proteins in this gel with the values reported for proteins in other gels, the density values were all decreased by this value. In addition, although the molecular weights and isoelectric points for many of these proteins could be determined accurately, there are also many instances where this could not be done. The locations of the internal protein standards used in the gel separations limited the area where molecular weights and pI's could accurately be determined. Although the protein standards used did provide adequate reference points for molecular weight and isoelectric point determinations and extrapolations, they could not provide a reference for the entire area of the gels. The isoelectric focusing strips did not resolve proteins that were close to the pH 3 and pH 10 regions of the gel and resulted in horizontal streaking at these locations. This also produced distortions that prevented the accurate matching of these protein spots between gels. These limitations prevented the

SSP	Mr	pI	MN WC NPS	MN WC PS	MN WC LAY
4319	34.4	6.1	1.0	0.0	112.8
5101	24.2	6.2	1.0	0.0	4.3
3411	39.2	5.5	1.0	0.0	16.4
415	42.2	4.5	1.0	0.1	1.6
2303	35.1	4.8	1.0	0.1	7.3
3313	35.4	5.7	1.0	0.1	54.8
2119	27.5	4.9	1.0	0.2	30.4
4115	27.6	6.1	1.0	0.2	23.8
8302	35.9	7.9	1.0	0.3	0.2
3416	44.2	5.7	1.0	0.3	50.9
2211	32.9	4.9	1.0	0.3	0.4
4314	37.2	6.0	1.0	0.4	3.4
2225	32.2	4.9	1.0	0.4	3.8
4202	29.6	5.8	1.0	0.4	9.4
5304	33.7	6.2	1.0	0.5	0.8
7216	29.6	6.9	1.0	0.5	7.5
3410	45.7	5.4	1.0	0.6	110.0
2214	33.4	4.9	1.0	0.6	1.8
5113	26.6	6.5	1.0	0.6	3.8
2302	35.8	4.8	1.0	0.6	4.0
7205	29.5	6.8	1.0	0.6	1.8
1311	35.9	4.8	1.0	0.7	2.2
3424	45.4	5.7	1.0	0.7	161.8
2215	31.6	4.9	1.0	0.7	7.5
7417	45.3	6.9	1.0	0.7	27.7
4327	34.9	5.9	1.0	0.7	48.0
4417	39.5	6.1	1.0	0.8	2.4
325	33.8	4.6	1.0	0.8	1.0
2313	33.8	4.9	1.0	0.8	3.9
2212	29.3	4.9	1.0	0.8	3.2
5221	30.8	6.5	1.0	0.9	86.6
3212	30.5	5.7	1.0	0.9	5.0
8428	45.0	7.3	1.0	0.9	1.5
2207	33.4	4.9	1.0	1.0	1.4
2316	35.2	4.9	1.0	1.0	1.6
6208	30.6	6.7	1.0	1.0	4.7
3320	35.5	5.0	1.0	1.0	0.5
4204	32.9	5.8	1.0	1.0	169.8
3319	36.0	5.0	1.0	1.0	0.6
4424	42.5	5.9	1.0	1.0	3.2

Table IV-8: Proteins with Largest Decrease in Expression from the NPS to PS Stage in MN Whole Cell Pituitary Extracts. The average for whole cell (WC), nonphotostimulated (NPS) extract triplicate set protein expression values were normalized to 1.00. The average relative change in expression of matched protein spots from each of the triplicate sets in the Higher Level matchset from MN pituitaries are shown. Rows with highlighting are proteins that show a similar change in expression in both the VT and MN matchsets. A density of 0.0 indicates a relative density between 0.00 and 0.05.

SSP	Mr	pI	MN WC NPS	MN WC PS	MN WC LAY
421	44.4	4.6	1.0	1.4	0.0
320	34.8	4.5	1.0	1.2	0.2
8302	35.9	7.9	1.0	0.3	0.2
2422	43.0	4.9	1.0	8.3	0.3
7423	45.3	6.9	1.0	1.2	0.3
2211	32.9	4.9	1.0	0.3	0.4
3320	35.5	5.0	1.0	1.0	0.5
5117	**26.1**	**6.6**	**1.0**	**1.6**	**0.5**
3319	36.0	5.0	1.0	1.0	0.6
6319	34.9	6.6	1.0	1.6	0.6
4402	45.0	5.8	1.0	3.1	0.7
227	32.1	4.6	1.0	1.7	0.7
3407	37.8	5.3	1.0	1.1	0.7
2226	31.5	5.0	1.0	5.2	0.8
5304	33.7	6.2	1.0	0.5	0.8
1312	37.3	4.8	1.0	1.2	0.8
5302	35.0	6.2	1.0	3.4	0.8
6226	31.3	6.5	1.0	1.7	0.8
2405	37.7	4.9	1.0	1.2	0.8
5315	35.7	6.4	1.0	3.0	0.8
5215	32.8	6.4	1.0	2.2	0.8
5204	33.4	6.3	1.0	4.0	0.8
4110	27.7	6.0	1.0	3.4	0.9
325	33.8	4.6	1.0	0.8	1.0
4413	39.1	6.0	1.0	1.8	1.0
3421	44.4	5.8	1.0	1.5	1.1
4414	44.5	6.0	1.0	1.3	1.1
3112	26.8	5.6	1.0	1.7	1.1
6223	31.0	6.7	1.0	2.4	1.2
6109	25.7	6.7	1.0	3.7	1.2
6306	35.8	6.6	1.0	2.6	1.2
3314	34.0	5.7	1.0	2.5	1.2
6310	35.5	6.7	1.0	17.5	1.2
4429	44.2	6.1	1.0	2.7	1.3
217	32.4	4.6	1.0	1.3	1.3
2425	40.5	4.9	1.0	1.8	1.3
5220	33.1	6.5	1.0	2.1	1.3
3409	41.9	5.4	1.0	1.5	1.3
2322	34.8	5.0	1.0	1.7	1.3
5318	35.5	6.5	1.0	8.5	1.3

Table IV-9: Proteins with Largest Decrease in Expression from the NPS to LAY Stage in MN Whole Cell Pituitary Extracts. The average for whole cell (WC), nonphotostimulated (NPS) extract triplicate set protein expression values were normalized to 1.00. The average relative change in expression of matched protein spots from each of the triplicate sets in the Higher Level matchset from MN pituitaries are shown. Rows with highlighting are proteins that show a similar change in expression in both the VT and MN matchsets. A density of 0.0 indicates a relative density between 0.00 and 0.05.

SSP	Mr	pI	MN WC NPS	MN WC PS	MN WC LAY
5401	41.1	6.2	1.0	41.8	2.6
5109	21.8	6.4	1.0	23.7	2.7
6310	35.5	6.7	1.0	17.5	1.2
4418	41.5	6.1	1.0	15.1	4.0
7220	31.2	7.0	1.0	12.2	232.7
5422	43.5	6.6	1.0	11.5	19.1
7110	**27.7**	**6.9**	**1.0**	**10.1**	**14.3**
1401	**44.9**	**4.6**	**1.0**	**9.3**	**10.8**
5318	35.5	6.5	1.0	8.5	1.3
2422	43.0	4.9	1.0	8.3	0.3
6224	29.6	6.7	1.0	6.9	2.0
3108	26.7	5.3	1.0	6.3	1.6
6406	45.3	6.6	1.0	5.9	2.3
2226	31.5	5.0	1.0	5.2	0.8
2329	35.0	5.0	1.0	5.0	6.4
4428	45.3	6.1	1.0	4.9	1.6
1118	26.3	4.8	1.0	4.3	144.6
5316	35.1	6.4	1.0	4.1	1.5
5204	33.4	6.3	1.0	4.0	0.8
6112	28.1	6.7	1.0	4.0	4.3
6109	25.7	6.7	1.0	3.7	1.2
5307	35.3	6.2	1.0	3.7	5.6
4410	39.6	6.0	1.0	3.6	3.5
5413	43.1	6.5	1.0	3.6	13.8
5414	44.5	6.5	1.0	3.4	2.2
5302	**35.0**	**6.2**	**1.0**	**3.4**	**0.8**
4110	27.7	6.0	1.0	3.4	0.9
223	30.4	4.5	1.0	3.1	4.2
2231	32.3	5.0	1.0	3.1	2.5
5321	35.3	6.6	1.0	3.1	1.5
4402	45.0	5.8	1.0	3.1	0.7
1107	**26.6**	**4.7**	**1.0**	**3.1**	**3.7**
7203	33.1	6.8	1.0	3.0	8.4
5315	35.7	6.4	1.0	3.0	0.8
6211	31.6	6.7	1.0	3.0	10.5
6302	**34.5**	**6.6**	**1.0**	**2.9**	**2.7**
1306	**35.8**	**4.7**	**1.0**	**2.9**	**2.0**
1214	30.8	4.6	1.0	2.8	3.2
7211	**29.5**	**6.9**	**1.0**	**2.8**	**1.4**
5408	43.7	6.3	1.0	2.8	1.9

Table IV-10: Proteins with Largest Increase in Expression from the NPS to PS Stage in MN Whole Cell Pituitary Extracts. The average for whole cell (WC), nonphotostimulated (NPS) extract triplicate set protein expression values were normalized to 1.00. The average relative change in expression of matched protein spots from each of the triplicate sets in the Higher Level matchset from MN pituitaries are shown. Rows with highlighting are proteins that show a similar change in expression in both the VT and MN matchsets.

SSP	Mr	pI	MN WC NPS	MN WC PS	MN WC LAY
5102	27.8	6.2	1.0	1.3	249.7
7220	31.2	7.0	1.0	12.2	232.7
4204	32.9	5.8	1.0	1.0	169.8
3424	45.4	5.7	1.0	0.7	161.8
1118	26.3	4.8	1.0	4.3	144.6
6317	35.8	6.5	1.0	1.7	131.8
7429	40.8	7.0	1.0	1.2	114.8
4319	34.4	6.1	1.0	0.0	112.8
3410	45.7	5.4	1.0	0.6	110.0
2227	32.8	4.8	1.0	1.1	87.7
5221	30.8	6.5	1.0	0.9	86.6
3313	35.4	5.7	1.0	0.1	54.8
3416	44.2	5.7	1.0	0.3	50.9
4327	34.9	5.9	1.0	0.7	48.0
2119	27.5	4.9	1.0	0.2	30.4
7417	45.3	6.9	1.0	0.7	27.7
4115	27.6	6.1	1.0	0.2	23.8
5422	43.5	6.6	1.0	11.5	19.1
3411	39.2	5.5	1.0	0.0	16.4
6110	**28.6**	**6.7**	**1.0**	**2.3**	**15.8**
7110	27.7	6.9	1.0	10.1	14.3
5413	43.1	6.5	1.0	3.6	13.8
1401	**44.9**	**4.6**	**1.0**	**9.3**	**10.8**
6211	31.6	6.7	1.0	3.0	10.5
4202	29.6	5.8	1.0	0.4	9.4
7203	33.1	6.8	1.0	3.0	8.4
1310	34.5	4.7	1.0	2.1	7.6
7216	29.6	6.9	1.0	0.5	7.5
2215	31.6	4.9	1.0	0.7	7.5
2303	35.1	4.8	1.0	0.1	7.3
5210	31.5	6.3	1.0	1.3	6.7
2329	35.0	5.0	1.0	5.0	6.4
6314	35.6	6.7	1.0	2.4	5.8
5307	35.3	6.2	1.0	3.7	5.6
6408	44.6	6.6	1.0	2.2	5.3
3212	30.5	5.7	1.0	0.9	5.0
3417	38.9	5.7	1.0	2.2	5.0
5223	29.3	6.5	1.0	1.9	4.7
6208	30.6	6.7	1.0	1.0	4.7
3117	28.9	5.7	1.0	1.8	4.6
5106	27.8	6.3	1.0	1.3	4.6

Table IV-11: Proteins with Largest Increase in Expression from the NPS to LAY Stage in MN Whole Cell Pituitary Extracts. The average for whole cell (WC), nonphotostimulated (NPS) extract triplicate set protein expression values were normalized to 1.00. The average relative change in expression of matched protein spots from each of the triplicate sets in the Higher Level matchset from MN pituitaries are shown. Rows with highlighting are proteins that show a similar change in expression in both the VT and MN matchsets.

PDQuest software from extrapolating accurate molecular weights and pI's for every detected protein spot. Therefore, detected proteins above 46.2 kDa. and a pI lower than 4.5 or below 21.5 kDa. and a pI above 8.0 could not be reliably determined. Of the 1,046 proteins detected in each of the two matchsets, 563 showed an increase in expression from the NPS to the LAY stage. Only 98 proteins decreased in expression from the NPS to the LAY stage and 483 maintained their level of expression.

Comparisons were made between the triplicate sets within each matchset and between the VT and MN matchsets. It was expected that the same SSP numbers for proteins would be seen on the tables for the VT and MN matchsets. This would indicate that the two matchsets had similar expression patterns for proteins at the same molecular weight and isoelectric point. Rows in Tables IV-4 to IV-11 that are highlighted meet this criteria. There are 12 proteins highlighted out of the 160 shown in all of the tables from one of the matchsets. Therefore, approximately 10% of the proteins matched between the Virginia Tech and Minnesota matchsets, with a molecular weight between 46.2 and 21.5 and an isoelectric point between 4.5 and 8.0, also showed similar protein expression patterns in at least one of the three triplicate sets. This percentage is relatively low and could be due to the fact that matching may not have been as accurate as necessary. It may also mean that each matchset had enough unique proteins with large expression changes that any matched SSP numbers were displaced from the top forty-lists.

The expression of some proteins showed a pattern that increased from NPS to PS and further increased from PS to LAY. For example, the protein spot represented by SSP 1401 found in Tables IV-7 and IV-11 has a molecular weight of 44.9 and an isoelectric point of 4.6. The density of the spot detected in the NPS stage of the whole cell (WC) extract

increased 4.1- and 9.3-fold in the VT and MN matchsets, respectively. The densities of the spots were 9.8- and 10.8-fold greater than the NPS stage in the LAY stage for the VT and MN matchsets, respectively. Another example of a protein showing a similar relative change in expression is protein spot SSP 6110 (Tables IV-7 and IV-11). In the VT matchset this protein increased 3.5-fold from NPS to PS and 16.5-fold from NPS to LAY. In the MN matchset, the increase is 2.3-fold and 15.8-fold from NPS to PS and NPS to LAY, respectively.

Protein spot SSP 2303 (Tables IV-4 and IV-8) showed a different pattern of expression, with a decrease from NPS to PS and then a rise from PS to LAY. In the VT matchset, protein SSP 2303 decreased to undetectable levels at PS but then rose 6-fold relative to NPS during LAY. In the MN matchset, protein SSP 2303 decreased to 10% of the NPS level during PS but then rose 7.3-fold relative to NPS during LAY. However, not all proteins showed similar patterns between the VT and MN matchsets. For example, protein SSP 2119 (Tables IV-4 and IV-8) in the VT matchset showed a decrease to undetectable levels during PS and a rise to almost NPS levels during LAY. In contrast, in the MN matchset, protein SSP 2119 decreased to 20% of the NPS level during PS and rose 30.4-fold relative to NPS during LAY.

V. Discussion

A. Affinity chromatography and southwestern screening

The procedures for using affinity chromatography and southwestern techniques to locate putative transcription factors were unsuccessful. However, the results of the affinity chromatography and southwestern screening assays support the possibility that there may be a complex of transcription factors involved in binding to the tPrl promoter region or some other method by which turkey Prl is being expressed. It is possible that tPrl protein expression is not initiated by the binding of a single protein to the promoter region. Instead, a complex of proteins may need to coordinate under specific environmental conditions to bind to the tPrl promoter region. The formation of this complex may depend upon very specific conditions that are not established in the *in vitro* experiment, and could have been disrupted with the drastically changing salt concentrations used to elute each fraction. Transcription factor complex formation is a possible explanation for the results of this experiment and would require alternative procedures to be designed to consider this possibility. There is also the possibility that the oligonucleotides used in these experiments may not be the correct sequences to be examined for DNA-protein interactions.

However, the most plausible reason why putative transcription factors were not isolated by this affinity chromatography procedure is that the experimental conditions were not optimal for the proper binding and isolation of the putative transcription factors. Assuming that the formation of a complex of proteins is not responsible for binding to the tPrl promoter region, and that a single protein product is responsible, significant adjustments to the procedure would need to be made to account for the functional characteristics of the putative transcription factors. In addition, the factors may not be

present in the LAY stage turkey hen's nuclear extract in high enough concentration to be isolated and detected. Given that time and resource -consuming adjustments were necessary to optimize the conditions of the affinity chromatography procedure, other approaches for isolating the putative transcription factors were explored.

Attempts to use the pituitary cDNA expression library in the southwestern screening technique were also not successful. Primary screening did result in many positive plaques. However, secondary screening of these positive plaques did not confirm the binding of the radioactively labeled tPrl promoter binding site probe to the proteins expressed by isolated phage. Despite adjustments made to the stringency of the washes and the probe incubation conditions, the positive results from the primary screenings could not be duplicated in the secondary screenings. This result indicates that the clones considered to be positive were representing nonspecific binding of the radioactive probe. This result may also indicate that aspects of the procedure were not optimized for putative transcription factor binding to the tPrl promoter region. One concern is that the guanidinium hydrocloride treatment of the membranes denatures all expressed proteins such that the DNA-protein interaction is disrupted. Although this does not explain the results of the secondary screening results, where no positive plaques were detected, there was no evidence of the probe specifically binding to plaques in either screening under the conditions described above. Another possibility is that the putative transcription factor contains multiple peptides. This would indicate that a complex of protein products would need to form, which then binds to the tPrl promoter region. Therefore, screening individual cloned products would not be effective in identifying these putative transcription factors. As a result of the southwestern screening experiments, a cDNA library for pituitary cells from LAY stage turkey hens was

successfully developed that can assist with future work toward characterizing specific proteins of interest.

B. Analysis of pituitary protein expression patterns

The objective to characterize changes in the overall protein expression pattern during the reproductive stages of the turkey hen did provide useful information. The experimental methods for the extraction, solubilization and 2D gel separation of proteins expressed during the NPS, PS and LAY stages of the cycle were successfully developed. The quality and quantity of protein extracted from turkey pituitary tissue was adequate for this study, according to the concentration determinations for each extract as well as western blot comparisons of nuclear and cytoplasmic extracts. Individual proteins appear, by the visual quality of the separation, to be well solubilized and well separated by these methods. As a result, increases and decreases in protein expression detected by the analysis of these gels using the PDQuest software did successfully provide information about proteins that may be involved in the regulation of reproductive cycling. Replicating these experimental methods resulted in a reproduced pattern of protein expression for each stage in the reproductive cycle and further suggested the importance of certain proteins, namely those listed near the top of Tables IV-4 through IV-11**,** during the reproductive cycle.

However, the experimental methods for 2D gel analysis developed in this study could be modified to improve the results that were obtained. On average, only 60% of the protein spots were matched between members of a given triplicate group. Considering that members of a triplicate group are supposed to be exact replicates, this percentage is much lower than desired. Precision must be achieved for generating quality triplicate groups. The

gels also had a considerable amount of horizontal and vertical streaking. By adjusting the detergent concentrations present while isoelectrically focusing proteins, these streaks may be further minimized or eliminated. In addition, there was considerable granularity in the background for many of the scanned gel images. Improvements must be made to the wash procedures for these gels to ensure background interference is consistently low for the entire matchset. The internal standard proteins used to establish MrpI gradients throughout the study were effective in providing a plot for a large portion of the gels. However, a wider range of standards would help to capture proteins on the fringes of the analyzed area. A thinner stack gel would also provide greater gel area for separation, and thus improve the resolution of protein separations. Improving the ability to accurately match protein spots within matchsets and between master gels would markedly improve the quality of the triplicate sets, the ability to verify replication of our results and to identify proteins that significantly change their expression during the reproductive cycle.

Although the experimental methods developed in this study may be modified to improve upon the results that were generated, valuable information was still acquired. Quality protein whole cell, cytoplasmic and nuclear extracts were repeatedly generated for turkey hen pituitary tissue, which were used to produce high quality 2D gel separations of expressed protein. These 2D gels indicate that the proteins were well solubilized and triplicate gels reveal a measurable level of protein pattern reproducibility. Overall trends in protein expression from the NPS to LAY stages in the reproductive cycle of the turkey hen could be determined from these gels.

In addition, many individual proteins were identified that changed at different phases of the reproductive cycle. Tables IV-4 through IV-11 list the top forty proteins with the

greatest changes in their density values, either increasing or decreasing in their level of expression from the NPS to PS and from the NPS to LAY stages in both matchsets. In each of these tables, the whole cell, NPS pituitary extract was used to compare relative changes in the protein spot density, which represents the level of expression of a protein. In some cases the change in expression pattern for a particular protein was similar between the VT and MN matchsets, whereas in other cases they were not.

By including values for the PS stage, changes in the expression of a given protein can be determined in greater detail than if changes between the NPS and LAY stages were only considered. Proteins with such changes may be of particular interest for future analysis, since such expression patterns may have physiologically relevant impacts on reproductive cycling. For example, the relative changes in density for protein number 6310, found in Table IV-9, indicates that the expression of the protein increased by 17.5-fold from the NPS to the PS stage, however, levels were close to those in the NPS stage during the LAY stage in the MN whole cell extracts. This change in expression would not have been detected if the PS stage was not considered in these assays.

Although the overall profile shows many proteins changed their expression in each of these stages, there are also a number of proteins that did not demonstrate great changes in expression in either matchset. This was expected given that not all proteins in the cells of the anterior pituitary are important for reproductive cycling and may instead serve a household function.Studies using two dimensional gels to examine changes in protein expression patterns for other cell types show comparable results to our own. A study comparing the total protein expression patterns for *Listeria monocytogenes* grown under two different conditions using 2D gels, resulted in the separation of 1,052 proteins. Only 31 significantly

changed in their spot densities (Tremoulet et al., 2002). In another study, the reaction of a luminal epithelial cell line transformed by mitogen treatment was analyzed using 2D gels, with a 3-10 isoelectric point gradient, and a 9-16% polyacrylamide gradient. Out of 2,607 separated proteins, 35 proteins showed significant increases or decreases in expression (Severine et al., 2001). These studies suggest that a relatively low percentage of proteins may be involved in cellular processes and the number of housekeeping proteins may be high. Each of these examples represent a very measured change in protein expression, with a homogeneous cell population and a highly controlled environment. Given the reproductive cycling of the turkey hen is a much more complex and involved physiological process than these systems suggests that a greater number of significant proteins is expected for our study. The fact that the pituitary tissue used in this study did not have a homogeneous cell population also increases the likelihood of detecting many more proteins with considerable changes in their expression.

VI. Implications for Future Studies

The next step for the separations that have been made in this study would be to isolate proteins from these gels with interesting protein expression patterns, as determined by the analysis described above, and identify them by mass spec. The proteins that were shown to have considerable expression changes between each stage, depicted in the Tables IV-4 through IV-11 represent potentially important proteins worth isolating from their individual gels for further analysis. Given the molecular weight and isoelectric point for GH and VIP, which are known to be involved in tPrl expression may also be isolated from these gels and characterized. Using bioinformatic sources and other experimental techniques, these proteins can be further characterized and the role they may play in turkey reproductive cycling can be determined. In addition, using the same extracts, the procedure for separating proteins on 2D gels can also be manipulated to improve the resolution of certain pI and Mr ranges. This may result in the isolation of even more proteins that are important to reproductive cycling.

Overall, future experiments will be focused on two objectives. One is to provide further information about specific signal transduction pathways and other regulatory proteins that have already been described by previous research efforts. Our laboratory is specifically interested in the signal transduction pathway responsible for VIP's regulatory function during reproductive cycling and the possible role(s) of second messengers and/or multiple protein factors in the tPrl gene expression and protein secretion. Therefore, initial efforts will be to further characterize this system. In addition, just as micoarrays are used to establish gene expression profiles for different physiological processes, our future goal will be to map the overall protein expression patterns. In this way, the important role that genes

expressed in the anterior pituitary play in the reproductive cycling of the turkey hen will be elucidated.

VII. References

Berghman, L.R., Grauwells, L., Vanhamme, L., Proudman, J.A., Foidart, A., Balthazart, J., and Vandesande, F. (1992). Immunocytochemistry and immunoblotting of avian prolactins using polyclonal and monoclonal antibodies toward a synthetic fragment of chicken prolactin. Gen. Comp. Endocrinol. 85:346-357

Burke, W.H. and Papkoff, H. (1980). Purification of turkey prolactin and the development of a homologous radioimmunoassay for its measurement. Gen. Comp Endocrinol. 40:297-307

Chaiseha, Y., Tong, Z., Youngren, O.M., and El Halawani, M.E. (1998). Transcriptional changes in hypothalamic vasoactive intestinal peptide during a photo-induced reproductive cycle in the turkey. J. Molec. Endocrinol. 21:267-275.

Chaiseha, Y. and El Halawani, M.E. (1999). Expression of Vasoactive Intestinal Peptide/Peptide Histidine Isoleucine in several hypothalamic areas during the turkey reproductive cycle: Relationship to prolactin secretion. Neuroendocrinology. 70:402-412.

Chatelain, A., Dupouy, J.P. and Dubois, M.P. (1979). Ontogenesis of cells producing polypeptide hormones (ACTH, MSH, LPH, GH, PRL) in the fetal hypophysis of the rat: influence of the hypothalamus. Cell Tissue Res. 196: 409-427

Crisostomo, S., Guemene, D., Garreau-Mills, M., Morvan, C. and Zadworny, D. (1998). Prevention of incubation behavior expression in turkey hens by active immunization against prolactin. Theriogenology. 50:675-690.

Dawson, A., Follett B.K., Goldsmith, A.R. and Nicholls T.J. (1985). Hypothalamic gonadotropin-releasing hormone and pituitary and plasma FSH and prolactin during photostimulation and photorefractoriness in intact and thyroidectomized starlings (Sturnus vulgaris). J. Endocrinol. 105:71-77.

El Halawani, M.E., Fehrer, S., Hargis, B.M., and Porter, T.E. (1998). Incubation Behavior in the Domestic Turkey: Physiological Correlates. CRC Critical Reviews in Poultry Biology. 1(4):285-315.

Gazzillo, L.C., (2000). The mapping of transcription factor binding sites in the turkey prolactin gene. Masters Thesis, Viginia Tech. Blacksburg, Virginia. 1-54.

Gharbi, S., Gaffey, P., Yang, A., Zvelebil, M.J., Cramer, R., Waterfeld, M. D., and Timms, J. F. (2001). Evaluation of two-dimensional differential gel electrophoresis for proteomic expression analysis of a model breast cancer cell system. Molec. Cell. Proteomics 1:91-98.

Gordon, D.F., Wagner, J., Atkinson, B.L., Chiono, M., Berry, R., Sikela, J. and Gutierrez-Hartmann, A. (1996) Human Cart-1: Structural organization, chromosomal localization, and functional analysis of a cartilage-specific homeodomain cDNA. DNA Cell Biology. 15:531-541.

Hansen, G.N. and Hansen, B.L. (1977). Light and electron microscopic identification of pituitary cells containing growth hormone and prolactin in the pigeon (Columba livia), using the immunoglobulin-enzyme bridge technique. Gen. Comp. Endocrinol. 32:99-107.

Hoeffler J.P., Broockfor, F.R. and Frawley, L.S. (1985). Ontogeny of prolactin cells in neonatal rats: initial prolactin secretors also release growth hormone. Endocrinology. 117:187-195.

Ingraham H.A., Chen, R., Mangalam, H.J., Elsholtz, H.P., Flynn, S.E., Lin, C.R., Simmons, D.M., Swanson, L., and Rosenfeld, M.G. (1988). A tissue-specific transcription factor containing a homeodomain specifies a pituitary phenotype. Cell. 5:521-529.

Josza, R., Scanes, C.G., Vigh, S. and Mess, B. (1979). Functional differentiation of the embryonic chicken pituitary gland studied by immunohistological approach. Gen. Comp. Endocrinol. 39:158-163.

Kadonaga, J.T. and Tjian, R. (1986). Affinity purification of sequence-specific DNA binding proteins. Proc. Natl Acad. Sci. (USA) 16:5889-5893

Karatzas, C.N., Guemene, D., Zadworny, D., and Kuhnlein, U. (1997). Changes in expression of the prolactin and growth hormone gene during different reproductive stages in the pituitary gland of turkeys. Reprod. Nutr. Dev. 37:69-79.

Kurima, K., Proudman, J.A., El Halawani, M.E., and Wong, E.A. (1995) The turkey prolactin-encoding gene and its regulatory region. Gene. 156:309-310.

Macnamee, M.C. and Sharp, P.J. (1989). The functional activity of hypothalamic dopamine in broody bantam hens. J. Endocrinol. 121:67-74.

Mangalam, H.J., Albert, V.R., Ingraham, H.A., Kapiloff, M., Wilson, L., Nelson, C., Elsholtz, H., Rosenfeld, M.G. (1989). A pituitary POU domain protein, Pit-1, activates both growth hormone and prolactin promoters transcriptionally. Genes Development. 3:946-958.

Mikami, S., Takagi T. and Farner D.S. (1980). Cytological differentiation of the interrenal tissue of the Japanese quail, Coturnix coturnix. Cell Tissue Res. 208:353-370.

Mikami, S. (1986). Immunocytochemistry of the avian hypothalamus and adenohypophysis. Int. Rev. Cytol. 103:189-248.

Molloy, M.P., Herbert, B.R., Walsh, B.J., Tyler, M.I., Traini, M., Sanchez, J.C., Hochstrasser, D.F., Williams, K.L. and Gooley, A.A. (1998). Extraction of membrane proteins by differential solubilization for separation using two-dimensional gel electrophoresis. Electrophoresis. 19:837-844.

Naik, D.R., Sirasawa, N., Shimada, T., and Ishikawa, H. (1990). Localization of prolactin in the pituitary gland: A comparative immunohistochemical study from fish to mammal. *In* "Kyoto Prolactin Conference Monograph: (K. Hoshino Ed.), Kyoto University, Kyoto. 5:83-97.

Nelson C., Albert V.R., Elsholtz H.P., Lu, L.I. and Rosenfeld, M.G. (1988). Activation of cell-specific expression of rat growth hormone and prolactin genes by a common transcription factor. Science. 239:1400-1405.

Onali, P., Eva C., Olianas, M.C., Schwartz, J.P. and Costa E. (1983). In GH3 pituitary cells, acetylcholine and vasoactive intestinal peptide antagonistically modulate adenylate cyclase, cyclic AMP content, and prolactin secretion. Mol. Pharmacol. PAGE

Opel, H., and Proudman, J.A. (1988). Stimulation of prolactin release in turkeys by vasoactive intestinal peptide. Proc. Soc. Exp. Biol. Med. 187:455-460.

Pitts, G.R., Youngren, O.M., Silsby, J.L., Rozenboim, I, Chaiseha, Y., Phillips, R.E., Foster, D.N. and El Halawani, M.E. (1994). Role of vasoactive intestinal peptide in the control of prolactin-induced turkey incubation behavior. I. Acute infusion of vasoactive intestinal peptide. Biol. Reprod. 50:1344-1349.

Porter, T.E., Silsby, J.L., Behnke, E.J., Knapp, T.R. and El Halawani, M.E. (1987). In vitro changes in granulosa (G) cell progesterone (P) production associated with the onset of incubation in the turkey. Poult. Sci. 66:161-167.

Iowa State Extension Service URL: www.econ.iastate.edu/outreach/agriculture/periodicals/chartbook/table22.html. March 20, 2003.

Proudman, J.A., Krishnan, K.A., Maruyama, K. (1995). Ontogeny of pituitary and serum growth hormone in growing turkeys as measured by radioimmunoassay and radioreceptor assay. Poult. Sci. 74:1201-1208.

Rabilloud T. (2000). Detecting proteins separated by 2-D gel electrophoresis Anal. Chem. 72:48A-55A.

Rabilloud, T., ed. (2000) "Proteome Research: Two-Dimensional Gel Electrophoresis and Identification Methodology", pp 5-7. Springer, New York

Ramesh, R., Solow, R., Proudman, J.A., and Kuenzel, W.J. (1998). Identification of mammosomatotrophs in the turkey hen: increase in abundance during hyperprolactinemia. Endocrinology. 139:781-786.

Ramesh, R., Kuenzel, W.J., Proudman, J.A. (1999). Increased proliferative activity and programmed cellular death in the turkey hen pituitary gland following interruption of incubation behavior. J. Endocrinol. 200:150-155.

Rozenboim, I. and El Halawani M.E., (1993). Characterization of vasoactive intestinal peptide pituitary membrane receptors in turkey hens during different stages of reproduction. Biol. Reprod. 48:1129-1134.

Roy, R.J., Gosselin, P. and Guerin, S.L. (1991). A short protocol for micro-purification of nuclear proteins from whole animal tissue. Biotechniques. 11:770-777

Sarapura, V.D., Strouth, H.L., Gordon, D.F., Wood, W.M., Ridgway, E.C. (1997). Msx1 is present in thyrotropic cells and binds to a consensus site on the glycoprotein hormone alpha-subunit promoter. Mol. Endocrinol. 11:1782-1794.

Scanes, C.G. (2000) Chapter 16: Introduction to Endocrinology: Pituitary Gland. *In* Whittow, G.C., ed. Sturkie's Avian Physiology 5th Edition. Academic Press, Washington, DC pp 437-458

Schnell, S.A., You, S. and El Halawani, M.E. (1999). D1 and D2 Dopamine receptor messenger ribonucleic acid in brain and pituitary during the reproductive cycle of the turkey hen. Biol. Reprod. 60:1378-1383.

Sharp, P.J., Macnamee, M.C., Sterling, R.J., Lea, R.W., and Pedersen, H.C. (1995) Relationships between prolactin, LH and broody behavior in bantam hens. J. Endocrinol. 118:279-286

Siopes, T.D. (2001). Temporal characteristics and incidences of photorefractoriness in turkey hens. Poult. Sci. 80:95-100

Sun, S. and El Halawani, M.E. (1995) Protein kinase C mediates chicken vasoactive intestinal peptide stimulated prolactin secretion and gene expression in turkey primary pituitary cells. Gen. Comp. Endocrinol. 99:289-297

Szabo, M., Staib, N.E., Collins, B.J. and Cuttler, L. (1990). Biphasic action of forskolin on growth hormone and prolactin secretion by rat anterior pituitary cells in vitro. Endocrinology. 127:1811-1817.

Talbot, R.T., Hanks, M.C., Sterling, R.J., Sang, H.M. and Sharp, P.J. (1991). Pituitary prolactin messenger ribonucleic acid levels in incubating and laying hens: Effects of manipulating plasma levels of vasoactive intestinal polypeptide. Endocrinology 129:496-502.

Tanaka, M., Yamamoto, I., Ohkubo, T., Wakita, M., Hoshino, S. and Nakasima, K. (1999) cDNA cloning and developmental alterations in gene expression of the two Pit-1/GHF-1 transcription factors in the chicken pituitary. Gen. Comp. Endocrinol. 114:441-448.

Thommes, R.C., Umporowicz, D.M., Leung, F.C. and Woods, J.E. (1987). Ontogenesis of immunocytochemically demonstrable somatotrophs in the adenohypophyseal pars distalis of the developing chicken embryo. Gen. Comp. Endocrinol. 67: 390-398.

Tong, Z., Pitts, G.R., You, S., Foster D.N., and El Halawani, M.E. (1998). Vasoactive intestinal peptide stimulates turkey prolactin gene expression by increasing transcription rate and enhancing mRNA stability. J. Mol. Endocrinol. 21:259-266

Tremoulet, F., Duche, O., Namane A., Martinie, B. and Labadie, J.C. (2002) Comparison of protein patterns of Listeria monocytogenes grown in biofilm or in planktonic mode by proteomic analysis. FEMS Microbiol Lett 210:25-31.

Warner, N. and Rutherford, C. (1999) Purification and cloning of TF2: A novel protein that binds a regulatory site of the gp2 promoter in Dictyostelium. Arch. Biochem. Biophys. 373:462-470.

Watanabe, Y.G. and Daikoku, S. (1979). An immunohistochemical study on the cytogenesis of adenohypophysial cells in fetal rats. Dev. Biol. 68:557-567.

Weatherly, K.L., Ramesh, R., Strange, H., Waite, K.L., Storrie, B., Proudman, J.A. and Wong, E.A. (2001). The turkey transcription factor Pit-1/GHF-1 can activate the turkey prolactin and growth hormone gene promoters in vitro but is not detectable in lactotrophs in vivo. Gen. Comp. Endocrinol. 123:244-253.

Wong, E.A., Ferrin, N.H., Silsby, J.L. and El Halawani, M.E. (1991). Cloning of a turkey prolactin cDNA: Expression of prolactin mRNA throughout the reproductive cycle of the domestic turkey (Meleagris gallapavo). Gen. Comp. Endocrinol. 83:18-26.

Woods, K.L. and Porter, T.E. (1998). Ontogeny of prolactin secreting cells during chicken embryonic development: Effect of vasoactive intestinal peptide. Gen. Comp. Endocrinol. 112:240-246.

Xu, M., Proudman, J.A., Pitts, G.R., Wong, E.A., Foster, D.N. and El Halawani, M.E. (1996). Vasoactive intestinal peptide stimulates prolactin mRNA expression in turkey pituitary cells: Effects of dopaminergic drugs. J. Soc. Exper. Biol. Med. 212:52-62.

You, S., Hsu, C.C., Kim, H., Kho, Y., Choi, Y.J., El Halawani, M.E. Farris, J. and Foster, D.N. (2001) Molecular cloning and expression analysis of the turkey vasoactive intestinal peptide receptor. Gen Comp Endocrinol. 124:53-65.

Youngren, O.M., Pitts, G.R., Phillips, RE. and El Halawani, M.E. (1996). Dopaminergic control of prolactin secrection in the turkey. Gen Comp Endocrinol. 104:225-230

Youngren, O.M., Pitts, G.R., Chaiseha, Y. and El Halawani, M.E. (1999). An opioid pathway in the hypothalamus of the turkey that stimulates prolactin secretion. Neuroendocrinology. 70:317-323

VITA

Amy M. Spellerberg was born in Fairfax, Viginia, the daughter of Mary E. and Peter A. Spellerberg. She graduated with a Bachelor of Science degree in Biology from Mary Washington College in May, 1996. She worked for two years at the Johns Hopkins Hospital, Baltimore, MD in the Pediatric Pathology Department, studying pediatric germ cell tumors. She then worked for two years a the Children's National Medical Center in Washington, DC in the Cancer and Transplantation Department studying hematopoietic stem cells. Amy began work with Dr. Eric A. Wong on a Master of Science degree in Animal and Poultry Sciences at Virginia Tech in August, 2000 and finished in May, 2004.