VITRIFICATION OF DAY OLD TURKEY TESTES AND OVARIES, AND SUBSEQUENT TRANSPLANTATION AND FOLLICULOGENESIS GROWTH RATES AND PATTERNS IN CHICKENS

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TITLE OF THESIS: Vitrification of day old turkey testes and ovaries, and subsequent transplantation and folliculogenesis growth rates and patterns in chickens

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CARE AND USE OF ANIMALS

The University of Saskatchewan's, University Committee on Animal Care and Supply (UCACS), approved all protocols and procedures mentioned in this thesis. With the UCACS number for these projects between 2013-2015 being 20120110. An on site veterinarian (Dr. Melanie van der Loop) was present for a portion of the surgeries as to be able to monitor the animals well being and to make sure the protocols were being followed. All animals were housed and maintained in the Western College of Veterinary Medicine's Animal Care Unit, unless otherwise mentioned.

ABSTRACT

The overall aim of this thesis was to determine if day old turkey gonads could be cryopreserved and transplanted into recipient poults. This would allow for grafts to develop and mature normally and potentially produce donor-derived offspring. In addition, the monitoring of folliculogenesis in chickens was studied to determine if ultrasonography would be a useful technique to study this biological process, with the intention of using this method in future studies on ovarian graft development. Three studies were conducted: cell and tissue viability of vitrified day old turkey gonads, transplantation of day old turkey gonads into recipient poults, and monitoring of follicle growth in chickens using ultrasonography.

The objective of the first study was to determine if day old turkey gonads were viable after vitrification using a standard protocol or with variation in equilibrium solution (ES) and/or vitrification solution (VS) absorption times. Three different ES time points were tested 10, 15 and 20 min (10ES, 15ES and 20ES) and two different VS time points 2 and 3 min (2VS and 3 VS). The cell and tissue viability was determined by Trypan Blue Assay and light microscopy, respectively. Testicular cell viability was conducted using three vitrification protocols and fresh tissue. All vitrification protocols along with fresh tissue were assessed by light microscopy, to evaluate histological alterations, in ovarian and testicular tissue. Protocols with the highest cell viability and best morphological scores were selected as being the most suitable for cryopreservation. Testicular tissue vitrified using 15ES or 20ES with 3VS had the highest cell viability. Ovarian and testicular tissue vitrified using 15ES with (3VS or 2VS), showed the best morphological scores, out of all the protocols.

The second study was broken down into two parts: Part A (Trial 1 & 2) was to determine the most suitable age group for poults pre-surgery to give the highest survivability; Part B (Trial 3) was to

determine if previously vitrified day old turkey gonads could develop and mature normally, by retrieving grafts post-surgery, at- different time points. In Trial 1, large white turkeys (LWT) 1, 3, 4 and 7 days of age were used and for Trial 2, LWT's aged 1, 3, 4, and 5 days of age were used. In Trial 3, bronze turkeys at 1 day of age were used, and graft tissue was used from day old LWT's previously vitrified (10ES/2VS) or fresh. For all Trials, the survivability at each time point was analyzed, and for the third Trial, the grafts recovered were histologically analysed. From Trials 1 and 2, seven and three day old poults had the highest survivability ratios (3/5 and 6/8) respectively. For Trial 3, day old male poults (96%) had a higher survivability than the females (68%). From Trial 3, transplants were only recovered in females and males up until 4 days and 4 weeks post-surgery respectively, with no fresh tissue grafts recovered. The histological analysis of testicular transplants showed deteriorating structure, with a steady progression away from normal morphology, post-surgery.

The third study's objective was to determine the growth rates and patterns of folliculogenesis in Barred Plymouth Rock (BPR) hens by using ultrasonography. Two ultrasound Trials were performed: the first to determine the optimum time interval between serial ultrasound scans to accurately map follicles, and the second to tackle the main objective of the third part of this thesis. For the first Trial, BPR hens were scanned periodically over 24 hours, follicle diameter and position were recorded and mapped with respect to the ovary and neighbouring follicles. Proportional follicle growth, compared to the first scanning session showed that the 24hr time point had the only significant ($P<0.001$) proportional follicle growth. It is recommended here that scans occur twice a day (morning and afternoon) to capture a more precise growth rate of follicles. In the second Trial, BPR hens were scanned twice a day, over an 11-day period. Follicle diameters (height and width) were recorded to calculate follicle area. The growth of each follicle's area was compared to the time before ovulation to determine the overall follicle growth rate. Additionally, it was determined if time (night, day) and type of preovulatory follicle (F1-F5)

played a significant role in follicle growth rates. The overall follicle growth rate was best described by a cubic equation (R^2=0.907, P<0.001). Follicle growth rates were influenced by both time (P=0.009) and type (P<0.001). With F2 and F3 follicles (P<0.05) having a higher growth rate than F1 and F5 types.

In conclusion, modifications to the standard vitrification protocol used on quail gonads were necessary to increase cell viability and lower morphological alterations for turkey gonads left whole. For future work it has been shown that day old turkey poults can survive gonad transplantation. The lack of development of grafts is most likely due to a combination of tissue damage after vitrification-warming procedures and insufficient immunosuppression of the host. This work has paved the way for the poultry industry to be able to cryogenically store turkey gonads and revive lines when required.

Additionally it was shown that serial ultrasound scans twice a day provided accurate monitoring of follicle growth in Barred Plymouth Rock hens. For BPR hen's follicle growth rates and patterns were successfully measured. This gives the industry another tool to better select superior laying hens and to create a more homogeneous laying flock. Future application of ultrasonography on gonad monitoring has the potential to show growth and maturation of grafted tissue before the production of donor-derived offspring, enabling earlier detection of successful transplantation.

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Thank you, and remember, never give up on your dreams.

George Hall

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LIST OF ABREVIATIONS

AA: absolute alcohol

AI: artificial insemination

am: anti meridiem

ANOVA: analysis of variance

App.: appendix

ART: artificial reproductive technologies

ASMT: air sac membrane transplantation

BC: blastodermal cell

BPM: breaths per minute

BPR: Barred Plymouth Rock

BU: Busulfan

bp: base pair

BT: bronze turkey

^{o}C: degrees Celsius

CAGR: Canadian animal genetic resource

CAM: chorioallantoic membrane

cm: centimeter

cm^2: centimeter squared

CP: cyclophosphamide

CPA: cryoprotectant agents

CsA: cyclosporin A

D: dead

DM: diameter

DMEM: Dulbecco modified eagle medium

DMSO: dimethyl sulfoxide

DNA: deoxyribonucleic acid

DPBS: Dulbecco phosphate buffered saline

DW: distilled water

E: euthanized

EDTA: ethylenediaminetetraacetic acid

EG: ethylene glycol

EODAS: erratic oviposition and defective egg syndrome

ES: equilibrium solution

ESC: embryonic stem cell

EtOH: ethanol

F: follicle

FAO: Food and Agriculture Organization

FBS: fetal bovine serum

Fig.: figure

FIGR: follicle internal growth rate

FSH: follicle stimulating hormone

G: granulosa cell

g: grams

Gly: glycerol

GnIH: gonad inhibiting hormone

GNR: gonad not removed

GnRH: gonad releasing hormone

H: radius height

hr: hour

HT: hybrid turkey

Hz: hertz

H1: first generation hybrid

H2: second generation hybrid

H5: fifth generation hybrid

ID: identification

IM: intramuscular

IS: immune suppressed

^{o}K: degrees Kelvin

kg: kilogram

L: liter

LD: light and dark

LDL: low density lipoprotein

LED: light-emitting diode

LH: luteinizing hormone

LN_2: liquid nitrogen

LT: left testectomized

LWT: large white turkey

M: molarity

MeOH: methanol

mg: milligram

min: minute

ml: millilitre

mm: millimeter

mm^2: millimeter squared

MMF: Mycophenolate mofetil

MS: microsatellite

mtDNA: mitochondrial deoxyribonucleic acid

n: number of units

NAGP: North America germplasm preservation

NIS: not immune suppressed

NS: not significant

O: ovariectomized

OT: orthotopic transplantation

OUP: orthotopicly under transplantation

P: probability

PG: propylene glycol

PGC: primordial germ cell

pm: post meridiem

PO: primary oocyte

POF: post ovulated follicle

PVP: polyvinylpyrrolidone

RNA: ribonucleic acid

R^2: R-squared

RT: room temperature

RTW: running tap water

S: survived

SD: standard deviation

SEM: standard error of the mean

Sg: spermatogonia

SGOT: surgery glue orthotopic transplantation

SNP: signal nucleotide polymorphism

SPSS: statistical package for the social sciences

St: Sertoli cell

T: testectomized

TSH: thyroid stimulating hormone

VLDL: very low density lipoprotein

VS: vitrification solution

VTG: vitellogenin

W: radius width

Xg: G-force

μl: microliter

μm: micrometer

π: pi

CHAPTER 1: INTRODUCTION

Conservation of wild species has become paramount, as the threat from man-induced extinction is now a real challenge for many species; both as an indirect result of climate change and from hunting and habitat destruction directly (Milner-Gulland et al., 2003; Root et al., 2003). For domestic animals, the picture might not seem as bleak superficially as we profit to a greater extent from their existence. Although with the industrialisation of the agricultural sector, pressure from extreme genetic selection has threatened many local breeds (Hoffmann, 2010). Indeed, there has been a reduction in local and rare domestic breeds in recent years; these breeds have been neglected in favour of superior commercial lines (FAO, 2007). With respect to protecting a species, there are two options, to protect the species in its native habit (*in situ*) or to remove it and propagate the species in a foreign environment (*ex situ*). There are many in situ (labeling species as endangered, legislating protection, habitat protection or manipulation) and ex situ (zoos, captive breeding programs, ART) models, which have been used to protect these endangered and threatened wild and domesticated species. The last resort, if all else fails, is an ex situ model called animal resource banking (Holt and Pickard, 1999). This works well for some classes (mammalia) by storing genetic material at an extremely low temperature to conserve the species or breeds for future generations.

With a view of conserving species, cryopreservation involves the freezing of tissue or cells, which contain germ cells, or cells close in linage. Their mature products, sperm and oocytes can also be cryopreserved along with embryos. Two major methods of cryopreservation and recovery are slow freezing with thawing and vitrification combined with warming (Vajta and Kuwayama, 2006; Saragusty and Arav, 2011). Predominantly in the literature slow freezing and vitrification methods have focused on mammalian species because of the direct application of these techniques and the reproductive anatomical similarity to humans (Fickel et al., 2007; Santos et al., 2010). With this focus, a gap lies in the literature with respect to cryopreservation of poultry breeds.

Physiological differences between mammals and birds, have limited the universal application of cryopreservation methods and protocols between these classes. Most mammals reproduce in a style called viviparity where gestation is internal and young are born alive, but this is not the main form of reproduction. Most vertebrates (birds, reptiles, amphibians and fish) reproduce oviparously with the production of eggs, which are laid, and gestation is subsequently external. In the case of birds, the challenge arises because the oocyte/ovum is almost the same size as the follicle and filled with yolk (Johnson and Woods, 2007). The sheer size of the fully mature ovum hinders its cryopreservation potential. In addition, the avian embryo develops as 'open cells' and is not separable from the yolk sac (Bellairs and Osmond, 2005). Even at the 64-cell stage only the most centrally situated cells have a complete plasma membrane. The peripheral cells still have an open cytoplasm with the yolk; this area is called the ooplasm. This ooplasm region remains as the axis of symmetry is being established. Removing the avian embryo while the ooplasm area is still present will cause damage to the embryo. The avian embryo then appears also to be a poor target for cryopreservation.

Due to these limitations, a reliance on living populations and backcrossing, aided by assisted reproductive technologies, have been the standard forms of conservation for the poultry industry. If the industry's goal is to preserve a species or breed, it would appear that backcrossing is sufficient as a short-term solution, even though the W chromosome, mtDNA and a portion of the genomic DNA is lost, when backcrossing is used (Blackburn et al., 2009). As a long-term permanent solution, backcrossing has a high risk associated with it due to the loss of the aforementioned valuable genetic material (Grant and Grant, 1992). When considering the conservation of wild avian species, sperm backcrossing is not a viable means if the goal is to preserve the entire genome. For the above reasons, a new technique is needed for the conservation of avian species. This new technique needs to utilize a compatible cell or tissue type with respect to cryopreservation restrictions and must preserve the entire genome. This led previous researchers to focus on using ovarian tissue transplantations (Grossman and Siegel, 1966; Song and Silversides, 2007a; Liptoi et al., 2013); or PGC transplantation between embryos, to overcome these

physiological cryopreservation problems (Reynaud, 1976; Wentworth et al., 1989; Petitte et al., 1991). Here in this thesis a focus was set on gonad transplantation due to its wide application and ability to use mature tissue.

An Agriculture & Agri-Food Canada research group was the first to show that it was possible to perform ovarian transplantation between chicken breeds (Song and Silversides, 2006). This allowed grafts to mature normally and the recipients were able to produce donor-derived offspring (Song and Silversides, 2007a). Their work expanded and looked at this technique from different angles, from mature tissue all the way to interspecific transplantation (Song et al., 2012; Liu et al., 2015). This research was conducted on domestic chicken (*Gallus gallus*), Japanese quail (*Coturnix japonica*), Muscovy duck (*Cairina moschata*), and Pekin duck (*Anas platyrhynchos*). These techniques have not been tested on the domestic turkey (*Meleagris gallopavo*) to date, even though it is the fifth most consumed meat in Canada, chicken being the first (TFC, 2014). Due to the significance of the domestic turkey to the Canadian agricultural system, and because we are seeing a reduction in population size of many local domestic breeds (*Beltsville Small White, Ridley Bronze, Chocolate, Jersey Buff, Lilac/Lavender, Midget White, Narragansett, White Holland, Bourbon Red, Royal Palm, Standard Bronze*) in North America, it is important to evaluate if this novel method can be a viable means of cryopreserving the domestic turkey and its genetic diversity.

CHAPTER 2: LITERATURE REVIEW

I. Preservation of Genetic Diversity in Turkeys

2.1 Importance of Genetic Diversity Preservation in Animals

Genetic diversity is the complexity of genetic variability in a species or breed. Genetic diversity increases with mutations and recombination of chromosomes, past on to offspring. It can also decrease from genetic drift (being the change in allele frequencies to the next generation due to random sampling of gametes) (Notter, 1999). Maintaining or increasing the genetic diversity of a species ensures the likelihood of its future existence (Hutter, 2002). Variability has historically been measured by phenotypic differences, but with modern technology it is now possible to look more closely at genotypic differences in the form of microsatellite and SNP profiles (Andersson, 2001). This technology provides a means of gathering large amounts of genetic data from populations, which can be stored in electronic databases. These population genetic profiles can be compared to newly identified populations to determine the overall genetic differences between them. These population genetic profiles can also determine the level of genetic diversity within a species, which can help in the decision making with respect to how resources will be distributed to protect the most valuable genetic populations within a species. A species with a greater genetic diversity is better suited when adapting to new environments. This concept is also part of "*The Origin of Species"*, the greater variance (in the supposed alleles) increases the potential of survival of the species (Darwin, 1859). This means that a population with a wide variance in alleles has the potential to produce offspring with a variety of different phenotypic traits; this allows the population to adapt better to changing conditions. However, this is not what the industrialized agricultural system requires; instead a uniformed production of offspring over generations is favoured. This means that certain traits are heavily selected for and the end result is an inbred population for those specific traits (Notter, 1999). Inbreeding is not harmful for the breed as long as the monozygotic form of the allele is not lethal or damaging to the

organism. However, it does mean that these agricultural lines have a reduced genetic diversity. A balance between allowing enough diversity and still selecting for significantly important traits will help resolving future unforeseen challenges in the agricultural industry.

The number of local breeds (breed that only occurs in one country) in a species is usually a good indicator of genetic diversity. Local breeds that have been isolated by geographical or ecological barriers for long periods of time differ due to significant mutations and recombinations in their genome (FAO, 2007). With globalization comes a greater amalgamation and hybridization between populations within a breed or species and between different breeds. This process is referred to as gene-flow; a normal phenomenon which can also be accelerated by man (Fulton, 2006). Gene flow increases genetic diversity with the addition of unique alleles from one population to another. Alternatively, the introduction of new individuals into a population can decrease genetic diversity when large high-performance breeds out-compete local breeds; this subsequently creates a mono-agricultural environment (FAO, 2007; Aslam et al., 2011a). This means that gene flow can facilitate the increase in genetic diversity but the introduction of new individuals could reduce the genetic diversity, depending on the nature of the individuals being introduced. As globalization removes borders, it is becoming increasingly challenging to hold specific countries, governments and industries responsible for maintaining sufficient diversity with respect to particular species and breeds (Fulton, 2006).

2.2 The State of Genetic Diversity in Poultry

Poultry stocks have been consolidated in part due to high economic pressures. High performance commercial lines have been favoured over their 'hobby farmer' counterparts. This has reduced the numbers of heritage breeds (FAO, 2007) and in so doing, has reduced the genetic diversity in many poultry species. Although creating commercial lines that are highly productive and more efficient at producing food. Universities have also seen a cut back in research poultry lines due to downsizing of

facilities and resources (Silversides et al., 2008). As an example it was reported in 1981 that there were 79 chicken lines being housed in 11 Canadian institutes (Crawford, 1981). Whereas in 2005 a survey conducted by the Silversides group (unpublished data) found that these numbers had been reduced to 33 lines (23 distinct populations) of chickens in 5 Canadian institutes (Silversides et al., 2008). These research lines can take decades to create and will be impossible to regenerate, as their parental strains are no longer available (Pisenti et al., 1999). By comparison to mammals, avian karyotypes are more conserved among genomes, showing that avian genomes are on the whole more stable than their mammal counterparts (Ellegren, 2010). If this stability at the karyotype level filters down to the genotype scale then this could mean that rare and unique genetic traits might still be present in the distant relatives of common poultry species. One of these unique genetic traits is the cold tolerance of the Chantecler chicken, which might appear as having little value in the present day to the poultry industry but has the potential to solve future industry challenges.

Even the definition of the word "breed" might be detrimental to the conservation of poultry. The definition from the FAO is based on variations in phenotype and geographical distribution; this does not take into consideration genotypic differences within a breed (FAO, 2007). Future work could focus on genotyping breeds to determine unique populations, which have a superior genetic diversity, and focus efforts to protect these unique populations. Focusing our efforts solely on identifying and protecting phenotypic differences might limit the scope of genetic diversity preservation when it comes to geographically isolated populations of the same breed or species; as a lack of phenotypic diversity doesn't necessarily mean a lack of genetic diversity. Looking at genotypic difference between populations would potentially widen the scope of preservation for certain species.

Out of the 1,970 avian domesticated breeds, 1,728 (88%) are designated local breeds (FAO, 2007). Out of these, there are 89 avian domesticated breeds in North America, and 26 (29%) of these are designated local breeds. This shows that North America is proportionately under-represented for local breeds,

compared to the rest of the world. This is in part due to the fact that most poultry breeds and species have been imported into North American but also in part due to the heavy commercialization of the industry (FAO, 2007) and the favouring of commercial strains over local breeds. When looking at the domesticated avian numbers, 85% of the North American domesticated local avian breeds are either, extinct (1), endangered (7) or critically endangered (15) (FAO, 2007).

2.3 The State of the Turkey and its Genetic Diversity in the Industry

There are 85 breeds of domesticated turkey in the world and North America has 12 of these local breeds. Most of these breeds have some designation referring to the population size: *Critical <100* (Beltsville Small White, Ridley Bronze), *Endangered 100-499* (Broad Breasted Bronze – naturally mating, Narragansett, Standard Bronze), *Vulnerable 500-999* (Bourbon Red), *At Risk 1000* + (Norfolk Black, Spanish) (FAO, 2007; RBC, 2012). The Ridley Bronze is the only turkey breed local to Canada, developed by the Ridley family of Saskatchewan (RBC, 2012). North America has 33% of the world's population of domesticated turkeys, representing 13% of the total turkey diversity in the world (FAO, 2007).

In Canada, turkey is the fifth most consumed meat behind chicken, beef, pork and fish (TFC, 2014). As of 2013, there were 527 registered turkey farms in Canada, down from 602 in 1983. In 2013 the production was estimated at 35 million birds a year, with a retail value of $395 million (TFC, 2014). As of 2014, turkey farmers in Canada were supplied by 8 hatcheries; down from 14 hatcheries in 2007. Interestingly, turkey production has increased over the last decade (TFC, 2011a; 2014), but has seen a reduction in farms and hatcheries. In Canada these hatcheries are supplied fertilized eggs from local industries. Just three significant primary breeders (Aviagen – Nicholas British United Turkeys, Hybrid) support these local industries.

These reductions have also been felt in the University setting due to shifting priorities; as of 2014, there are no Canadian Universities that routinely house breeding turkey flocks for genetic conservation purposes (Dr. Schwean-Lardner, personal communication, 2015). There were 6 turkey lines as of 1984, reducing to 2 lines in 1998, and now are eliminated entirely (Pisenti et al., 1999). This drastic reduction and stream lining has not only been at the commercial and university level, but the turkey itself has seen a drastic change in physiological characteristics and stream lining for commercial benefits. The turkey itself has undergone extensive physiological changes due to immense selective pressure from the industry (Havenstein, 2006). In one study the phenotypic selection for increased body weight, commercial turkeys over sixteen generations, achieved a 50% increase in body weight at 16 weeks of age (Nestor et al., 1969; Nestor, 1984). This rapid growth has led to many issues surrounding the welfare and reproductive fitness of turkeys (Marchewka et al., 2013). Their size and weight impedes them from mating naturally, which means all commercial turkeys are artificially inseminated (van Wambeke and Huyghebaert, 1989). Commercial turkeys also have a decreased life expectancy, although breeder turkeys are rarely kept more than a year for production economics and lowered reproductive capacity, if they were to be kept for a greater time period it is doubtful they would live longer (Marchewka, et al., 2013). Although this is only the case for heavily selected domestic turkey lines; local and heritage breeds are defined by the "American Livestock Breeds Conservancy" as being able to mate naturally and having a far superior longevity (5-7 years). If in the future genetic issues of commercial domestic turkeys were to become unfavourable, the genetic solutions might still be located in local and heritage breeds.

2.4 Canadian Organizations and Industries involved in Turkey Genetic Preservation

This section provides more detail about the specific organizations that play a role in the preservation of genetic diversity in the turkey industry. Two large players in poultry preservation in Canada are Agriculture and Agri-Food Canada and Universities, although over recent years shifting priorities have forced the downsizing, and depopulation of turkey research lines between 1984 and 1998. Around 63% of

the Canadian university poultry stocks were eliminated, with a third of those losses being between 1995 and 1998, illustrating an exponential elimination of stocks up until 1998 (Pisenti et al., 1999). The main goal of governmental agencies and university programs still in effect is to preserve industry lines, but it appears more funding is required to be able to adequately protect heritage breeds. These industry lines are not the most genetically diverse and are unlikely to contain novel genes useful for future challenges (Fulton, 2006; Aslam et al., 2011a). The Canadian Poultry Research Council along with the Turkey Farmers of Canada represent the Canadian poultry and turkey sectors. In recent years, both organizations have shown an interest in protecting genetic diversity, and they are now looking toward partnering with universities and government agencies to accomplish these goals (TFC, 2011b). Rare Breeds Canada and the American Livestock Breeds Conservancy are two associations who have collaborated with their own local poultry fanciers to maintain accurate records of local breeds and population numbers. Their main priority is to focus on accurate population genetic profiles, which are good resources to act from, with reliable counts, but these organizations don't have cryobanks of their own. Instead individual poultry fanciers can use government and industry cryobanks but due to the potential political and proprietary deterrence poultry fanciers might not utilize these resources to their full potential.

2.5 Resources available for genetic preservation

Resources can be divided into information (statistics and electronic databases) and biological samples either in the ex situ, *in vivo* form as individuals or in the ex situ *in vitro* form as germplasm, with these germplasm samples usually being cryopreserved. Both are needed to preserve genetic diversity. Since living turkey lines preserved in a research setting are non-existent in Canada, industry and local turkey fanciers are the players maintaining these breeds *in vivo* (Fulton, 2006). The two major cryobanks in North America maintained by government agencies, which have a diverse sampling of cryogenically stored germplasm, are the Canada Animal Genetic Resources Program (CAGR) and the National Animal Germplasm Program (NAGP). The NAGP is operated by the United States Department of Agriculture

(Blackburn, 2006; USDA-ARS, 2009) and the CAGR is managed by Agriculture and Agri-Food Canada. These two federal organizations work together to optimise their systems. Both the CAGR and the NAGP have a variety of turkey and chicken breeds cryopreserved, using a number of different biological materials (sperm, gonads and blood) from a larger pool of individuals (USDA-ARS, 2010).

Genomic sequencing to form genetic profile databases is becoming more attainable as the cost for the processing drops. It is now becoming a reality that we can use these databases for all species and breeds (USDA-ARS, 2012). Two major genetic tools that can be used for genome analysis are Single Nucleotide Polymorphisms (SNP's), and Microsatellites (MS). They allow for the comparison of individuals without sequencing their entire genome, which would be extremely costly and inefficient to repeat each time. SNP's are polymorphic markers, typically biallelic (Aitken et al., 2004). A SNP usually occurs every 400-1000 base pairs (Brouillette et al., 2000; Sachidanandam et al., 2001). SNP based linkage maps for turkey genomic studies are becoming increasingly popular, and can be used as a sentinel for biodiversity (Muir et al., 2008). Informative SNP's are highly conserved between species, which means they can be more easily related to ancestral lines. As these are single nucleotide differences, they can have no effect or can have a drastic effect on phenotypes. The position of the SNP in non-coding or coding areas of DNA doesn't predict the effect. SNP's in non-coding areas can have large effects on gene expression, whereas SNPs in coding areas may have no effect at all depending if there is a change in amino acid sequence. In recent years, these genetic markers have been associated with potential disease phenotypes (Aslam et al., 2011b). MS are usually polymorphic, if they are not polymorphic to start with their structure allows for insertions and/or deletions that allow for polymorphisms to occur. These polymorphisms are made up of tandem nucleotide repeats bi- (ACACAC), tri- (CAGCAG), quad- (TAACTAAC), and so forth. Due to their considerable variance in allelic forms less are required to show genetic differences in individuals (Reed et al., 2000; Reed et al., 2006). MS are usually located in noncoding DNA, and are not located in regulatory regions. If this does occur it usually results in genetic disorders (Almeida et al., 2013). The strength of MS lies in their ability to be used for cross-species amplification. For instance, a chicken MS

library can be used on a turkey breed with relatively high success, even though these species are separated by more than 40 million years (Reed et al., 2000; Chaves et al., 2006). This information can be used to show how closely related individuals are, or if a population is highly inbred (Reed et al., 2005). SNP's and MS databases have a greater scope and complexity compared to simply looking at allelic differences in genes. This is due in part, to the very nature of allelic differences, which tend to be less stable with a higher mutation rate. Allelic differences also tend to be fewer in genomic quantity when compared to SNP's or MS (Reed et al., 2005; Aslam et al., 2010). The biggest factor though involved in the creation of these databases and application, will be the cost associated with them. Although, these costs have been dropping steeply in recent years, the most noticeable drop, was with the introduction of the next generation sequencers, into the market (Hayden, 2014). The next generation sequencers high-throughput capability and speed are due to its ability to sequence millions of fragments of DNA in unison, instead of the previous models which could only sequence one fragment at a time (Grada and Weinbrecht, 2013).

2.6 Mutant Stocks, Specialized Strains, Standard Breeds and Elite High-Performance Breeds

Instead of looking at poultry stocks as a whole unit that has equal genetic preservation value, Fulton (2006) suggested breaking them down into types of strains and breeds; mutant stocks, specialized strains, standard breeds and elite high-performance breeds. Mutant stocks are birds that typically share one mutant phenotype, a result of a single mutant allele. These stocks tend to have the most drastic phenotypic variance such as naked neck and dwarfism. Most of these mutations are not suitable for commercial production, but have high value as research models. Specialized strains have usually been created in a research setting for a specific purpose, and have taken years to develop, and should be protected as most of the parental strains are no longer available. Once lost, it would be impossible to regenerate. Standard breeds are populations of birds that have been isolated, artificially selected for, and steered towards certain traits, which are deemed desirable at the time of selection. Standard breeds are made of a unique set of genes with a variety of alleles for each gene, which can produce a uniformed or variable plumage

color, these phenotypes can be re-made by appropriate crosses, this usually means that these breeds hold the most genetic diversity. Elite high-performance breeds were in the past made up of crosses from standard breeds; these crosses were designed to blend the important traits and at the same time protect the genetics of standard breed for the company. Nowadays high-performance breeds are no longer crosses of standard breeds, and are selected on their own for a variety of important traits. It is hard to tell whether all the important traits have already been integrated, but as public demand changes, so will the importance of certain traits (Fulton, 2006). Common traits today might lose economic value, and other non-implemented traits might gain economic value in the future. By protecting and safeguarding the genetic stocks and genetic diversity in commercial and heritage/local breeds at present, the industry will be better prepared for future challenges, and to respond to shifting consumer demands for a specific product.

II. Avian Preservation Techniques

Although artificial reproductive techniques and cryopreservation strategies are quite advanced for mammalians, the same cannot be said for avian species. This is due to the physiological difference between the mammalian viviparous forms of reproduction versus the avian oviparous styles. For birds the difference starts at the beginning with yolk formation in the oocyte to a degree far greater than mammals, and utilization of open cells in embryo development. This ends with external gestation in the form of eggs versus internal gestation exploited by viviparous animals. In the second part of this review the focus will be on reproductive anatomy and types of preservation techniques. This means looking at the process of both folliculogenesis and spermatogenesis, which occurs in the ovary and testis respectively.

2.7 Folliculogenesis and Spermatogenesis

Although folliculogenesis and spermatogenesis occur in their respective gonads, similar hormonal pathways via external and internal signals, stimulate them. An example of an internal signal would be

nutritional status of the bird. On the other hand, external signals can be visual stimulation from potential mates, temperature/humidity and most importantly, photoperiod (Morton et al., 1985; Gwinner, 2003). Photoperiod involves the recognition that a temporal dark period is now light or vice versa. This allows the animal to determine if the days are shortening or elongating. In mammals, the only photoreceptor is the eye (Reiter, 1980) when it is stimulated by light a nerve signal is sent to the pineal gland, in the absence of this signal (dark periods) the pineal gland releases melatonin (Ikegami and Yoshimura, 2013). In birds, the eye is not required to experience the effect of light with respect to a photoperiodic response (Benoit, 1964) instead light penetrating through the skull is perceived by deep brain photoreceptors. Although in birds the pineal gland releases melatonin in the same way it is not responsible for stimulating the reproductive season (Juss et al., 1993). Instead melatonin, plays a greater role in determining the circadian rhythm and changes to it, as an internal zietgeber (Gwinner et al., 1997). The exact chemical which signals the reproductive season is unclear, it does though stimulate a cascade of chemical signals (TSH as an example), which is finally detected by the hypothalamus producing a certain set of gonadotropins (GnRH-I, Lamprey III and GnIH) that are released into the hypophyseal portal blood (Ikegami and Yoshimura, 2013). These hormones are detected by the anterior pituitary gland, which in response produces gonadotropins (LH and FSH) (Johnson and Woods, 2007). These gonadotropins pass into the bloodstream, and influence the development of the germ cells in the testes or ovaries.

2.7.1 Folliculogenesis and Ovum/Embryo Cryopreservation Potential

Folliculogenesis in poultry, which starts shortly after hatch, is the process by which a follicle and the primary oocyte within, grows symbiotically until the follicle ruptures (ovulation). In birds, folliculogenesis is divided into four follicle stages: primordial, primary, prehierarchal and preovulatory (Johnson and Woods, 2007). This process involves three growth phases: slow (primary), intermediate (prehierarchal) and rapid (preovulatory) (Marza and Marza, 1935). Shortly after hatching there are around 480,000 primary oocytes located in the ovary (Johnson and Woods, 2007). Primary oocytes are

surrounded by a single layer of granulosa cells, and are in an arrested state (first meiotic prophase). These cells change in number and shape when the follicle is selected to grow, this structure is called a primordial follicle (Chalana and Guraya, 1979a). Granulosa cells, which are only separated from the oocyte by a small vitelline and perivitelline membrane, have been shown to be crucial for the development of the primary oocyte (Dorrington et al., 1983). Granulosa cells are vital for gonadotropin-induced hormone production, mediation of yolk protein uptake and maternal RNA transfer (Johnson and Woods, 2007). Primordial follicles do not grow and are in an arrested state, they can stay in an arrested state for years before becoming active. In chickens primordial follicles are up to 80μm in diameter (Johnson and Woods, 2007). This pool of primordial follicles in the ovary will shrink over time as follicles are selected to enter the slow growth phase.

After the arrested stage, FSH activates individual primordial follicles, to enter a slow growth phase and are now designated primary follicles. Not all primordial follicles are activated at the same time, producing a constant supply of potential follicles (Johnson and Woods, 2007). The activation of primordial follicles occurs with the recruitment of the mesenchymal cells to create a theca cell layer, which is separated from the granulosa cells by a basal lamina membrane (Asem et al., 2000). This slow growth phase can last for years, and involves the slow uptake of yolk precursors by the primary oocyte (Carlson et al., 1996). Although the growth is independent of gonadotropins and growth factors, follicle viability still remains dependent on these factors (Johnson et al., 1996). Primary follicles in chickens are 0.04-2.0 mm in diameter (Marza and Marza, 1935). Primary follicles are located within the ovary cortex (Johnson and Woods, 2007).

The primary follicles then enter the intermediate growth phase and are designated prehierarchal follicles, with the uptake of white yolk precursors. Prehierarchal follicles range between 2-4 mm in diameter in chicken (Marza and Marza, 1935); this phase can last for weeks to months (Johnson and Woods, 2007). The prehierarchal follicle is surrounded by smooth muscle, blood vessels and epithelium, which allow the

follicle to protrude out of the cortex and stromal tissue layer, and can now be seen externally of the ovary (Chalana and Guraya, 1979b). This growth is due to uptake of lipoprotein-rich white yolk, made up of vitellogenin (VTG), very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) by the primary oocyte. The liver produces these factors in response to estrogen, which is released by the granulosa cells, and other cells surrounding the oocyte (Johnson and Woods, 2007). More follicles enter this intermediate growth phase than are actually needed to continue the supply of preovulatory follicles. Prehierarchal follicles, which are not selected (almost 20%) become atretic, and die through apoptosis (Gilbert et al., 1983).

The number of prehierarchal follicles selected to transition into the preovulatory follicle stage, determines the clutch size, follicles are usually ovulated on sequential days. Preovulatory follicles (4-40 mm in diameter) reach this size in about 7 -11 days in the domestic chicken (Gilbert, 1972). A gap junction protein (occludin) and an oocyte receptor (VLDL/VTG) have been identified as key in the oocytes ability to rapidly absorb yolk precursors (Shen et al., 1993; Schuster et al., 2004). The VTG/VLDL receptor binds both of the yolk lipoprotein precursors vitellogenin (VTG) and very low-density lipoprotein (VLDL) and transfers these components across the oocytes plasma membrane (Shen et al., 1993). The VLDL/VTG receptor found on the chicken oocyte is part of the LDL receptor family (Bujo et al., 1994). A pool of these receptors are located in the centre of quiescent follicles and recruited to the plasma membrane at the start of the rapid growth phase (Shen et al., 1993). Occludin, which is located on the oocyte-facing side of the granulosa cells, is mediated by FSH and Activin A. An increase in FSH level reduces the expression of occludin (Schuster et al., 2004). As follicles mature, occludin levels drop allowing for easier passage of these precursors (VLDL, VTG) to pass by the granulosa cell layers.

The largest preovulatory follicle is ovulated, in response to a surge in pituitary-derived LH. This surge occurs 4 to 6 hours before ovulation (Johnson and Woods, 2007). The fully mature ovum enters the reproductive tract, where fertilization can occur in the infundibulum. The ruptured post-ovulated follicle

(POF), consisting of granulosa and theca tissue is left behind. The site of fertilization occurs at the germinal disc. This is a small area of cytoplasm, appearing as white plaque 2-3mm in diameter (Romanoff, 1960) and is positioned on top of a column of white yolk, which continues from the latebra located in the centre of the ovum to the disc (Perry et al., 1978). POF is not homologous to the mammalian corpus luteum, as it does not maintain hormonal control over the developing embryo. It does produce relaxin; its role in oviposition is still unclear (Brackett et al., 1997). Instead, the POF is rapidly reabsorbed, although it has been suggested that for one day it still remains active, producing progesterone (Johnson and Woods, 2007).

The challenge with cryopreservation of the mature avian ovum is the sheer size of the large yolk filled egg (Fulton, 2006). The plasma membrane of the mature ovum is too weak, relative to the volume of yolk, and so is unable to withstand the harshness of cryogenic storage either through current vitrification or slow freezing procedures. There are also currently no methods in vitro, which will mimic the natural process whereby yolk precursors are taken up by the developing oocyte allowing for maturation to occur. Secondly, after fertilization in mammals, the oocyte would normally go through cleavage. The subsequent cells are called blastomeres and eventually give rise to the blastocyst. However, in birds, the fertilized zygote goes through partial cleavage (Patterson, 1910; Kochav et al., 1980). The avian zygote continues to proceed through partial asynchronous cleavage, and develops on the surface of the yolk, now referred to as the yolk sac (Eyal-Giladi and Kochav, 1976). The avian zygotic cells are defined as being open blastomeres (Kochav et al., 1980), as there is no membrane between their cytoplasm and the yolk sac. This means that at the beginning of development, these cells cannot be separated from the yolk sac. By the 64-cell stage only the most centrally situated cells have a complete plasma membrane, separating them from the yolk sac (Bellairs and Osmond, 2005). At this point, part of the early avian embryo could theoretically be separated from the yolk sac. It is unclear if enough could be removed to still produce a viable embryo. Also, separation of the embryo past the 64-cell stage would lead to disturbance in the axis

of symmetry and would not be a viable target for cryogenic storage and/or transplantation (Johnson and Woods, 2007).

2.7.2 Spermatogenesis and Spermatid Cryopreservation Potential

Spermatogenesis, is the process by which spermatogonium (A_1), the most immature of the male germline cells, develop into fully developed spermatids (Aire, 2007). This process begins weeks to months after hatching. In birds, this process occurs in the seminiferous epithelium of mature testes, located in the abdominal cavity, ventral to the kidneys (Fig. 2.1). In mammals, spermatogonia first proliferate through mitosis into furthering stages of spermatogonia A_1-A_4, I, B; while at the same time maintaining a stem cell line, replenishing the germline (Lin and Jones, 1992). In birds, the number and designation of spermatogonia has been a hotly debated topic in the literature (Aire, 2007), it was first reported that in birds there was only one stage of spermatogonium (Zlotnik, 1947; Kumaran and Turner, 1949). Later reports described two stages (Gupta, 1955; Lake, 1956). It was shown that there are in fact four stages of avian spermatogonia given the designation Ad, Ap1, Ap2 and B (Lin and Jones, 1992). Spermatogonia then transition through meiosis developing into primary spermatocytes and then secondary spermatocytes (Aire, 2007). Secondary spermatocytes finally differentiate into spermatids through a process called spermiogenesis by which a spherical spermatocyte goes through four phases (Golgi, Cap, Acrosomal and Maturation), to become a fully differentiated spermatid. At the end of this process, spermatids have only one copy of each chromosome and have the characteristic, sperm-like form. This process takes place under the control of Sertoli cells (intratubular somatic cells), which facilitate nutrient supply to the developing sperm cells, and creates the blood testes barrier. Sertoli cells in response to pituitary pulses of FSH, signal the spermatogonia to begin developing (Aire, 2007). A pituitary LH pulse follows the FSH pulse shortly after. Leydig cells, located around the seminiferous tubules produce testosterone in response to this LH pulse. The production of testosterone inhibits FSH and LH by the repression of the release of GnRH. This inhibition is relatively short lived, and the cycle repeats itself soon after. This means that

spermatogonia are frequently being signalled by the Sertoli cells to proceed to develop into spermatids, and allowing for a continuous supply of spermatids ready for ejaculation when required. In Japanese quail, spermatogonia start to appear 11 days after hatch (Purcell, 1970) and the first spermatozoon are produced 25 days later (Jones and Jackson, 1972), allowing for fully developed spermatozoon to be produced 36 days after hatch (Mather and Wilson, 1964). Later it was shown that the average cycle (each cycle can be broken down into 10 stages) lasts 64 hrs with 4.5 cycles before spermatozoon are produced 12 days later for Japanese quail (Lin et al., 1990).This process is a lot faster than spermatogenesis for bovine, which takes roughly 61 days, 4.5 cycles, a cycle being 13.5 days (Amann and Schanbacher, 1983). This process continues all through the season, and is not stimulated in exactly the same way as in folliculogenesis for birds; the effect of changing light conditions is not so abrupt in the testis (Aire, 2007).

The male genetics can be preserved via cryogenic storage of spermatozoon. Issues arise due to the fact that avian sperm morphology and chemical characteristics are very different compared to mammalian sperm (Whittow, 2000). Cryoprotectants, which work for mammals do not always have the same effect for birds. Glycerol is a good example of a mammalian cryoprotectant with contraceptive qualities for avian sperm; glycerol appears to affect sperm morphology (cytoplasm, membrane bilayers and protein attachment to the surface) rendering the sperm ineffective (Hammerstedt and Graham, 1992). If glycerol is removed from the sperm upon recovery from cryopreservation before artificial insemination, fertility can be regained (Purdy et al., 2009). With respect to wild birds or rare heritage breed's, semen collection through cooperative, massage or electroejaculation might not be possible (Gee and Temple, 1978), due to physical limitations or seasonal parameters. Semen can also be of to poor a quality to be useful in AI (Blanco et al., 2009). This means that another technique needs to be investigated for cryostorage of the male germ line.

2.8 Ex situ, *In-vivo* Living Specimens

Live commercial poultry preservation has two major security hurdles: biological and economical security. Biological security is assured by placing an elite commercial line in multiple locations. So, that if one flock is compromised, the entire genetic reserve is not affected. Economic security is managed by placing stocks in multiple countries. If one border is shut down due to an epidemic, business can continue in other countries without concern for importation hurdles (Fulton, 2006). For the industry or conservation groups to create these flocks all over the world means removing individuals from an original population and relocating them to start a new population. Propagation of the new population occurs in an isolated environment away from the original population. There is a genetic concern when a group of individuals is isolated from a large population, to start a new flock. These two genetic concerns are genetic drift and the founder effect (Kimura and Otha, 1971 ; Chakraborty and Deka, 2002). With respect to the founder effect the frequencies of alleles in the individuals, which start the new flock will be the limited pool of alleles that the new population will have to work from. The fewer individuals used, to start the new population, the less of an accurate representation the new population will be to the original population. If measures aren't taken to make sure that breeding is random and that all individuals' genetics are passed on as the new population grows then genetic drift will diverge the new population further away from the original populations allele frequencies. The Ex-situ population method of preservation is also the most expensive and labour intensive. An increase in funding might alleviate the problem in the short term, but this would not address the possible loss of diversity from genetic drift and the founder effect (Blackburn, 2006; Fulton, 2006; Silversides et al., 2012).

2.9 Sperm Backcrossing

The cryopreservation of the first avian sperm (Shaffner et al., 1942) unlocked the door allowing for sperm backcrossing to be the first method that breeders could use to rejuvenate poultry breeds by AI. Sperm

backcrossing works by fertilizing a female (recipient) of a different breed or species with fresh/cryopreserved semen (donor) from the original breed or species, which is trying to be rejuvenated, to create a hybrid (H1). Two different species could be used in the case of sperm backcrossing as long as the hybrids are fertile, which isn't always the case for interspecies crosses. This hybrid (H1) shares 50% of its genome with the donor semen and the recipient. The hybrid (H1) is then inseminated with the original cryopreserved semen to create a hybrid (H2), which has 75% of the original genome. After five consecutive generations and inseminations with the original cryopreserved semen, a hybrid (H5) is created having the potential to carry 97% of the original genome of the rare or endangered species (Blesbois et al., 2007). The major downside of this process is that the maternal mtDNA and W sex chromosome are lost. Although sperm do possess mtDNA, it was generally accepted that paternal mtDNA is not passed on to the oocyte upon fertilization. In the 90's, this dogma was replaced with the view that partial paternal mtDNA is leaked into the oocyte, although the inheritance is not as strong as with maternal mtDNA (Gyllensten et al., 1991). Apart from the loss of genetic material through backcrossing, there are other hurdles: a suitable recipient to cross with the donor might not be available if the species is extremely unique (Petitte, 2006; Wernery et al., 2010). The time frame for restoring a chicken line through backcrossing is about 1089 days, which allows for 5 generations (Blesbois et al., 2007; Silversides et al., 2012). It is almost 7 times more cost effective to backcross when looking at preserving 10 lines over 20 years; when compared to *In situ* living lines for preservation (Silversides et al., 2012).

2.10 Primordial Germs Cells

The most important cells to preserve when maintaining species are germ line cells these possess the ability to produce the next generation. Any cell type that can differentiate into germ line are potential targets to be cryogenically stored (Petitte, 2006). Embryonic stem cells (ESC) are pluripotent cells, which can differentiate into all cell types in the developing embryo, including the germ line. Another target would be the early blastodermal cell (BC) (Petitte et al., 1990; Petitte, 2006). These aforementioned cells

are limited in biological numbers and are not always easily available. Harvesting primordial germ cells (PGCs), a cell line which can differentiated into germ line is feasible by taking advantage of a key avian physiology, that PGCs migrate from the yolk sac through the circulatory system to the gonadal ridge (Meyer, 1964). This means that in early embryonic development, PGC's can be isolated from the circulatory system at embryo stages 14 to 16 and can also be isolated from the germinal ridge at stages 26 to 28 (Petitte, 2006). The period of development from fertilization through to hypoblast has been classified into 14 stages (Eyal-Giladi and Kochav, 1976), and for the subsequent development until hatch (stages 15 - 46) laid out by a different system (Hamburger and Hamilton, 1951). Primordial germ cells can be harvested from a donor embryo and injected into a recipient embryo, these PGC's will migrate to the recipients gonads (Simkiss et al., 1989). This process creates germ line chimeras (Tajima et al., 1993). Chimeras consist of cells from two individuals. Successful germline chimeras can be crossed to create offspring that match the genome of the original donor cells. This is a technically challenging procedure and is primarily used by the biotechnology industry to create genetically modified poultry lines (Petitte et al., 1990; Liu et al., 2012a). However, these cells are not prolific in the developing embryo, which means that a method of amplifying *in vitro* needs to be developed (Petitte, 2006). PGCs are the last precursors to the fully functioning germ line, and so are the last potential cells for cryopreservation, before gonadal tissue itself (Wernery et al., 2010).

In contrast to backcrossing, which loses mitochondria DNA and the W chromosome, PGCs conserve the entire genome of the donor bird (Petitte et al., 1990). There are three main challenges when creating chimeras: the physical manipulation of the avian embryo, the selection of the recipient embryo to the donor cells has to be a symbiotic match, and finally the cost of reconstitution has to be taken into consideration (Petitte, 2006; Wernery et al., 2010). Another consideration is that in the case of rare and endangered breeds this method uses the manipulation and sacrifice of embryos for cryopreservation. The inevitable conflict in this method is that embryos are needed for conservation of living populations with respect to propagation. By the targeted removal of the key age group needed for recruitment, there is an

inherent risk in the survival of the living population. This could jeopardise the living population, whilst at the same time trying to cryopreserve the species. Instead, another method needs to be addressed which can avoid the use of embryos in cryopreservation of endangered or threatened poultry breeds.

2.11 Gonad Tissue Transplantation

Another option to preserve poultry genetic resources is to cryopreserve gonadal tissue. Gonadal tissue does not have to be collected from embryos, and is in greater biological quantity. When comparing gonad tissue cryopreservation to in situ populations, in order to preserve genetics, it is almost 20 times more cost effective to cryopreserve and transplant gonads (Silversides et al., 2012); when the goal is to preserve 10 lines over 20 years, and then recover all lines. Gonadal tissue transplantation could be the most cost effective method of reconstituting a line, and keeps the entire genome of a breed.

2.11.1 Anatomical overview of day old avian gonads

A brief overview in the abdominal anatomy of immature chick, within the proximity of the gonads will help in the comprehension of the challenging nature of gonad transplantation, in future sections. For birds, gonads are located in the abdominal cavity, in the far dorsal cranial position (Fig. 2.1), and directly below the lungs (King, 1993). For day old female chicks, only the left ovary develops and is functional in adulthood (Bellairs and Osmond, 2005). In males, both testes develop with the left testis being slightly larger than the right one (Fig. 2.1). For male day old chicks, the testes are located in close proximity to the left and right trunks of the vein *illaca communis*, both trunks lead into the vein *cava caudalis* and back to the heart (King, 1993). Removal of the testes (testectomy) or ovary (ovariectomy) in day old chicks is complicated by the close proximity of the gonads to the *illaca communis* vein. If these veins are cut or nicked by the surgeon in anyway, the likelihood that clotting occurs fast enough to prevent massive haemorrhaging is unlikely (Song and Silversides, 2007b).

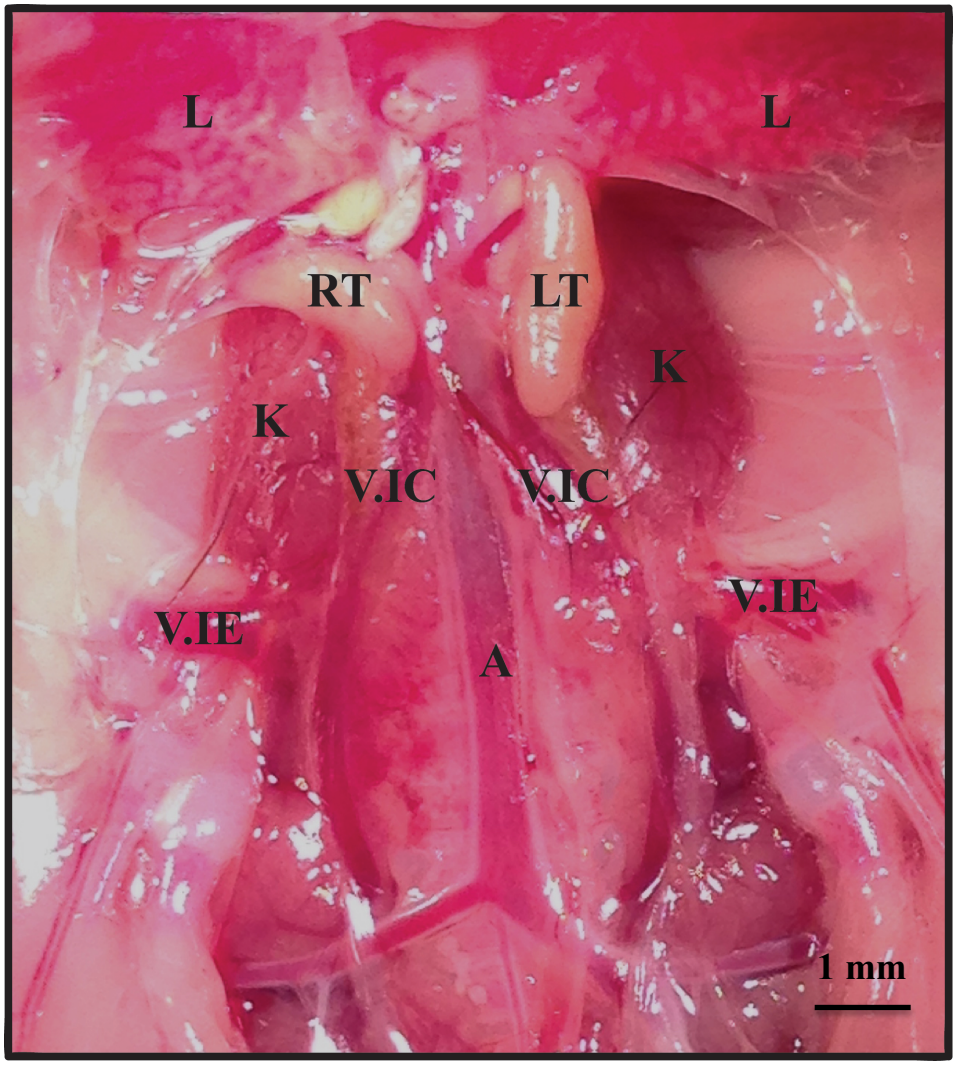

Figure 2.1 Internal view from the ventral side of the abdomen cavity in a day old Barred Plymouth Rock male chick. (L = lung; RT = right testis; LT = left testis; K = kidney; V.IC = vein illaca communis; V.IE = vein illaca externa; A = aorta).

2.11.2 Ovarian Tissue

Ovarian transplantation involving the chicken (*Gallus gallus domesticus*) was first tried on, 24 - 30 day old chicks (Grossman and Siegel, 1966). These ovarian transplants failed to produce donor-derived offspring, when the recipients reached maturity. The chicks used appeared to be too mature, and the grafts failed most likely due to rejection from the recipients adaptive immune system, which reaches competency a couple days post hatch (Mast and Goddeeris, 1999). Transplantation of ovaries to produce donor-derived offspring was first shown to be possible by the Silversides group in 2007. The ovaries of day old chicks (Barred Plymouth Rock) were removed, and orthotopicly (*in the original position*), transplanted into the abdominal cavity of day old chicks (White Leghorn) that had previously had their own ovaries removed (Song and Silversides, 2006; 2007a). The donor ovaries were not sutured into place, but positioned underneath the abdominal cavity organs. Suturing of the ovary is theoretically not necessary; as the site heals, the circulatory system connected to the donor ovary. The major hurdle with transplantation is overcoming the host's adaptive immune system, which detects foreign antigens with novel antibodies from B cells. Activation of B cells occurs when a correct match is made between an antigen and antibody, and is facilitated by T cells. If this occurs, the immune system will subsequently attack cells expressing this specific antigen and rejection will occur. Day old chicks were used because their adaptive immune system is not fully functional (Song and Silversides, 2006). The chicks were treated with Mycophenolate Mofetil (Cellcept, *Roche*), which inhibits inosine monophosphate dehydrogenase (IMPDH), the enzyme in mammals controlling the purine synthesis pathway used by T and B cells during proliferation (Allison and Eugui, 2000). B cells in birds originate in the Bursa of Fabricius and when mature are able to detect foreign antigens. T cells originate from the thymus and are more general in their immune response, but are key in allowing for the activation of B cells. Both cell types are key components in graft rejection, which is why it is necessary to knockdown both to supress a response of the immune system (Heidt et al., 2010). It was shown that immunosuppressed chickens were not more likely to produce donor-derived offspring over their non-immunosuppressed counterparts, but

were more likely to produce a higher number of donor-derived offspring (Song and Silversides, 2007a). The lower production of chicks from the non-immunosuppressed group was from poor fertility. However, in quail, immunosuppressed individuals were more likely to produce donor-derived offspring (Song and Silversides, 2008a). Another challenge is that over time residual host germ cells that were not completely removed on the day of surgery have the potential to overwhelm donor germ cells located in the transplant. This would explain why over time the amount of donor-derived offspring produced begins to drop (Song and Silversides, 2008b). This was overcome by administrating Busulfan (BU) to pre-recipient embryos after 24 hours of incubation, well before hatching and surgery. This drug is effective in the sterilization of avian embryos (Aige-Gil and Simkiss, 1991). Busulfan appears to target slow proliferating cells (primordial germ cells) more than rapidly proliferating ones (Marsh, 1976). Busulfan is a bifunctional alkylating agent (Dunn, 1974) able to cross-link DNA or Protein (Tong and Ludlum, 1980). This causes DNA replication and repairing problems and eventual cell death, through cell apoptosis and this knocks down the host PGCs (Galaup and Paci, 2013). This procedure produced a higher ratio of donor derived offspring, and produced donor derived offspring for a longer period (Song and Silversides, 2008b). To show that this technique could potentially work on other avian species, the same procedure was conducted on Japanese quail (Song and Silversides, 2008a). Due to the size of Japanese quail, it was shown that surgery was more successful on week-old than day-old chicks. The week-old quail chicks were able to produce donor-derived offspring after reaching maturity. This also showed that for some poultry species the chick's immune system is still receptive to ovarian transplantation past the first day of age (Song and Silversides, 2008a). All the work so far mentioned on chickens and quail was conducted using fresh ovarian tissue that was immediately transplanted into a recipient. The next parameter, which was tested, was the ability for the ovarian tissue to survive cryopreservation (slow-freezing or vitrification) and then subsequently transplanted, to produce donor-derived offspring. Quail were used again, most likely because they reach sexual maturity faster (6-7 weeks when compared to 20-22 weeks for chickens). Ovaries were frozen in two different ways, slow freezing and vitrification (Liu et al., 2010). Cryopreserved tissue post-warming or post-thawing can be evaluated in a variety of ways (cell viability

assays, apoptotic marker analysis, light microscopy and cell/tissue culture), even before transplantation occurs. For Japanese quail, cell viability was slightly higher in vitrified ovaries compared to slow-frozen ovaries, but both were able to produce donor-derived offspring after successful transplantation (Liu et al., 2010). In subsequent work by this group, a different method of evaluating quail ovarian tissue was utilized. This method uses the chorioallantoic membrane (CAM) of a developing embryo, and grafts gonads to this well-vascularized membrane. This allows for the evaluation of follicles and additionally potential vascularization of ovarian tissue, which was not covered by the previous cell and tissue viability tests, this method was also used to trouble shoot new vitrification protocols (Liu et al., 2012b).

It is going to be impractical in the future to have the host and donor from the same breed or species, especially if the goal is to restore an endangered or a rare breed. It is then paramount to show this procedure can be done between species. To show that this was possible, ovarian transplantation was conducted between Muscovy (*Cairina moschata*) and Pekin (*Anas platyrhynchos*) ducks (Song et al., 2012). Muscovy ducks were used as the donors, and the Pekin ducks were used as the hosts. Pekin ducks that had Muscovy ovaries successfully produced Muscovy ducklings (Song et al., 2012). The Silversides group went one step further and questioned whether adult ovarian tissue could be used as transplant tissue, to produce donor-derived offspring (Liu et al., 2015). This was shown by harvesting ovarian tissue from 13 week-old Japanese quail hens, ovaries were trimmed of preovulatory and prehierarchal follicles and dissected into pieces, for transplantation into week old chicks. Out of the 7 recipient birds, 2 produced donor derived offspring (Liu et al., 2015). This brings the question: how does a mature ovary either reset its 'clock' or put it on hold while the recipient's own biological clock catches up and then proceeds to become active again? Mature ovaries when transplanted, did not remain active in producing donor-derived offspring as long as their immature counterparts (Liu et al., 2015).

2.11.3 Testicular Tissue

For testicular transplants, most of the results obtained from the work done so far, mirrors the results from the ovarian transplanted tissue. Both slow freezing and vitrification of testicular tissue has led to the production of spermatozoa able to produce donor-derived offspring (Song and Silversides, 2007c; Liu et al., 2013a). One of the major differences between testicular and ovary transplantation is that testes have to be connected to the vas deferens to be able to ejaculate sperm for collection. Based on the size and structure of testes and vas deferens, it would be extremely difficult to connect the transplants in day or week old chicks. Instead, the testes are simply grown in the recipient, and then removed. Fluid is subsequently collected, and used for intramagnal insemination. Avian spermatozoa do not need a period of time in the epididymis to become fertile for the purposes of intramagnal insemination, although vaginal insemination of sperm requires epididymal factors to promote motility (Howarth, 1983). Avian spermatozoa collected from testes, epididymis and vas deferens in this work showed the same fertilization rates of around 85-90% (first week) after intramagnal insemination. For testicular transplantation, the exact placement of the grafts was tested to determine the best location for maturation. Testes were transplanted orthotopically in the abdominal cavity, and under the abdominal skin, and also under the skin on the back (Song and Silversides, 2007b). Testes transplanted in the abdominal cavity and under the skin on the back produced mature spermatozoa. It has also been shown that testes do not have to be whole to develop, and that they can develop from pieces of testicular tissue (Song and Silversides, 2007b) or even from cell suspensions injected under the skin (Song et al., 2010). The cell suspension work was not conducted between individuals but rather the cell suspensions were injected under the skin of the same chick that donated the testis material. One of the major surgical challenges with testis transplantation is massive haemorrhaging causing most male chicks not to survive surgery. This is overcome by removing the host's testes in pieces (Song and Silversides, 2007b; Song et al., 2010). Even when testes were grafted onto the back of chicks, their host testes were still surgically removed. Haemorrhaging likely occurs due to the close proximity that the right and left testes has to the vein *iliaca communis* (Fig. 2.1) and artery

truncus coeliacus, just at the branch point from the vein *cava caudalis;* any tear or nick of these blood vessels leads to imminent death. For male chickens instead of using day old chicks (as for the females) for transplantation, the same surgery was conducted on 2- and 6-day old male chicks (Silversides et al., 2013). Although both age groups led to successful grafts, the casualties from surgery were drastically lower in the 6 day-old group. This is most likely due to a change in position of the testes to these major blood vessels as chicks mature. As the chick develops, the testes begin to migrate posterior away from these blood vessels. Recent findings have shown that the testes of the host do not in fact need to be removed to facilitate proper donor testicular tissue graft development and maturation (Liptoi et al., 2013).

CHAPTER 3: HYPOTHESES AND OBJECTIVES

Thesis Hypothesis (Chapters 4 and 5):

Meleagris gonads can be cryogenically stored, and subsequently transplanted into host day old poults, to produce donor-derived offspring.

Chapter 4

Hypothesis:

Day old large white turkey poult gonads are viable post-warming after vitrification.

Objective:

To confirm that under the standard vitrification protocol commonly used on quail gonads or with modifications to this protocol turkey gonads are viable after vitrification. Plasma membrane damage of cells and tissue morphological scores will determine viability of gonads.

Chapter 5

Hypothesis:

Day old large white turkey gonads, which were previously vitrified and subsequently warmed, can be successfully transplanted into recipient bronze poults, and the transplanted gonadal tissue can mature and develop normally.

Objective A:

To determine the appropriate conditions of poults pre-surgery, the best age at surgery and the best method of gonad attachment, for transplantation in day old bronze poults. Analysing survivability in different age groups and observing grafts post-surgery to determine if they have stayed in an appropriate position will assess these conditions and parameters.

Objective B:

To determine if grafts have taken and are developing normally, tissue morphology over time will determine if the grafts are maturing normally.

Chapter 6

Hypothesis:

Ultrasonography can be used on chickens to map folliculogenesis during the laying period.

Objective:

To study follicle growth in Barred Plymouth Rock chickens, and determine growth rates and patterns of follicles, with the potential of using this technique to monitor grafts later in development.

CHAPTER 4: VITRIFICATION OF DAY OLD TURKEY POULT GONADS AND SUBSEQUENT ANALYSIS OF VIABILITY POST-WARMING

4.1 Abstract

Cryopreservation of gonads allows for the conservation of a wide array of individual genetic traits, and as a whole, a large pool of genetic diversity at a fraction of the cost of living specimens. Vitrification has been used to store quail gonads, these gonads once transplanted were able to mature and produce gametes, giving rise to progeny. The objective of this work was to determine if day old turkey gonads are viable after vitrification by using the standard protocol used on quail gonads, or with modifications to this protocol. Gonads were submerged in equilibrium solution (ES) and then vitrification solution (VS) for a defined time (10, 15 or 20 min for ES and 2 or 3 mins for VS), before being plunged directly into LN_2 and stored. Upon subsequent warming of gonads viability was determined by two methods: a cell viability assay, which measured the percentage of cells with intact cellular membranes; and by a histological analysis of tissue, which graded tissue based on morphological alterations at the cellular level. The grading was done on peripheral and centre seminiferous cords for testes, and the cortex for ovaries. Testicular tissue cell viability was conducted on three vitrification protocols (10ES/2VS, 15ES/3VS and 20ES/3VS) along with fresh tissue. Histological assessment of morphological alterations was conducted on ovarian and testicular tissue from all possible protocol combinations (10ES, 15ES, 20ES with either 2VS or 3VS) along with fresh tissue and a no cryoprotectant group. Statistical analysis was conducted on both cell viability (ANOVA, Tukey test) and histological scores (Mann-Whitney *U*-test) to determine which treatment protocols had higher viability after vitrification. Vitrified testicular tissue treatment groups (15ES/3VS and 20ES/3VS) had the highest cell viability. For morphological alterations in testes, the treatment groups with the lowest peripheral and centre seminiferous cord scores were (15ES/2VS and 15ES/3VS). For ovarian cortex, treatment group 15ES/3VS was the only group that had no significant difference (P=0.818) compared to fresh tissue, and had an average under the acceptable threshold (score ≤

1). Based on our method of determining viability after vitrification, this study proposes that vitrification protocol 15ES/3VS is more suitable than standard protocol (10ES/2VS) for the vitrification of turkey testicular and ovarian tissues.

4.2 Introduction

Cryopreservation is the storage of biological material at extremely low temperatures and can be used to store germplasm from a wide array of species. Cryopreservation has been shown to be a successful method of ex-situ conservation for poultry breeds (Blesbois et al., 2007). Although, at present the female gametes (ovum), cannot be cryopreserved, due to biological limitations. Without the ability to freeze the female gametes, the W chromosome and mitochondrial DNA (mtDNA) cannot be cryopreserved and regenerated. This is not the case for male gametes (sperm), which can be stored with the intention of using backcrossing, but this technique does not revive a hundred percent of the original breed's genome. Due to the lack of female gamete storage, it is not possible to preserve the entire genome of turkeys through cryopreservation, and so once a line or breed is lost, it is lost for the foreseeable future. With the global reduction in local breeds and rare turkey breeds in North American being threatened (FAO, 2007; RBC, 2012), a suitable technique for storing the entire turkey genome is required. This study used a vitrification method, which has already been proven successful in chicken and quail (Song and Silversides, 2007c; Liu et al., 2010; Liu et al., 2013a), to freeze immature testes and ovaries from turkeys.

Cryopreservation is the immortalization of cells in a glass like state or frozen, which can be recovered at a future time. A common storage medium is liquid nitrogen (-196^{o}C, 77^{o}K). These extreme temperatures would be lethal for cells without the use of cryoprotectant agents (CPAs). Two common cryopreservation techniques are slow freezing and vitrification, which respectively use low levels of CPAs with a gradual drop in temperature or high levels of CPAs with a drastic drop in temperature. These CPAs dehydrate cells and stabilize the remaining water molecules, limiting the formation of intracellular ice crystals,

which upon freezing or vitrification, damage cells leading to necrotic tissue upon recovery (Meryman, 1971). Cryopreservation of avian gonads is a relatively new area of research and has only been reported in chicken and quail (Song and Silversides, 2007c; Liu et al., 2010). In chickens, this work has only been reported for Barred Plymouth Rock roosters, where testes were stored using the slow freezing method (Song and Silversides, 2007c). Successful storage of testes and ovaries, have also been reported in Japanese quail (Liu et al., 2010; Liu et al., 2013a) from both vitrification and slow freezing methods. For quail gonads vitrification was the preferred method over slow freezing due to its higher viability and simplistic procedural nature (Liu et al., 2010). Viability of avian gonads upon recovery has been tested via cell viability (Liu et al., 2010), and also by the eventual production of gametes after the gonads were transplanted into a recipient and allowed to grow and mature normally, producing donor-derived offspring.

The objective of this study was to determine if day old turkey gonads are viable after vitrification using the standard protocol used on quail gonads, or by a new protocol, which varied the time spent in cryoprotectant solutions before vitrification. Cell and tissue viability will be determined by Trypan blue, and light microscopy to visualize morphological alterations, respectively. These viability measurements were able to show that the standard protocol used on quail gonads was not suitable for turkey gonads left whole. Instead modifications to the standard protocol were able to increase cell and tissue viability.

4.3 Materials and Methods

4.3.1 Chemicals and Reagents

Dulbecco's phosphate buffered saline (DPBS), fetal bovine serum (FBS) and Dulbecco's modified eagle medium + [1g/L D-glucose, L-glutamine and 110mg/ml sodium pyruvate] DMEM were purchased from Life Technologies Inc. (Burlington, ON, Canada). Hematoxylin and Eosin were purchased from Fisher Scientific Company. (Ottawa, ON, Canada). All other chemicals and reagents were purchased from Sigma-Aldrich (Oakville, ON, Canada), unless otherwise stated.

4.3.2 Vitrification of day old turkey poult gonads and subsequent analysis of viability post-warming

4.3.2.1 Vitrification

Gonads were harvested from day old large white turkey (LWT) poults (Hybrid Turkey, *Kitchener, ON, Canada)*, which were euthanized by cervical dislocation and subsequently decapitated to confirm proper spinal cord severing. Gonads were removed from the body cavity, testes by forceps and ovaries by cutting along the dorsal connective tissue in the body cavity using small scissors. Gonads were kept in **holding media** [Dulbecco's Phosphate Buffered Saline Solution with: 20% Fetal Bovine Serum (FBS)] for a maximum of 4 hrs. Past this point it is unclear if gonads are still viable after cryopreservation, while being stored on ice (Liu et al., 2010). Gonads were cleaned of connective tissue and external blood vessels. Gonads were left whole before being mounted on to an acupuncture needle as previously described (Liu et al., 2012c; Liu et al., 2012b). Three CPA's were used: ethylene glycol, DMSO (permeable) and sucrose (non-permeable). The exposure time to these cryoprotectants was varied based on the premise that day old turkey gonads are larger than week old quail gonads; and so might require a longer time in solutions to allow for an adequate amount of CPA's to penetrate the gonadal tissue. The

gonads were secured to needles, and were placed in equilibrium solution [Holding media with: 7.5% Ethylene Glycol, 7.5% DMSO] at RT for 10, 15 or 20 mins depending on the treatment group, now referred to as 10ES, 15ES and 20ES. The gonads were then moved into a vitrification solution [Holding media with: 15% Ethylene Glycol, 15% DMSO and 1M Sucrose] at RT for 2 or 3 mins, once again depending on the treatment group now referred to as 2VS and 3VS. Needles with gonads still secured were removed from the vitrification solution, and were blotted on a kimwipe to remove excess solution, and then submerged directly into LN_2. They were then placed in vials and capped. The vials were stored in LN_2 for a minimal 24 hours before being warmed.

4.3.2.2 Post-vitrification warming

Vitrified gonads were placed directly into holding media with 1M Sucrose for 5 mins at RT, and subsequently transferred to holding media solutions with decreasing concentrations of sucrose (0.5M, 0.25M and 0M) for the same duration and temperature. Gonads were then removed from the needles, placed in nutrient rich media [Dulbecco's Modified Eagle Medium + (1g/L D-glucose, L-glutamine and 110mg/ml sodium pyruvate) with, 20% FBS] for a maximum of 4 hrs, while being kept on ice.

4.3.2.3 Cell viability assay

Trypan Blue assay was done to determine the viability of the gonadal cells before and after the vitrification procedures (Liu et al., 2010). Fresh testicular tissue (control group) was compared to vitrification treatment protocols 10ES/2VS, 15ES/3VS and 20ES/3VS. Fresh testes were analyzed immediately after removal from poults, vitrified tissue was warmed following section 4.3.2.2 and kept in nutrient rich media for 4 hrs before analysis. The tissue was then finely chopped using a scalpel blade and incubated in 0.25% Trypsin-EDTA acid solution with 1.5mg/ml Collagenase. Tubes were incubated at 37^{o}C in a water bath for 1 hour, with gentle pipetting every 10 mins. At the end of incubation, FBS was

added to each tube to make the final concentration of 50% FBS, inhibiting further digestion of the tissue. The samples were then centrifuged at 500 X g for 10 min, and the pellets were re-suspended in 100 µl of nutrient rich media. Subsequently, a portion of the sample was removed and combined with 1/2 a portion of 0.4% Trypan Blue and allowed to stand for 10 mins before loading onto a Hemocytometer. Cells were recorded as non-stained, or stained, and then the total (non-stained + stained) was calculated. All nine fields from the Hemocytometer were counted; the lower limit of cells counted for all fields combined, was set at 100. If this was not reached the sample was discarded and the count was not recorded. The cell viability of a sample was calculated as the non-stained cells divided by the total number of cells. Four samples were counted and recorded for each testis. This was repeated four times for fresh tissue and six times for each vitrification treatment protocol.

4.3.2.4 Histology and light microscopy

Fresh control ovarian and testicular tissues were compared to all possible vitrification treatment protocol combinations (20ES, 15ES, 10ES with 3VS, 2VS) and a negative control (no cryoprotectant) group. For ovarian tissue, three ovaries were analysed for each treatment protocol including the control. For testicular tissue group, five testes were analysed for each treatment protocol with the exception of the no cryoprotectant group, which only had four testes. Fresh gonads were fixed in Bouin's solution (5% Acetic Acid, 9% Formaldehyde and 0.9% Picric Acid); vitrified treatment groups were warmed using the protocol from section 4.3.2.2, and were kept in nutrient rich media for 4 hrs before being placed in Bouin's solution. This was done to mimic the normal time lapse before potential use (transplantation). Tissue was kept in Bouin's solution for 24 hrs at 4^{o}C before being washed and stored in DPBS again at 4^{o}C until processed. Samples were loaded into biopsy embedding cassettes and stored in 70% EtOH until placed into the tissue processer, a MVPI modular vacuum processor (Instrumentation Laboratory, Bedford, MA, United States). A 6-hour cycle was run [3 × 70% ethanol for 30 mins; 80% ethanol for 30 mins; 3 × 100% ethanol for 30 mins; 2 × 100% xylene for 30 mins; 2 × paraffin for 30 mins at 60^{o}C].

After the run cycle, tissue was mounted using paraffin wax and sectioned using a Microm HM 310 microtome (GMI, Ramsey, Minnesota, USA) and 5µm sections were then placed onto slides. Two slides from each gonad were selected, based on the largest appearance of the section, deemed to be the centre of the gonad. Sections were stained using a H&E (Hematoxylin & Eosin) protocol [xylene (1) for 2 min; xylene (2) for 2 min; absolute alcohol (AA) 1-3 for 2 mins each; 95% ethanol for 2 min; 70% ethanol for 2 min; running tap water (RTW) for a few seconds; distilled water (DW) for a few seconds; Hematoxylin for 5 mins; RTW for a few seconds; one dip in acid alcohol; RTW for 10 mins; DW for one dip; Eosin for 3 min; RTW for two dips; one dip in 70% ethanol, 95% ethanol, AA (3), AA (2); AA (1) for 1 min; xylene (2) for 2 min; xylene (3) store until ready to mount]. After being cover slipped and allowed to dry overnight they were then analyzed using a conventional light microscope (Axioskop 40, Carl Zeiss Microscopy, LLC, United States) at x1000 magnification. Images of sections were captured using a microscopy camera (Infinity 3, Lumenera Corporation, ON, Canada).

The morphological alterations grading used here was adapted from the Milazzo group's scheme (Milazzo et al., 2008; Milazzo et al., 2010). For our study, this grading scheme was directly applied to testicular tissue and was modified for the ovarian tissue. Other groups have also used this grading scheme successfully to evaluate morphology from previously vitrified tissue (Travers et al., 2011; Yildiz et al., 2013). This evaluation technique looks at intracellular compartments and structures such as nuclei and nucleoli, extracellular connections and difference in cell types. All of these can be affected by improper cryopreservation technique. For testicular tissue the peripheral and centre seminiferous cords were graded separately, along the lines of a hot spot methodology, which focuses on the least and most affected areas. Only the cortex of the ovary was graded due to the presence of primary oocytes in this area of the ovary.

4.3.2.5 Testicular tissue grading scheme

Seminiferous cords were segregated into peripheral or centre cord groups, and five cords in each area were graded from each testis section. Cords were graded on five morphological changes: Sertoli cell distinction from spermatogonia, observation of the nucleoli, nuclei condensation, intratubular cell detachment from the basement membrane and gap formation and/or shrinkage (Milazzo et al., 2008; Milazzo et al., 2010). The scoring system was as follows: (i) Sertoli cell distinction from spermatogonia: easy 0, difficult 1 and impossible 2 (ii) observation of the nucleoli: easy 0 (observed in more than 40% of the cells), indistinguishable 1 (observed in less than 40% of the cells, in the case of pyknotic and condensed nuclei) (iii) nuclei condensation: absent 0, present in <40% 1 and present in >40% 2 (again where pyknotic nuclei are very common) (iv) intratubular cell detachment from the basement membrane: absent 0, partial 2 and total 3 (v) intratubular cell gap formation and/or shrinkage: absent 0, slight 1 and obvious 2. Each seminiferous cord received a score between 0 and 10 for morphological alterations. The global score for each testis peripheral and centre cords were the average of the 10 seminiferous cord scores from each area (five areas per section, two sections for each testis). For each vitrification protocol, the value is the average of the global scores from these testes. A score of 1 or less was desired (Milazzo et al., 2008; Milazzo et al., 2010).

4.3.2.6 Ovarian tissue grading scheme

Ovarian cortex area (defined as the field of vision for the observer) was evaluated because of the presence of primordial germ cells. Five areas were graded from each ovary section. Cortex areas were graded on five morphological alterations based on a modified grading system of Milazzo's group (Milazzo et al., 2008; Milazzo et al., 2010). Primordial germ cell distinction from immature granulosa cells, observation of the nucleoli, nuclei condensation, peripheral breakdown of cortex cells and gap formation and/or shrinkage. The scoring system was as follows: (i) primary oocyte distinction from immature granulosa

cells: easy 0, difficult 1 and impossible 2 (ii) observation of the nucleoli: easy 0 (observed in more than 40% of the cells), indistinguishable 1 (observed in less than 40% of the cells, in the case of pyknotic and condensed nuclei) (iii) nuclei condensation: absent 0, present in <40% 1 and present in >40% 2 (again where pyknotic nuclei are very common) (iv) peripheral breakdown of cortex cells: absent 0, minor 1 (with 25% or less of the peripheral cortex appearing disturbed) and major 2 (with 25% or more of the peripheral cortex disturbed) (v) cortex cells gap formation and/or shrinkage: absent 0, slight 2 and obvious 3. Each cortex area received a score between 0 and 10, for morphological alterations. The global score for each ovary was the average of the 10 cortex area scores (five per section, two sections for each ovary). For each vitrification protocol, the value is the average of the global scores from these ovaries. As for the testes grading scheme, a score of 1 or less was desired.

4.3.2.7 Statistical analysis

For the testicular cell viability, the average (Mean ± SD) for each treatment (Control, 10ES/2VS, 15ES/3VS and 20ES/3VS) was analyzed by an ANOVA test to determine if any of the groups were significantly ($P<0.05$) different. When significance was shown, a further Post-Hoc (Tukey) test was run to determine which groups were different to each other and p-values were estimated. For global score analysis of morphological alterations, the average (Mean ± SD) was used for both ovarian and testicular tissue values. These averages were compared using a Mann-Whitney *U*-test, due to the fact that the data were non-parametric. Significance was set at $P<0.05$. For ovarian global scores all treatment groups were compared to the control group. For testicular tissue, global scores (centre seminiferous cords) were compared from all treatments to the positive control (0ES/0VS), after which all treatment groups were compared amongst each other. The global scores from peripheral seminiferous cords were not analysed, due to the apparent sharp drop in global score averages and that they all fell below or were close to the acceptable threshold; they were still shown graphically, to demonstrate the extreme difference in values to

the centre seminiferous cords. All data were analyzed using SPSS (IBM SPSS Statistics, Version 21, Armonk, NY).

4.4 Results

4.4.1 Vitrification of day old turkey poult gonads and subsequent analysis of viability post-warming

The testicular cell viability of control (fresh) and vitrified treatment groups (10ES/2VS, 15ES/3VS, 20ES/3VS) were respectively 88.3 ± 1.8%, 63.2 ± 1.5%, 75.7 ± 1.5% and 76.3 ± 1.5% (Fig. 4.1). The fresh tissue cell viability was higher ($P<0.001$) than the three treatment groups. Treatment group 10ES/2VS had the lowest cell viability. Cell viability averages for 15ES/3VS and 20ES/3VS treatment groups were no different ($P=0.988$) but were higher ($P<0.001$) compared to 10ES/2VS treatment group. Cell viability for treatment group's 15ES/3VS and 20ES/3VS (Fig. 4.1) were in proximity to previous published research (*dotted line*). This level of viability has been shown to produce viable quail ovarian tissue after vitrification (Liu et al., 2010).

For testicular tissue (peripheral and centre seminiferous cords) morphological alterations were evaluated from all vitrification protocols and the positive control (0ES/0VS). Examples of seminiferous cords from various (fresh, 15ES/3VS, 10ES/2VS, 0ES/0VS) protocols are shown in Fig 4.2. Panels **(A)** and **(B)** have normal testicular morphology. Protocol 10ES/2VS **(C)** has morphological damage in the centre of the testis identified by a darker appearance. The 0ES/0VS testis **(D)** has a uniformed appearance although cryopreservation damage is present. For fresh centre seminiferous cords **(A')**, Sertoli cells have large spherical nuclei and cytoplasm, which fills the future lumen of the cord. Type A spermatogonia are present with smaller spherical nuclei close to the basal lamina [score = 0]. For vitrification protocol 15ES/3VS, **(B')** centre cord Sertoli cells and spermatogonia can not be distinguished apart, pyknotic nuclei are rare but present, the basement membrane is attached to intratubular cells but shrinkage and gap

formation is slightly present [score = 3]. For vitrification protocol 10ES/2VS **(C')** centre cord Sertoli cells and spermatogonia cannot be distinguished apart, condensation of nuclei are common with partial detachment of basement membrane and gap formation with shrinkage [score = 9]. For vitrification protocol 0ES/0VS **(D')** the intratubular cells cannot be distinguish apart in the centre cords. Total nuclei condensation with full detachment of basement membrane is present [score = 10]. For fresh peripheral seminiferous cords **(A'')** the score [0] matches that for the centre cords. For vitrification protocol 15ES/3VS, peripheral cords **(B'')** have normal morphology [score = 0]. For vitrification protocol 10ES/2VS peripheral cords **(C'')** have slight gap formation with distinction between intratubular cells difficult [score = 2]. For vitrification protocol 0ES/0VS peripheral cords **(D'')** description and score [10] are the same as centre cords for this protocol.

Morphological alteration global scores for peripheral seminiferous cords from all vitrification treatment groups were lower ($P<0.05$) than the positive (0ES, 0VS) control (Fig. 4.3). All vitrification treatment groups' were under the acceptable threshold (score ≤ 1), except for protocol 20ES/2VS. Due to this fact, peripheral seminiferous cord morphology was not statistically evaluated further. For centre seminiferous cords, treatment group 15ES/2VS had the only global score, which was lower than 10ES/2VS ($P<0.05$) and 20ES/2VS ($P<0.01$) treatment groups (Fig. 4.3). Treatment group 20ES/2VS global score was higher ($P<0.05$) than treatment protocols 20ES/3VS and 15ES/3VS. Global scores from treatment protocols 15ES/3VS and 15ES/2VS were no different ($P=0.119$). None of the centre seminiferous cord morphological scores were under the threshold (score ≤ 1) for acceptable damage.

Ovarian cortex tissue morphological alterations were evaluated from all vitrification protocols and a negative control (fresh tissue). Examples of the ovarian cortex evaluated from each protocol (fresh, 15ES/3VS, 10ES/2VS, 0ES/0VS) are shown (Fig 4.4). For fresh ovarian cortex **(A, A')**, primary oocytes have a large cytoplasm with no distinct nuclei outline. They are easily differentiated from *immature* granulosa cells, which have distinct spherical nuclei, within a small cytoplasm. There was no gap

formation, shrinkage or peripheral break down [score = 0]. For vitrification protocol 15ES/3VS **(B, B')** cellular differentiation was possible with minor nuclei condensation and pyknotic appearance in some of the cells. Slight gap formation and shrinkage was present with no overall peripheral breakdown [score = 3]. For vitrification protocol 10ES/2VS **(C, C')** differentiation between primary oocytes and *immature* granulosa cells, was not possible. Minor nuclei condensation was present with gap formation, shrinkage and minor peripheral breakdown [score = 6]. For the positive control, 0ES/0VS **(D, D')** primary oocytes and *immature* granulosa cells could not be differentiated. Pyknotic nuclei were apparent across the entire sample, with major peripheral break down, and obvious gap formation and shrinkage [score = 10].

For ovarian cortex, vitrification treatment group's 20ES/3VS, 10ES/2VS, 0ES/0VS ($P<0.01$), and 10ES/3VS ($P<0.05$) had higher global score's than the fresh control (Fig. 4.5). Vitrification treatment group's 20ES/2VS ($P=0.053$), 15ES/3VS ($P=0.747$) and 15ES/2VS ($P=0.192$) had global score's which were not higher than the fresh control. The only vitrification treatment group that had an average value under the allowable threshold (score ≤ 1) was 15ES/3VS.

4.5 Figures and Tables

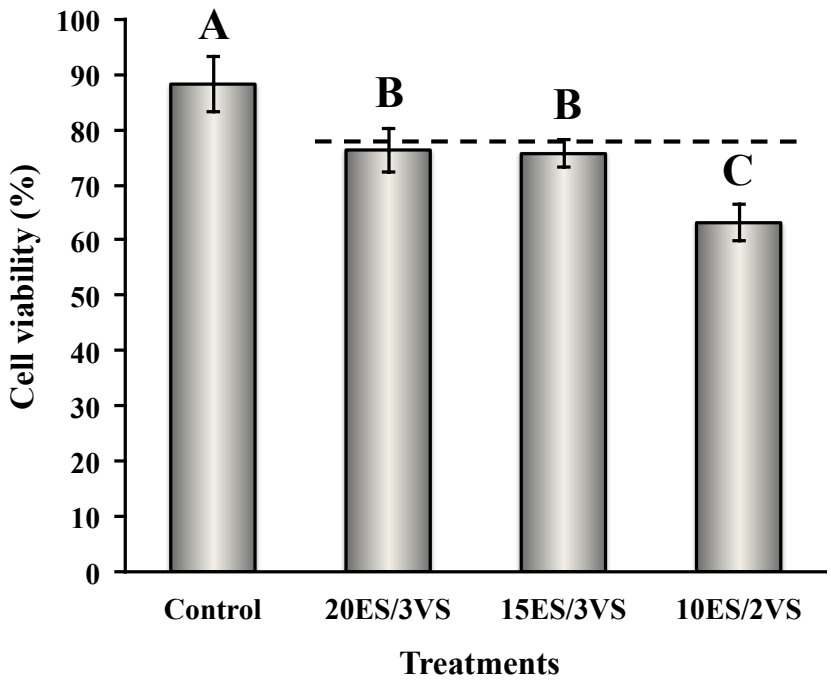

Figure 4.1 Proportion of testicular cells with intact plasma membranes (Mean ± SD), from day old Large White turkey fresh (control) or previously vitrified testes. Treatments are represented by time (min) spent in equilibrium solution (ES) followed by vitrification solution (VS). For control (n=4) and for all other treatments (n=6); *dotted line* represents the cell viability value reported in the literature for successful cryopreservation of quail ovarian tissue. Values with different characters (A-C) are significant ($P<0.001$).

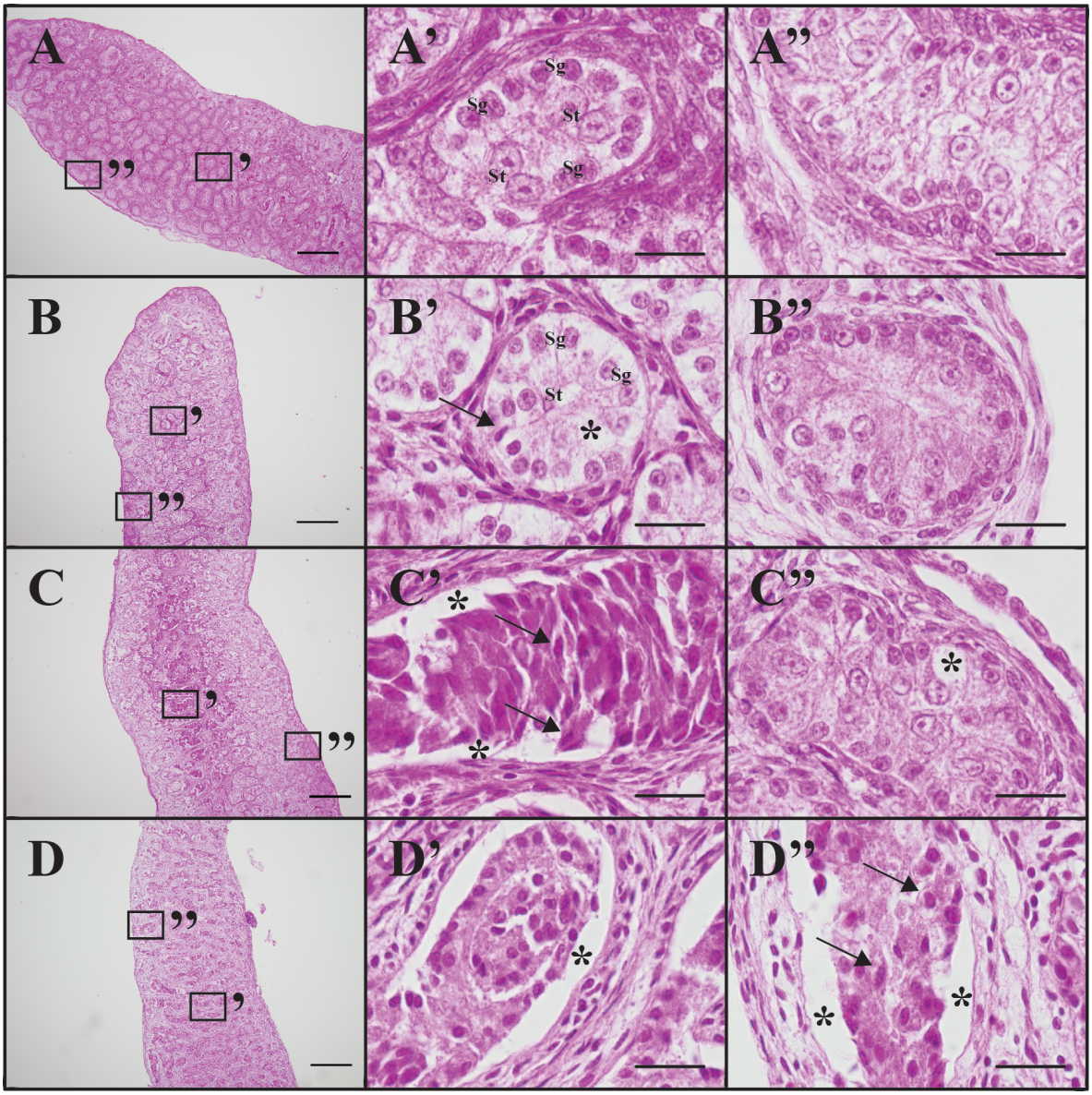

Figure 4.2 An example of varying degrees of morphological alterations in testes and seminiferous cords (peripheral and centre) from day old turkeys, after cryopreservation with varying times in equilibrium and vitrification solution. **(A-A'')** Fresh; **(B-B'')** 15ES/3VS; **(C-C'')** 10ES/2VS; **(D-D'')** 0ES/0VS, panels with **(')** show centre cords, whereas **('')** are peripheral seminiferous cords, from their respective panel on the left. **(A)** Uniformed structure of seminiferous cords across testis; **(A')** spermatogonia **(Sg)**, small nuclei in close proximity to the basal lamina, sertoli cells **(St)** large nuclei with cytoplasm filling the future lumen; **(A'')** peripheral cord matches centre cord morphology. **(B)** Slightly altered cords in the centre; **(B')** spermatogonia **(Sg)** and sertoli cells **(St)** are harder to differentiated apart, pyknotic nuclei **(arrow)** are rare, with a small amount of gap formation **(*)** and shrinkage; **(B'')** peripheral cord shows normal morphology. **(C)** Altered seminiferous cords in centre of testis; **(C')** pyknotic and condensed nuclei **(arrows)** are common throughout, partial detachment **(*)** of the intratubular cells from the basal lamina; **(C'')** gap formation **(*)** and shrinkage. **(D)** Altered cords across the entire testis; **(D')** total detachment **(*)** of the intratubular cells; **(D'')** partial detachment **(*)** of the intratubular cells in peripheral cords, with pyknotic and condensed nuclei **(arrows)** also present. Scale bars: **(A-D)** 200 μm; **(A-D')** and **(A-D'')** 15 μm.

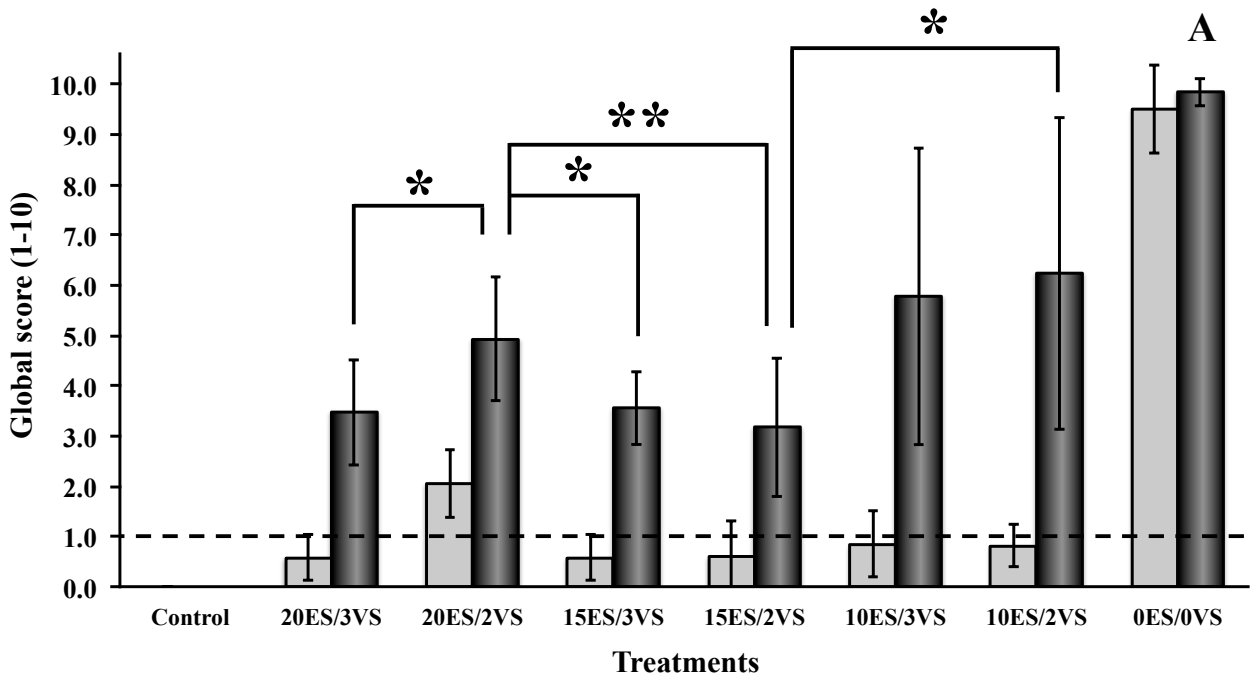

Figure 4.3 Global scores grading morphological alteration from seminiferous cords located on the peripheral (**light grey**) or centre (**dark grey**) of testes, from day old Large White turkeys vitrified with various treatments, or fresh (control). Treatments are represented by time (min) spent in equilibrium solution (ES) followed by vitrification solution (VS). Global scores (mean ± SD) are from 50 seminiferous cords located on the periphery or centre of testes (n=5), testes were randomly assigned into treatment groups. Global scores ≤ 1 (*Dotted line, score=1*) are the target, as this threshold has been defined by previous groups to be viable. Peripheral global scores were not analyzed further; they were under or close to the defined threshold. For centre global scores all treatment groups were significantly ($P<0.05$) lower than the 0ES/0VS, no cryopreservation group denoted by the letter A. The remaining treatment's centre global scores were compared against each other, except for the control group; **$P<0.01$, *$P<0.05$.

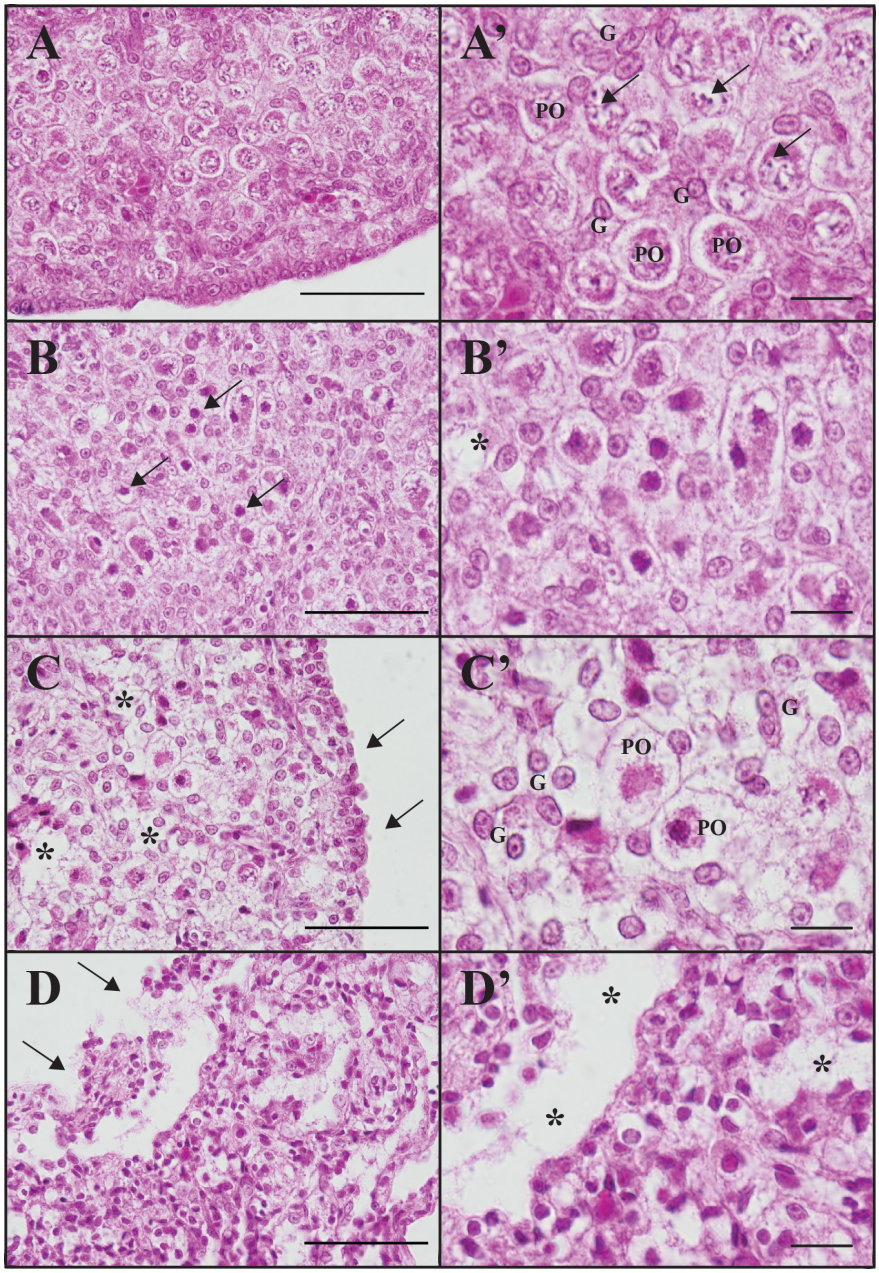

Figure 4.4 An example of varying degrees of morphological alterations in the ovarian cortex from day old turkeys, after cryopreservation with varying times in equilibrium and vitrification solution. **(A, A')** Fresh; **(B, B')** 15ES/3VS; **(C, C')** 10ES/2VS; **(D, D')** 0ES/0VS, panels with **(')** show an enlarged imagine from its' respective left panel. **(A)** The peripheral edge of the ovaries cortex is sharp and clear; **(A')** primary oocyte **(PO)** have a large nuclei with no distinct outline, the nucleolus's **(arrow)** are easily seen, *immature* granulosa cells **(G)** have a spherical nuclei and are beginning to invade and surround the primary oocytes. **(B)** Pyknotic nuclei **(arrow)** are rare; **(B')** primary oocyte and *immature* granulosa cell differentiation remains easy to determine. **(C)** The peripheral edge of the ovaries cortex is becoming less defined **(arrow)** along with gap formation **(*)** and shrinkage; **(C')** primary oocyte **(PO)** and *immature* granulosa cell **(G)** differentiation is difficult. **(D)** The peripheral edge of the ovaries cortex is completely disrupted **(arrow)** with large holes protruding into the cortex; **(D')** gap formation and shrinkage **(*)** is present throughout the whole cortex. Scale bars: **(A-D)** 50 µm; **(A'-D')** 10 µm.

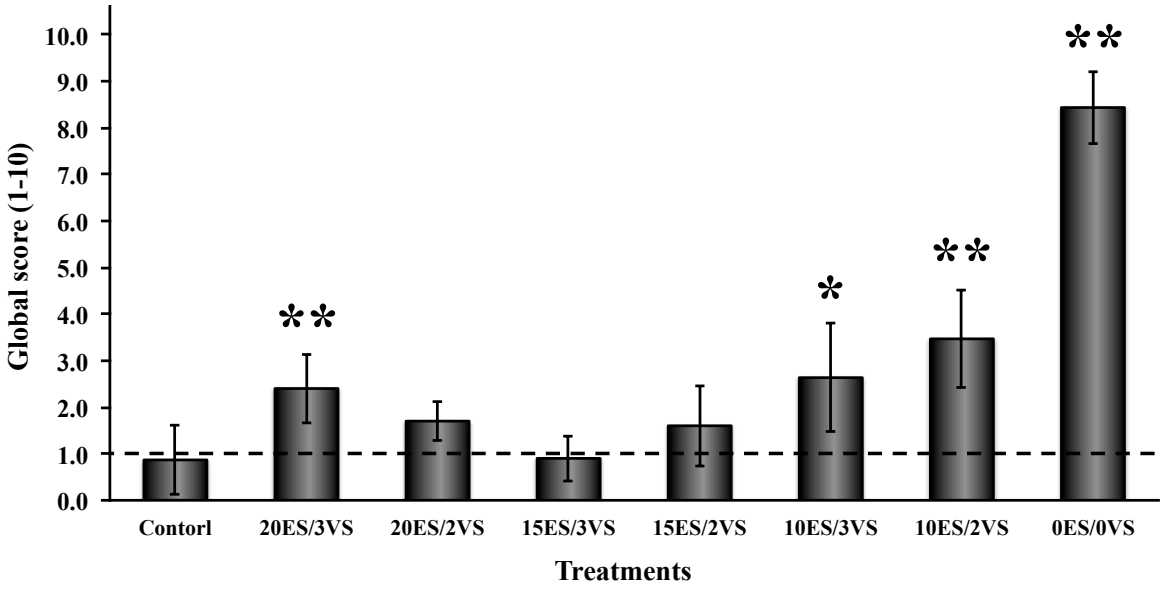

Figure 4.5 Global scores (Mean ± SD) for morphological alterations in the cortex from day old Large White turkey ovaries previously vitrified with various treatment conditions. Treatments are represented by time spent in equilibrium solution (ES) followed by vitrification solution (VS). Global scores are means of global scores from 30 observations from three different samples cryopreserved with the same protocol; ovaries were randomly assigned into treatment groups. Global scores ≤ 1 (*Dotted line, score=1*) are the target, as this threshold has been defined by previous groups to be viable; *P<0.05; **P<0.01 versus fresh control.

4.6 Discussion

The goal of this study was to determine if day old turkey gonads after vitrification and warming were viable using standard or modified protocols. This study demonstrated that for cryopreserved ovarian tissue left whole from day old turkeys, vitrification protocol 15ES/3VS produced viable tissue post-warming. On the other hand for testicular tissue left whole, none of the protocols used demonstrated adequate cryopreservation. Although an improvement over the standard protocol was seen for the modified protocol 15ES/3VS. Here we have shown that a longer time in cryoprotectant solutions is required, than the standard protocol calls for, for vitrification of day old whole turkey gonads.

For day old turkey ovarian tissue left whole for cryopreservation, protocol 15ES/3VS is recommended due to its low cortex morphological scores (Fig. 4.5). The new protocol 15ES/3VS had a morphological score no higher ($P=0.747$) than fresh tissue and was also under the acceptable threshold [score ≤ 1] (Milazzo et al., 2008; Milazzo et al., 2010). While two of the other modified protocols (20ES/2VS and 15ES/2VS) were not statistically different compared to fresh tissue, their averages were above the allowable threshold (Fig. 4.5). It should also be noted that the standard protocol (10ES/2VS) had a much higher ($P<0.01$) level of morphological damage than the fresh ovarian tissue. For testicular tissue left whole, none of the cryopreservation protocols matched the success of the ovarian tissue but protocol 15ES/3VS was shown to be better than the standard protocol due to the fact it was able to improve the cell viability score into the targeted range (Fig 4.1), unlike the standard protocol (10ES/2VS), which showed subpar cell viability. In fact both of the newly tested vitrification protocols (20ES/3VS and 15ES/3VS) tested for cell viability had values within the range for quail ovarian tissue $77.1 \pm 1.9\%$ (Liu et al., 2010), which has already been shown to lead to viable tissue. Based solely on this data it is unclear, which new protocol is more favourable. By evaluating the global scores for morphological damage of seminiferous cords located in the centre it was shown that not one of the averages were below the defined threshold [score ≤ 1] (Milazzo et al., 2008; Milazzo et al., 2010). Even though none of the protocols were ideal the

lowest global scores came from treatment groups' 20S/3VS, 15ES/3VS and 15ES/ 2VS. The fact that 15ES/2VS had a lower global score ($P<0.05$) than the 10ES/2VS showed that increasing the time spent in CPA's had a positive effect on morphological alteration scores. Though the effects from CPA's was not able to lower the global scores below the acceptable threshold, this would suggest further development of the protocol to optimize the preservation of turkey gonads.

The assessment of poultry gonad viability after cryopreservation procedures focused at the start, on the production of donor-derived offspring after transplantation (Song and Silversides, 2007c). In later work this was accompanied by cell viability and follicle grading schemes, although basic (Liu et al., 2010). In mammals these morphological grading schemes are better developed and have focused on a number of cellular parameters (Milazzo et al., 2008). The morphological grading scheme used here has been well tested and has been shown to lead to viable tissue after cryopreservation (Milazzo et al., 2010; Travers et al., 2011; Yildiz et al., 2013). In the most recent work on testing vitrification protocols of day old avian gonads, the transplantation method of evaluating has been dropped and instead tissue was analyzed after being grafted to the CAM (Liu et al., 2012c; Liu et al., 2012b). With respect domestic turkey, the time for birds to mature is around 30-35 weeks before the production of young. This is a long waiting period to test vitrification protocols through the transplantation method. This is why our method of evaluating gonads was most suitable to determine cell and tissue viability in a timely and more cost effective means.

To date the protocol used to first vitrify quail gonads (Liu et al., 2010) has not changed (Liu et al., 2013a). Here this standard protocol used on quail gonads was varied when working on turkey gonads with respect to the time spent in either equilibrium and/or vitrification solutions. These longer periods in cryoprotecting solutions were used to counteract the larger size of the day old turkey gonad. It should be noted that not all CPA's were increased to the same extent. The greatest time increase (5-10 mins), was seen in the equilibrium solution step, which lacked sucrose. If furthering optimization of testes is required, increasing the time spent in non-permeable cryoprotectants is one potential option. The increase

of sucrose in vitrification protocols has not be shown to be toxic to cells (Kuleshova et al., 1999). Increasing the exposure time of all the CPA's had a beneficial effect until the later time points. As ovarian (20ES/3VS, Fig. 4.5) and testicular (20ES/2VS, Fig. 4.3) global scores showed worse scores than protocols with less exposure to CPA's. These observations suggest that these protocols are starting to reach a toxic level of CPA's by overexposure to DMSO and/or ethylene glycol. The standard protocol aforementioned has been shown to work on testes left whole. The difference between this work and previous work is that the tunica albuginea and tunica vaginalis that was torn open for quail testes (Liu et al., 2012c; Liu et al., 2013a), this was not done here, instead the gonads were left whole, to limit the amount of testicular damage. Another possible way of increasing the exposure of the tissue to CPA's would be to tear open both of these membranes as reported by the quail work.

This work shows the need for better optimization of vitrification protocols for gonad cryopreservation of new poultry species. We also show that the standard vitrification protocol used on quail ovarian tissue is not acceptable for turkey gonads, left whole. These new conditions demonstrated here for the first time were able to lead to higher viability for testicular tissue, although not at an acceptable level yet. For ovarian tissue the 15ES/3ES protocol demonstrated successful cryopreservation. This technique will allow for the successful cryostorage of turkey gonads, and will reduce the cost for industries that wish to store large numbers of turkey genetic resources. This research also paves the way to successfully produce donor-derived offspring after these gonads are transplanted into recipient birds.

CHAPTER 5: THE OVARIECTOMY OR TESTECTOMY OF TURKEY POULTS AND SUBSEQUENT TRANSPLANTATION OF DONOR GONADS, FRESH AND PREVIOUSLY VITRIFIED

5.1 Abstract

Gonad transplantation between breeds or species allows for the production of donor-derived offspring by the recipient, which in turn ensures the entire genome of the donor is passed on. This procedure paired with gonad vitrification protocols, which allows for gonad tissue to be stored at lower temperatures immortalizing the tissue, will ensure that turkey lines can be cryopreserved and revived. The objective of the current study was to determine the most appropriate age group and conditions of poults before surgery; and whether previously vitrified gonads could be transplanted and develop normally. In Trial 1, vitrified gonads from day old hybrid turkey (HT) were transplanted orthotopicly into large white turkey (LWT) recipients at 1, 3, 4 and 7 days of age, and food was withheld until after surgery. The body weight, duration of surgery, and the survivability at each time point were analysed. Recipient poults were sacrificed at 8 weeks post-surgery, grafted gonads were retried if present. For Trial 2, the previous set of parameters was repeated, but with LWT at 1, 3, 4 and 5 days of age, and grafts were placed orthotopicly underneath the left abdominal air sac. The poults were supplied, food ad libitum before surgery. In Trial 3, LWT fresh or vitrified gonads were transplanted into day old BT. Transplants were orthotopicly placed under the left abdominal air sac, the peritoneum, or attached using surgical glue. The aforementioned output analyses were conducted, and birds were euthanized at various stages, from 24 hours to 18 weeks post-surgery. The body weight and duration of surgery did not vary to any considerable degree throughout the three Trials. The survivability rate increased between Trial 1 (44%) and Trial 2 (65%) poults, and between male (96%) and female (68%) Trial 3 poults. Only previously vitrified grafts from Trial 3 were recovered from birds euthanized on or before 4 weeks of age. Histological analysis of testicular grafts showed that over time the morphology of transplants was lost when compared to host tissue. Based on

these results, day old poults are the most suitable for gonad transplantation, with respect to survivability. Fresh and vitrified gonads from LWT did not grow or mature normally in BT.

5.2 Introduction

Cryopreservation of avian gonads enables the whole genome to be stored, which can be utilized at a future date. This storage of genetic material allows for the protection of avian populations, breeds, species and the genetic diversity, which is present in these groups. Gonads can be harvested and cryopreserved from a wide range of age groups (immature day old chicks to adults). Even though immature gonads can be cryopreserved, these gonads and germplasm still need to mature to produce gametes. This maturation could occur in vivo or in vitro. The in vivo (allotransplantation or xenotransplantation) method has been well documented allowing for proper gonad maturation (Song and Silversides, 2007a; 2008a; Song et al., 2012). Transplantation, involves the selection of an appropriate recipient (breed, species), surgery age, surgical procedure, and immunosuppressant used.

Gonad transplantation in avian species was first shown to be possible and practical in 2006 with the successful transfer of chicken ovaries between two different breeds (Song and Silversides, 2006). Ovaries were evaluated after two weeks post-surgery. This was shortly followed up by the production of donor-derived offspring, from ovarian grafts (Song and Silversides, 2007a). Following these results, testicular transplants between chicken breeds was successful in being able to produce gametes (immature spermatids), and via intramagnal insemination donor-derived offspring were produced (Song and Silversides, 2007b). For Japanese quail, gonadal transplantation has been demonstrated in both genders (Song and Silversides, 2008a; Liu et al., 2013a). For Muscovy duck, only transplantation of the ovary has been successful (Song et al., 2012). In all cases these grafts were able to produce donor-derived offspring from these species.

An appropriate transplant recipient is one in which the physiological characteristics of the reproductive tract and endocrine signals are compatible to the donors gonad. This will allow the gonad to mature temporarily within the recipient, and with respect to ovaries, in the correct position to the reproductive tract (close to the opening of the infundibulum). Recent studies suggest that not all poultry breeds are compatible to receive a transplant (Liptoi et al., 2013). When selecting the optimal recipient, inevitably it has to be determined whether the future offspring are in fact donor-derived or originate from the host's remaining primordial germ cells. This can be done by either genotyping or phenotypic observations, with the latter being more cost effective, and producing offspring. An appropriate age group for surgery is one in which the chick can survive at the earliest age, as a means to by-pass the response of the adaptive immune system of the host. At 30 and 45 days of age chickens are deemed to be too old as they have a well-established immune system capable of fully rejecting transplants (Grossman and Siegel, 1966). Day old chicks and ducklings have been shown to be able to survive this type of surgery (Song and Silversides, 2006; 2007b; Song et al., 2012). For quail, it appears that 7 day-old chicks are more appropriate due to their size and fragile nature at younger ages (Song and Silversides, 2008a). When transplanting ovaries, the surgical position of the graft is required to be orthotopic, for anatomical reasons. For testes the position of the graft is not limited to an orthotopic position. In fact testes transplanted orthotopicly, abdominally and onto the back have all produced mature testes able to produce immature spermatozoon (Song and Silversides, 2007b). In all gonad transplant cases, the mechanical immobilization of the grafts appears unnecessary (Song and Silversides, 2006).

The goal of this study was to show that gonad transplantation in turkeys is a viable means of preserving turkey lines, which has already been proven successful in chickens, quails and ducks (Song and Silversides, 2007a; 2008a; Song et al., 2012). This coupled with cryopreservation protocols will allow for the storage of testes and ovaries and reconstitution of the entire turkey genome. Here we have documented the best age for transplantation to occur with respect to survivability of poults, and the best conditions and parameters to have the poults in pre-surgery and throughout the surgery procedure, while

also testing surgical procedures. It was determined that, one-day old poults were most suited to transplantation, although graft development and morphological analysis showed inadequate progression towards maturing of the testes.

5.3 Materials and Methods

5.3.1 Chemicals and Reagents

Isoflurane (99.9%) was purchased from Fresenius Kabi Animal Health (Richmond Hill, ON, Canada), Xylazine, *Rompun* was purchased from Bayer Inc. (Toronto, ON, Canada). Ceftiofur, *Excenel* was purchased from Zoetis Canada Inc. (Kirkland, QB, Canada). Meloxicam, *Metacam* was purchased from Boehringer Ingelheim. (Burlington, ON, Canada). Mycophenolate mofetil, *CellCept* was purchased from Roche. (Mississauga, ON, Canada). Mycophenolate mofetil, *generic* was purchased from Mylan (Canonsburg, PA, USA). All other chemicals and reagents were purchased from Sigma-Aldrich (Oakville, ON, Canada), unless otherwise stated.

5.3.2 Transplantation of hybrid turkey gonads previously vitrified, into large white turkey poults

5.3.2.1 Pre-Surgery

As turkey genetic resources are limited, the most suitable commercial choice for recipient breed was the bronze turkey **(BT)**, which is phenotypically dominant over the large white turkey **(LWT)** with respect to plumage coloration (Robertson et al., 1943). As BT eggs were not seasonally available, the LWT was used as the host with the hybrid turkey **(HT)** supplying the donor vitrified tissue. Fertilized eggs from LWT were set in a 1502 sportsman (GQF) incubator and were subsequently transferred to a 1550 hatcher (GQF) 3-4 days before hatch date. Additional poults were also ordered these poults were flown to the university after hatching and arrived on the next day. All birds were provided by Hybrid Turkeys a (Hendrix's Genetics Company, Kitchener, Ontario, Canada). Poults that hatched on campus or were delivered were given 24 hours to acclimatize in the hatcher before undergoing surgery. Poults were given water but no food before surgery, relying on the yolk sac for nutrients.

5.3.2.2 Surgery procedure

Large white turkeys at 1, 3, 4 and 7 days of age were used as recipients for transplantation. Poults were first placed in a rodent anaesthesia box and anaesthetized using Isoflurane through a vaporizer, at a starting level of 4-5%, with an oxygen flow of 0.5 L/min. When poults appeared sedated, they were removed from the box and a rodent mask covering only their beak was secured in place. Isoflurane was reduced to 3% during the transition. The left side of the abdomen was plucked/shaved and cleaned using germi-stat gel 4% Chlorhexidine Gluconate (Germiphene Corporation, Branford, ON, Canada). The poult was then moved to the surgery table, onto a heated water pad (42^{o}C). In addition, a heating lamp was also used to regulate body temperature. An electronic rectal thermometer was used to monitor core body temperature; breathing rate (breaths per min) was also recorded every five minutes. The Isoflurane was adjusted in response to breathing and vital signs, keeping the poult in the plain of anaesthesia while the surgery was being performed. First, an incision was made 0.5 cm below and parallel to the left side of the rib cage, through all the epithelia layers (approximately 2-3 cm in length). This incision was used to make a further incision through the abdomen muscle wall, again below the left rib cage, exposing the liver, gizzard and yolk sack. The peritoneal membrane was torn allowing for access to the yolk sac. The yolk sac was carefully removed ensuring the fragile sac in 1 and 3 day-old poults was not torn (it was not removed in 4 and 7 day old poults). The yolk sac connecting stalk was sutured to prevent bleeding before being cut and removed. The intestine and gizzard were gently displaced and pushed lower into the abdomen allowing for access to the gonads. A Novaflex, fiber light (World Precision Instruments, Sarasota, FL, USA) was used to illuminate the abdominal cavity. The ovary was located in the extreme cranial part of the left abdominal cavity, and the testes were slightly lower in the cranial part of the abdominal cavity and on the right and left sides (Fig 2.1). Gonads were carefully removed by crushing, and then slowly tearing away in pieces, until the whole or a majority (>90%) of the gonad was removed. Vitrified-warmed gonadal tissue from HT was transplanted orthotopicly and was not secured (tissue was vitrified using protocol 10ES/2VS as per section 4.3.2.1, and warmed using the protocol found in section

4.3.2.2) and was kept in holding media [DPBS, with 20% FBS] for up to 4hrs before being transplanted. The gizzard and intestines were then gently pushed back into position without disturbing the position of the graft. The abdominal muscle wall and epithelia skin layer were then suture closed with monocryl multipass needles (4-0, PC-5, 19mm 3/8c) (Johnson and Johnson, Markham, ON, Canada) together at the same time due to the fragile nature of the tissue. Poults, which did not recover within 15-30 mins of surgery were euthanized by cervical dislocation.

5.3.2.3 Post-surgery care and donor tissue recovery

Immediately upon recovery, poults were given an oral dose of Meloxicam (0.5mg/kg of bird) for analgesic purposes and an IM dose of Ceftiofur (0.2mg/bird) as an antibiotic. Poults were identified by a number code, a letter after this number signified the poult died before recovery. Poults were also given an oral dose of Mycophenolate mofetil *generic* (100mg/kg of bird) once a day for two weeks and then once a week for an additional 6 weeks, allowing for 8 weeks of immunosuppression in total. Turkeys were then subsequently euthanized at 8 weeks post-surgery, Isoflurane at 5% was used to anaesthetize the birds and then cervical dislocation was performed. Birds were subsequently dissected; body cavities were photographed and potential grafts were removed.

5.3.2.4 Statistical analysis

Body weights at surgery and duration of the procedure were statistically analyzed for each age group. To determine if there were any trends which could predict eventual survivability of poults. The means and standard error of means were calculated for both factors, although individuals not surviving surgery were excluded from the mean for *duration of surgery*, as these individuals would lower the value incorrectly. A one-way ANOVA test was run combined with a post-hoc Tukey test to identify which days were significantly ($P<0.05$) different compared to others. The survival of poults for each age group were

calculated by dividing the individuals who survived by the total operated on. All analyses were conducted using the statistical program SPSS (IBM SPSS Statistics, version 21).

5.3.3 Transplantation of hybrid turkey gonads under the abdominal air sac membrane of large white turkey poults

5.3.3.1 Pre-Surgery

As BT eggs were not seasonally available, the LWT was used as the host with the HT supplying the donor vitrified tissue. Large white turkey fertilized eggs were set in the same way as section (5.3.2.1) with the following changes. Poults, which hatched, were given 24 hours to acclimatize in the hatcher before being moved to the Animal Care Unit. Poults were housed on shavings and were given water and food before surgery.

5.3.3.2 Surgery procedure

The surgery procedure was the same as for section 5.3.2.2 with the following modifications. White turkeys were used as recipients at 1, 3, 4 and 5 days of age. In addition to a fiber light to illuminate the abdominal cavity, the surgeon wore SurgiTel prism loupes with 8x magnification and an LED light (MS Meditech Solutions Inc., Richmond, British Columbia, Canada), which allowed for greater precision and illumination of the abdominal cavity. After ovariectomy or partial to full testectomy in certain poults the left abdominal air sac was cut open starting at the furthest abdominal point, and cut in a curve along the ventral side of the abdomen. This allowed for the abdominal air sac to be folded over the newly transplanted donor gonads, securing them in place. The gizzard and intestine were then gently moved back over the newly torn abdominal air sac, and the abdomen was sutured closed, as previously described.

5.3.3.3 Post-surgery care and donor tissue recovery

The post-surgery care and tissue recovery was the same as section 5.3.2.3.

5.3.3.4 Statistical analysis

The statistical analysis was conducted in the same manner as section 5.3.2.4.

5.3.4 Transplantation of white turkey gonads previously vitrified or fresh, secured in the abdomen of bronze turkey poults using three different techniques

5.3.4.1 Pre-Surgery

Bronze turkey fertilized eggs were set in the same way as section 5.3.2.1 with the following changes. Poults, which were hatched, were given 24 hours to acclimatize in the hatcher before undergoing surgery. No food or water was given to poults before surgery.

5.3.4.2 Surgery procedure

The surgery procedure was the same as for sections 5.3.2.2 & 5.3.3.2 with the following modifications: day old BT were used as recipients; LWT fresh donor tissue was used as a control alongside previously vitrified tissue. The vitrification method was the same as the aforementioned sections. Both graft types were kept in nutrient media (DMEM + 20% FBS) no longer than 4 hours before transplantation. Due to anaesthetic complications, an additional sedative to isoflurane gas was needed; this was an IM injection of xylazine (2mg/kg of bird) and was administered before the gas anaesthesia. After ovariectomy or partial to full testectomy, grafts were secured in three ways: by using the abdominal air sac (section 5.3.2.2),

with surgical glue (3M Vetbond) which was used to adhere the graft in place, or by placing the gonad(s) with extreme precision under the peritoneum membrane situated where the host's original gonad was located. This was accomplished by first tearing the peritoneum at the most posterior position to the host's gonad. The ovary or left testis was then removed through this hole keeping the extremely fragile membrane intact. The right testis was then removed or kept, depending if a partial or full testectomy was being conducted. The ovary or both testes were then positioned under the peritoneum. The gizzard and intestine were then gently moved back into place and the abdomen was sutured closed.

5.3.4.3 Post-surgery care and donor tissue recovery

The post-surgery care and tissue recovery was the same as described in section 5.3.2.3. The change in immune suppression drug brand and end points are as follows. The analgesic Meloxicam (0.5mg/kg of bird) was administered on the day of surgery and subsequently for 3 days after surgery. Poults were given an oral dose of Mycophenolate mofetil (100mg/kg of bird), in the same manner as the previous Trial except generic tablets were replaced with CellCept liquid suspension version. Bronze turkeys were subsequently euthanized at 24 hours, 2-4, 6 days, 2, 4, 6, 8 and 18 weeks of age, and grafts were recovered.

5.3.4.4 Histological and Statistical analysis

If grafts were present in recipients, they were removed and histological analysis was conducted in the same manner as section 4.3.2.4. The statistical analysis was conducted in the same manner as section 5.3.2.4.

5.4 Results

5.4.1 Transplantation of hybrid turkey gonads previously vitrified, into large white turkey poults

The survivability ratios of LWT poults at 1, 3, 4 and 7 days of age were 1/3, 2/4, 2/6 and 3/5 respectively. The combined total of survivability for Trial 1 was 44%. There was a (P=0.012) difference in body weight between 4 and 7 day-old poults, with 4-day old poults having the lower body weight (46.7g ± 1.6g), and 7-day old poults having the higher body weight (58.6g ± 4.0g). The 7-day old weight is much lower (150-160g) than the performance guide states for this age (HHGC, 2015). There was no significant difference between the age groups with respect to duration of surgery (Table 5.1). All poults were euthanized at 8 weeks post-surgery. In LWT 3 and 9, which only underwent ovariectomy, the hosts ovary was absent or severely reduced in size, compared to control birds. In LWT 5, 6, 8, 10 and 11, which received a transplant; ovarian tissue was absent or matched the physiological characteristic of an ovariectomized bird.

5.4.2 Transplantation of hybrid turkey gonads under the abdominal air sac membrane of large white turkey poults

The survivability ratios of LWT poults at 1, 3, 4 and 5 days of age was 5/7, 6/8, 1/4 and 1/2 respectively; the combined total for survivability for Trial 2 was 65%. There was a (P<0.02) difference in body weight between day old poults and the other three age groups. With day old poults having the lowest body weight (51.3g ± 0.9g). There was no significant difference between 1 and 3 day old poults with respect to duration of surgery (Table 5.2). All poults were euthanized 8 weeks post-surgery. In LWT 14, which underwent only a left testectomy, the left testis was absent and the right testis was present. In LWT 15, 19, 23, 25, 26, and 28 that were ovariectomized and received a transplant, ovarian tissue was absent or matched the physiological characteristic of ovariectomized birds from Trial 1. In LWT 16, 18, 22, 24 and

27 that were partially or fully testectomized and received a transplant; testicular tissue was absent in the position of the host's left testis. For LWT poults where the left abdominal air sac covered the gonad after transplantation, the air sac was still open to the body cavity and the membrane was retracted into the very furthest left cranial position of the abdomen, away from the typical position of the ovary or left testis.

5.4.3 Transplantation of white turkey gonads previously vitrified or fresh, secured in the abdomen of bronze turkey poults using three different techniques

The survivability of day old male and female bronze poults was 96% and 68% respectively. There was no significant difference between the sexes with respect to body weight or duration of surgery (Table 5.3 & 5.5). In BT 33, 34 and 68, which were euthanized at 24 hours post-surgery, testicular grafts were located in all individuals. For 33 and 68 their testes were still in the correct position underneath their respective membranes. For BT 39, 46, 36, 67 and 40 which were euthanized between 2 and 6 days post-surgery testicular grafts were found in 4 out of the 5 individuals; and for BT 37 and 55 which were euthanized at 2 and 4 weeks respectively testicular grafts were present in both. Grafts appeared white to creamy with little size difference to when they were transplanted, all testes were located underneath their respective membranes. All the aforementioned birds were part of the vitrified tissue transplantation group. For BT birds euthanized after 6 weeks post-surgery, no testicular grafts were located in the vitrified and fresh tissue groups (Table 5.4).

In Trial 3, three different gonad placement methods were tried based on the observation that grafts did not appear to stay in the same position over a 24-hour period (data not shown). The surgical glue appeared to be the most abrasive and possibly counterproductive (data not shown). However, it does guarantee full attachment. The gonads retrieved after attachment using surgical glue were embedded in the surgical glue and not attached to the recipient (data not shown). Male poults which had the left abdominal air sac torn open to cover the transplants, the air sac, was still open to the body cavity, upon post-surgery gonad

recovery. The membrane was retracted into the left cranial position of the abdomen (data not shown); away from the typical position of the left testis. Transplants placed under the peritoneum membrane stayed in place, with the evidence that upon recovery days later, gonads were still located in the same position (data not shown). The membrane was still attached and was located where the left testis of the host would normally be seated, whether or not a graft was present.

Between the 3 days and 4 weeks post-surgery vitrified testicular transplants showed a gradual loss of seminiferous cords. In the first panel (A) from day old fresh tissue, seminiferous cords are clearly present (Fig. 5.1). In panel B, 3-day old post surgery graft, normal testicular structure is present, for C & D panels from 4-day and 2-week post surgery grafts respectively, have only a couple seminiferous cords present. In the E panel, there are no seminiferous cords present from 4-week old post surgery grafts.

For BT females, the only ovarian graft present post-surgery was retrieved from BT 44 (which was euthanized 4 days post-surgery) and was part of the vitrified ovarian tissue group. For BT birds euthanized after 4 days post-surgery, no ovarian tissue were located for both the vitrified and fresh tissue groups (Table 5.6). For birds, which had transplanted tissue placed under the left abdominal air sac membrane or the peritoneum, the physiology of the membrane was the same as the aforementioned Trial 3 BT males.

5.5 Figures and Tables

Table 5.1. Survival ratios of 1, 3, 4 and 7 day old Large White turkey poults from surgery Trial 1 are shown with average body weight at the time of surgery and duration of surgery. Body weight and duration of surgery are broken down for each age group, whereas survivability is shown for the entire Trial. GNR = Gonad Not Removed, O = Ovariectomized, OT = Orthotopic Transplantation, S = Survived, D = Died. A-B (P=0.012) between 4 and 7 day old poults at the time of surgery

Sex	Day of Surgery	WT (ID#)	Weight (g)	Duration of Surgery (min)	Surgery Procedure	Outcome (S/S+D)
Male		2	57.6	28	GNR	S
	1	1	52.1	30	-	-
		3A	57.9	29	O	D
		3B	53.6	23	O	D
	Mean ± SEM		**55.3 ± 1.4^{AB}**	**29.0 ± 1.0**		**(1/3)**
	3	3	55.0	32	O	S
		4A	53.1	22	O + OT	D
		4B	53.1	26	O	D
		5	50.0	24	O + OT	S
	Mean ± SEM		**52.8 ± 1.0^{AB}**	**28.0 ± 4.0**		**(2/4)**
Female	**4**	6A	42.3	16	O + OT	D
		6B	48.7	28	O	D
		6	42.8	26	O + OT	S
		7A	50.6	23	O	D
		7B	50.8	25	O + OT	D
		8	45.0	23	O + OT	S
	Mean ± SEM		**46.7 ± 1.6^{A}**	**24.5 ± 1.5**		**(2/6)**
	7	9	49.2	50	O	S
		10A	49.4	6	-	D
		10	59.5	27	O + OT	S
		11	66.7	35	O + OT	S
		12A	68.0	7	-	D
	Mean ± SEM		**58.6 ± 4.0^{B}**	**37.3 ± 6.7**		**(3/5)**
Total	**Mean ± SEM**		**52.9 ± 1.6**	**30.6 ± 2.7**	Survivability %	**(8/18)** 44%

Table 5.2. Survival ratios of 1, 3, 4 and 5 day old Large White turkey poults from surgery Trial 2 are shown with average body weight at the time of surgery and duration of surgery. Body weight and duration of surgery, are broken down by age group, whereas survivability is shown for the entire Trial. O = Ovariectomized, T = Testectomized, LT = Left Testectomized, OT = Orthotopic Transplantation, ASMT = Air Sac Membrane Transplantation, S = Survived, D = Died, E = Euthanized. A-B ($P<0.02$) between 1 and 3, 4, 5 day-old poults at the time of surgery, (*) Due to n=1 the Mean and SEM cannot be calculated.

Sex	Day of Surgery	WT (ID#)	Weight (g)	Duration of Surgery (min)	Surgery Procedure	Outcome (S/S+D+E)
Male	1	14	52.1	27	LT	S
		16	52.6	26	T + OT	S
		17B	52.2	18	T + ASMT	D
		18	48.3	31	T + OT	S
Female		15	54.4	27	O + OT	S
		17A	52.3	30	O + ASMT	D
		19	47.5	32	O + ASMT	S
	Mean ± SEM		**51.3 ± 0.9^{A}**	**28.6 ± 1.2**		**(5/7)**
Male	3	22	65.1	27	LT + ASMT	S
		24	66.1	28	T + ASMT	S
Female		23	59.2	20	O + ASMT	S
		25	65.1	32	O + ASMT	S
		26	61.5	50	O + OT	S
		20	61.5	3	-	D
		21	57.4	11	-	S
unknown		20A	63.3	-	-	D
	Mean ± SEM		**62.4 ± 1.1^{B}**	**31.4 ± 5.0**		**(6/8)**
Male	4	27A	70.9	24	-	E
		27	54.9	24	LT + OT	S
		28B	65.7	20	T	E
Female		28A	58.9	50	O + OT	E
	Mean ± SEM		**62.6 ± 3.6^{B}**	*		**(1/4)**
Female	5	28	64.7	42	O + ASMT	S
		29A	60.5	8	-	E
	Mean ± SEM		**62.6 ± 2.1^{B}**	*		**(1/2)**
Total	**Mean ± SEM**		**58.8 ± 1.4**	**30.5 ± 2.4**	Survivability %	**(13/20)** 65%

Table 5.3. The percent survivability of day old Bronze turkey male poults from Trial 3; average, body weight and duration of surgery are also shown, in addition to the type of surgery performed. T = Testectomized, LT = Left Testectomized, SGOT = Surgery Glue Orthotopic Transplantation, ASMT = Air Sac Membrane Transplantation, OUP = Orthotopicly Under Peritoneum, S = Survived, D = Died, E = Euthanized.

BT (ID#)	Weight (g)	Duration of Surgery (min)	Surgery Procedure	Outcome
30C	67.4	31	T	E
30	65.9	41	T	S
32	56.0	24	T + SGOT	S
33	64.8	41	T + ASMT	S
34	66.4	29	T + SGOT	S
35	67.7	25	T + SGOT	S
36	61.2	31	T + ASMT	S
37	64.0	35	T + ASMT	S
39	58.0	32	LT + ASMT	S
40	76.0	38	T + ASMT	S
45	74.3	29	T + ASMT	S
46	67.5	48	T + ASMT	S
53	71.5	30	T + OUP	S
54	71.6	25	T + OUP	S
55	69.3	37	T + OUP	S
58	73.7	28	LT + OUP	S
59	62.3	28	T + OUP	S
60	56.1	32	T + OUP	S
63	67.0	25	LT + OUP	S
67	68.1	43	LT + OUP	S
68	57.8	39	LT + OUP	S
70	64.5	32	LT + OUP	S
72	68.0	30	LT + OUP	S
Mean ± SEM	**66.0 ± 1.2**	**32.7 ± 1.4**	**(S/S+E) Survivability**	**(22/23) 96%**

Table 5.4. Number of Large White turkey grafts present in Bronze turkey recipient males by age of euthanization and graft recovery and graft type for surgery Trial 3. Types of procedures performed are shown. For fresh and vitrified grafts ages are broken down by weeks. T = Testectomized, LT = Left Testectomized, SGOT = Surgery Glue, Orthotopic Transplantation, ASMT = Air Sac Membrane Transplantation, OUP = Orthotopicly Under Peritoneum.

Graft Type	Euthanized	BT (ID#)	Surgery Procedure	Graft Present	Number of Grafts present
Fresh	8 weeks	58	T + OUP	NO	(0/1)
	18 weeks	53	T + OUP	NO	
		59	T + OUP	NO	
		60	T + OUP	NO	(0/3)
Total grafts present					**(0/4)**
Vitrified	24 hours	33	T + ASMT	YES	
		34	T + SGOT	YES	
		68	LT + OUP	YES	(3/3)
	1 week	39	LT + ASMT	YES	
		46	T + ASMT	YES	
		36	T + ASMT	YES	
		67	T + OUP	YES	
		40	T + ASMT	NO	(4/5)
	2 weeks	37	T + ASMT	YES	(1/1)
	4 weeks	55	T + OUP	YES	(1/1)
	6 weeks	32	T + SGOT	NO	
		35	T + SGOT	NO	(0/2)
	8 weeks	45	T + ASMT	NO	
		63	T + OUP	NO	
		70	LT + OUP	NO	(0/5)
	18 weeks	54	T + OUP	NO	
		72	LT + OUP	NO	(0/2)
Total grafts present					**(9/19)**

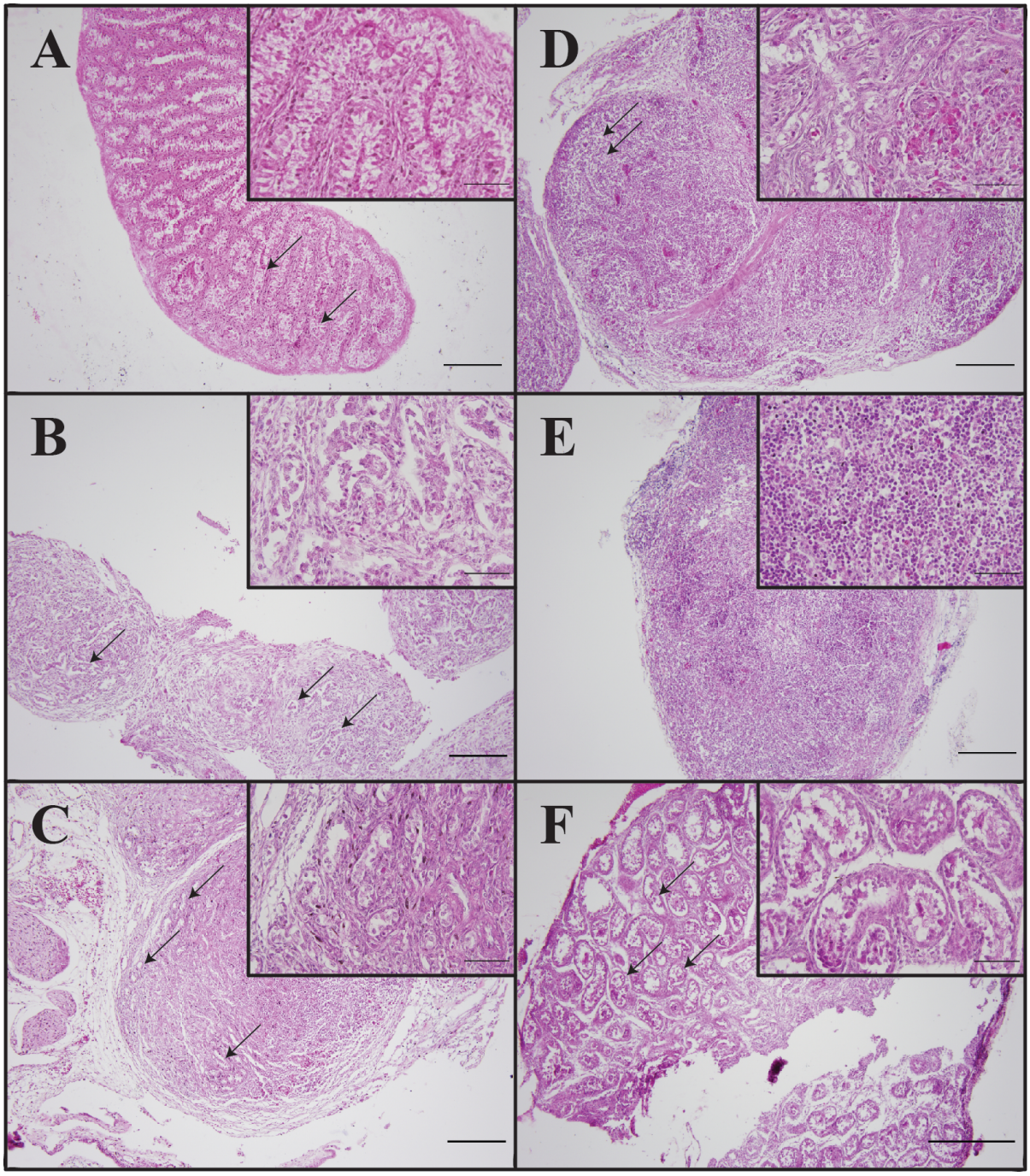

Figure 5.1 Morphological appearances of testes, from host (Bronze turkey) or transplants from (Large White turkey) post-surgery. Inlets in each window are a close up of the area mentioned and directed to by the arrows. **(A)** 1-day old host, normal seminiferous cords **(arrows)**. **(B)** 3-days post-surgery severely altered seminiferous cords **(arrows)** still present all over the testes. **(C)** 4-days post-surgery almost unrecognizable seminiferous cords **(arrows)** located around the periphery of the testes. **(D)** 2-weeks post-surgery no seminiferous cords **(arrows)** located, although cells morphology appears similar to that of cells constituting cords. **(E)** 4-weeks post-surgery no testicular structures or cells present, which appear to have been derived from seminiferous cord cell types. **(F)** 4-weeks host normal seminiferous cord **(arrows)** morphology. Scale bars **(A-F)** 200µm, **(Inlets)** 50µm.

Table 5.5. The percent survivability of day old Bronze turkey female poults from Trial 3; average, body weight and duration of surgery are also shown, in addition to the type of surgery performed. Types of surgery performed are also shown. O = Ovariectomized, ASMT = Air Sac Membrane Transplantation, OUP = Orthotopicly Under Peritoneum, S = Survived, D = Died, E = Euthanized.

BT (ID#)	Weight (g)	Duration of Surgery (min)	Surgery Procedure	Outcome
30A	77.1	33	O	D
30B	79.8	25	O	E
41	60.4	34	O + ASMT	S
42	57.8	31	O + ASMT	S
43	68.0	27	O + OUP	S
44	68.4	32	O + OUP	S
52A	65.4	29	O	D
52	63.1	23	O + OUP	S
56	67.5	27	O + OUP	S
57A	65.3	49	O + OUP	D
57	67.2	33	O + OUP	S
61A	56.7	35	O + OUP	D
61B	73.2	43	O + OUP	D
61	67.7	32	O + OUP	S
62	58.8	32	O + OUP	S
64	64.0	32	O + OUP	S
65	63.9	39	O + OUP	S
69	54.9	34	O + OUP	S
71	65.5	35	O + OUP	S
Mean ± SEM	**65.5 ± 1.5**	**31.6 ± 1.1**	**S/S +D (% Survived)**	**13/19 (68)**

Table 5.6. Number of Large White turkey grafts present in Bronze turkey recipient females by age at euthanasia and graft type (fresh or vitrified), for surgery Trial 3. Also shown are the types of procedures performed. O = Ovariectomized, ASMT = Air Sac Membrane Transplantation, OUP = Orthotopicly Under Peritoneum.

Graft Type	Euthanized	BT (ID#)	Surgery Procedure	Graft Present	Number of Grafts present
Fresh	18 weeks	52	O + OUP	NO	
		56	O + OUP	NO	(0/2)
Total grafts present					**(0/2)**
Vitrified	4 days	44	O + OUP	YES	(1/1)
	12 days	65	O + OUP	NO	(0/1)
	8 weeks	57	O + OUP	NO	(0/1)
	18 weeks	41	O + ASMT	NO	
		42	O + ASMT	NO	
		43	O + OUP	NO	
		61	O + OUP	NO	
		62	O + OUP	NO	
		64	O + OUP	NO	
		69	O + OUP	NO	
		71	O + OUP	NO	(0/8)
Total grafts present					**(1/11)**

5.6 Discussion

The goal of this study was to determine if gonad transplantation in poults is a viable approach of retrieving cryopreserved gonads, and facilitate in vivo maturation. This would allow for the potential success of future work to produce donor-derived offspring from cryopreserved turkey gonads.
No mature turkey gonad grafts were recovered from any of the surgery Trials. No HT previously vitrified gonads, transplanted in Trial 1 & 2 were found in LWT euthanized 8 weeks post-surgery. From Trial 3, donor LWT testicular and ovarian tissue previously vitrified were only recovered in BT euthanized up to 4-weeks and 4-days post-surgery, respectively (Table 5.4 & 5.6). No fresh tissue grafts from Trial 3 were found, in recipients at 8 or 18 weeks post-surgery. Recovered testes in the vitrified group from Trial 3 were histologically analysed and showed that the structure of the testes and seminiferous cords had deteriorated over time between 3-days and 4-weeks post-surgery (Fig. 5.1). We cannot determine if the morphological damages were induced by vitrification protocols and/or inadequate immunosuppression of the recipient. Here we have shown that day old poults like day old chicks and ducklings can survive gonad transplantation, but proper graft development was not obtained, for fresh or vitrified tissue.

Survivability of poults is inevitably influenced by two factors: the condition of the poult and the surgical procedure itself. The condition of the poult is based on the intrinsic properties of the poult with respect to the physical fitness and tolerance to surgery. The influence of the surgical procedure itself is based on the surgeon's ability and the type of equipment accessible. An improvement of survivability over time from Trial 1 (44%) to Trial 3 (83%) shows this effect that the surgeon played in determining survivability, as the surgeon became experienced with the surgical procedure (Table 5.1, 5.3, 5.4). This highlights the need for an appropriate amount of training and skill set before this challenging procedure is undertaken. The numbers of poults, which underwent gonad transplantation each day for Trial 1, was proportionally small which makes comparison between days challenging (Table 5.1). There was a drop ($P=0.012$) in pre-surgery body weight on day 4 for poults withheld from food before surgery. Even though the yolk sac was

still present at this age, the body weight was being affected, which could have a negative consequence on survivability. For Trial 2 the number of poults operated on the first couple of days was larger than the later time points, as poults became increasingly challenging to work with at 4 and 5 days of age. Anaesthetic complications compounded by an expanded digestive track were the reasons for these challenges. The expanded digestive track was a consequence of allowing poults food *ad libitum* before surgery; this obstructed the view of the gonads. Poults at 4 days of age or older having food *ad libitum* are not suitable candidates for surgery based on these observations.

The survival ratios at 1 and 3 days, from Trial 2 were both high (Table. 5.2), either age could be suitable for future surgery Trials. Due to the fact that the younger the recipient, the less chance of immune response, day old poults are preferred over 3-day old poults (Mast and Goddeeris, 1999). Day old poults from Trial 3 had the highest survivability out of all the Trials with the male poults having a considerable increase in survivability over their female counterparts (Table 5.3 and 5.5). Here, the surgeon was most experienced to perform the procedure, which explains the high survivability. The rationale behind the spread in survivability between male and female poults is that a larger proportion of male chicks underwent only a left testectomy (Table 5.3), reducing the probability of haemorrhaging. There is evidence to suggest that the removal of testes is not necessary for successful testicular transplantation. In a recent study on domestic chicken recipient testes were left in place and the donor tissue was grafted in close proximity to the recipients (Liptoi et al., 2013). Donor tissue recovered at 8 weeks of age had well-developed seminiferous cords. Due to the risk of haemorrhaging by removing the right testis the recommendation here would be to only remove the left testis in poults. The fact that grafts either did not grow or in the case of Trial 3 testes showed sub-par morphology, and were not present past 4 weeks post-surgery, shows suboptimal conditions to sustain gonadal development. As aforementioned this lack of success could have originated from morphological damaged induced by vitrification protocols and/or inadequate immunosuppression.

Fresh ovarian and testicular tissue also failed to grow pointing to an immune system problem. It has been shown in chickens that an immune suppression is not vital for graft development (Song and Silversides, 2007a). Mycophenolate mofetil (MMF) has been used to block the adaptive immune system in chicken, quail and ducks after gonad transplantation (Song and Silversides, 2007a; 2008a; Liptoi et al., 2013). In quail MMF was able to increase the success of gonad transplantation (Song and Silversides, 2008a), this drug though did not have a significant effect on success of transplantation in chickens (Song and Silversides, 2007a). It should be noted that the sample sizes here of fresh testicular (n=4) and ovarian (n=2) transplants were lower than for the vitrified groups, which makes comparison challenging. There could be two possible reasons the immunosuppression failed: the dose was below an effective level or this drug is simply not effective on Bronze and Large White turkeys.

With regard to the anaesthesia, isoflurane used alone was sufficient for LWT poults. A combination of isoflurane and xylazine was used for BT poults. Poults with or without food before surgery reacted well to isoflurane up until 3 and 7 days of age, respectively, with a couple of exceptions. The combination of isoflurane and xylazine was used on day old BT poults kept from food. In previous work, a combination of ketamine and xylazine was used to sedate chicks (chicken and quail) and ducklings (Song and Silversides, 2007a; 2008a; Song et al., 2012). In subsequent work isoflurane on it's own was used on chicks (Liu et al., 2013b; Silversides et al., 2013). It is unclear why there was a difference in anaesthia requirements between these similar breeds of turkeys. With the addition of xylazine, the amount of isoflurane needed was less for BT poults (App. B.3 and B.4) when compared to LWT poults from Trial 1 and 2 (App. B.1 and B.4). The breathing rate also decreased for Trial 3 poults compared to Trial 1 and 2 poults. This was expected as xylazine is a sedative but also a muscle relaxant capable of reducing breathing rate (Greene and Thurmon, 1988). With these results, a combination of isoflurane and xylazine anaesthetic is the most appropriate for working on turkey poults.

Immobilization of graft allows for vascularization to occur and can also allow for angiogenesis. In the literature to date, synthetic immobilization (e.g. suturing) of the graft is not required (Song and Silversides, 2006); it would appear that the size of the tissue renders it stationary in the abdominal cavity. This was corroborated by our own observations that grafts remained in the same position in the abdominal cavity over a 24-hour period. When two non-synthetic (abdominal air sac membrane and peritoneum) and one synthetic (surgical glue), immobilization techniques were used. The surgical glue appeared the most abrasive and possibly counterproductive. As the gonads retrieved after attachment using surgical glue were embedded in the surgical glue (data not shown). Attachment using the left abdominal air sac technique showed that the torn abdominal air sac membrane was located in the left cranial dorsal part of the abdomen. Although the membrane was still attached, the movement away from an orthotopic position is not favourable (data not shown). As this reduces the chance the ovary and future ovulated ova, will be in the correct proximity to the infundibulum. One of the key physiological problems with this technique is that opening up an air sac hinders the gas anaesthesia effectiveness (Lierz and Korbel, 2012). If air from the environment is pulled through the opening and into the lungs, a combination of gas and liquid anaesthetic is recommended. In the case of transplants placed under the peritoneum membrane, these grafts stayed in place, as these transplanted gonads were recovered in the same position as to when they were grafted (data not shown). Placing transplants underneath the peritoneum membrane of the abdominal side orthotopicly is the most suitable method.

In conclusion, gonad transplantation has been shown to be feasible in turkey poults, although the viability of the grafted tissue was poor and showed insufficient growth and maturation. We recommend that one-day old poults should be used for gonad transplantation as they have a high survivability rate and are most likely to lack immunocompetencey. We also recommended that gonads be transplanted underneath the dorsal peritoneum membrane, orthotopicly as to allow for the grafts to remain immobilized; and that while isoflurane is adequate for sedation of LWT poults. For anaesthesia procedures the addition of xylazine is required for the successful sedation of BT poults. These different recommendations will

improve the success of gonadal transfer and could lead to the production of donor-derived off spring from recipient birds.

CHAPTER 6: DETERMINING PRE-OVULATORY FOLLICLE (F1-F5) GROWTH RATES AND PATTERNS IN BARRED PLYMOUTH ROCK HENS USING ULTRASONOGRAPHY

6.1 Abstract

The rapid follicle growth phase in avian pre-ovulatory follicles (F1-F5) is the fourth stage in folliculogenesis, which is marked by an accelerated uptake of yellow yolk by the ovum. Usually, it is evaluated by post-mortem analysis, or after oviposition by studying the concentric rings of yolk deposited. Here we used ultrasonography to map follicle (size and position) to determine growth rates and patterns of folliculogenesis in Barred Plymouth Rock (BPR) hens. When determining the optimal period between ultrasound scans, 6 BPR hens 9-10 months of age and acclimatized to a LD (16:8) photoperiod were scanned multiple times over a 24-hour period, and follicle diameter and position were recorded. Proportional follicle growth to the initial scan was calculated to determine the most appropriate serial scanning period to accurately track F1-F5 follicle growth. From this Trial, proportional follicle growth was only significant at 24hr ($P<0.001$). Based on this finding, ultrasound scans can be performed twice a day (morning and evening). When determining follicle growth rates and patterns, ten BPR hens at 14 month of age were acclimatized to a LD (14:10) photoperiod and were scanned twice a day for ten and a half days. Follicle diameters (height and width) were recorded along with position; these were used to calculate the follicle area. Three birds were then euthanized after the final scan to determine how accurate follicle detection was for this method. Follicle maps were constructed to track F5 follicles all the way to ovulation and to determine the follicle growth curve. Multiple follicle interval growth rates (FIGR) were then calculated for each follicle, and analysed based on time (day verse night) and type (F5-F1) to determine if any factor or interaction were significant ($P<0.05$) with respect to FIGR. From Trial 2, the post-mortem count of follicles from three hens showed that only one F5 follicle was missed (12/13) showing a 92.3% detection rate of preovulatory follicles. The cubic equation $y = -1.75\times10^{-6}(x)^3 + -3.98\times10^{-4}(x)^2 + 0.04(x) + 9.46$ (y = follicle area, x = hours before oviposition), best described the follicle

growth rate for 9 out of the 10 hens, with one hen (#9) having a subpar follicle growth rate. Follicle growth was higher at night than in the daytime ($P=0.009$), with follicle types F2 and F3 having a higher ($P<0.05$) growth rate than F1 and F5 types. For the first time, ultrasonography has been shown to be a valid method of determining folliculogenesis growth rates and patterns in BPR hens and it can be used by the poultry industry as another means to select superior laying hens.

6.2 Introduction

The poultry industry has placed an enormous selective pressure on domesticated chicken and turkeys to improve production traits. Total egg production in a year for laying lines, or time to market weight for broiler lines, are both economic and quantitative traits impacting competitive markets. Total egg production for laying lines is nearing a biological plateau as commercial lines approach an egg a day rates (Johnson et al., 2015), this egg production can easily be measured with the collection of eggs. In chicken, a follicle ruptures on average every 24-25hrs during the laying period, and the yolk filled ovum is released into the infundibulum, enabling albumen to surround the ovum and eventual eggshell calcification (Warren and Scott, 1935). This means that a follicle must be recruited from the pool of slow growth phase follicles every day to enter the rapid growth hierarchy phase (Etches et al., 1983). Although collecting of eggs is an accurate way of measuring egg production, it doesn't accurate measure folliculogenesis. Previous studies on folliculogenesis in poultry have analysed follicles collected after post-mortem or oviposition (Grau, 1976; Etches et al., 1983; Astheimer and Grau, 1989). Post-mortem analysis, while valuable, has a high animal cost associated with it and cannot allow for repeated measuring from the same bird.

The need for a technique to monitor folliculogenesis in chickens in a non-terminal way, which allows for repeated data collection, is apparent; ultrasonography meets both of these conditions. The non-invasive technique of ultrasonography has already been successfully used on a wide array of species (cow, llama,

camel) by demonstrating folliculogenesis growth rates and patterns (Pierson and Ginther, 1987; Bravo et al., 1990; Vyas and Sahani, 2000). Ultrasonography has been used to study a number of biological processes in birds: development of chicken embryos *In Ovo* (Peebles et al., 1993; Pugh et al., 1993); evaluation of the urogenital tract in a wide variety of pet birds (Hofbauer and Krautwald-Junghanns, 1999); diagnosis of erratic oviposition and defective egg syndrome (EODES) in broiler hens (Melnychuk et al., 2002); diagnosis of atretic follicles in mature female ostriches by looking at follicles that had a hypoechoic centre and hyperechoic wall with an irregular follicle shape (Gonzales and Acorda, 2012); determining the temporal relationship between oviposition, ovulation and plasma concentrations of gonadotropins (luteinizing hormone) and sex steroids (progesterone, and estradiol-17β) in ostriches (Bronneberg et al., 2007). Mature female ostriches, where also used to validate ultrasonography by comparing the number of preovulatory follicles 1-9cm in diameter, detected (in vivo) via ultrasound scanning. With follicles observed post-mortem (in vitro) this showed that only 58% of the follicles present post-mortem were detected by ultrasound (Bronneberg and Taverne, 2003). Ultrasound analysis was used to evaluate the size of follicles in chicken (Melnychuk et al., 2002), but the follicular growth rates and patterns have not been evaluated.

In birds, folliculogenesis can be broken down into four follicle stages: primordial, primary, pre-hierarchal and pre-ovulatory follicles. Primordial follicles (0-80μm), which are arrested are selected periodically to enter the slow growth phases and transition into primary follicles (0.04-2.0mm), both of these follicle types remain visually concealed within the cortex of the ovary (Marza and Marza, 1935; Johnson and Woods, 2007). Pre-hierarchal follicles (2-4mm) protrude out of the ovary's cortex with the uptake of white yolk; this stage is also known as the intermediate phase (Marza and Marza, 1935). These follicles are then selected to enter the final stage, rapid growth development with the uptake of yellow yolk. This final stage (pre-ovulatory follicles, 4-40mm) can be further categorized by size relative to the hierarchy, with the largest follicle designated F1, and subsequently smaller follicles receiving F2, F3 and so forth (Marza and Marza, 1935; Johnson and Woods, 2007). For chickens, the rapid growth phase takes around

7-11 days (Warren and Conrad, 1939). At the end of the rapid growth phase there is a rest period, where the follicle doesn't grow (Bacon and Skala, 1968). This means the F1 follicle has a growth period, and a slight rest period before eventual ovulation, with ovulation the follicle ruptures along the stigma (Johnson and Woods, 2007). Ultrasound technique in cows using trans-rectal probing can visualize follicles as small 2-3mm in diameter (Pierson and Ginther, 1987).

Ultrasonography as a way to track folliculogenesis in poultry will give the industry and academia a new tool to evaluate the follicle status, of the ovary in a non-invasive manner. The overall goal of this study was to determine the growth rates and patterns of pre-ovulatory follicles as they progress from F5 to F1 stage, in Barred Plymouth Rock hens, using ultrasonography. The objective of Trial 1 was to determine the most appropriate time interval between serial ultrasound scans in order to track and map F1-F5 follicles accurately and precisely. For Trial 2 the objective was to measure and track follicle growth from several hens to obtain follicle maps and growth curves, for each follicle. The folliculogenesis was successfully measured using ultrasonography, and showed a growth rate similar to reported growth curves for chickens, with follicle patterns being strongly correlated to follicle type and time of day.

6.3 Materials and Methods

6.3.1 Equipment and Software

The ultrasound machine used for these studies was a MyLab30Vet and the ultrasound transducer was a CA123: Neonatal, Abdominal, Vascular Micro-convex array (5.0-8.0Hz). The software used by the ultrasound machine was RES 1.01 and VET 9.13. All of these products were bought from Esaote Canada Inc. (Georgetown, ON, Canada).

6.3.2 Determining the optimal period between ultrasound scanning sessions in chickens

6.3.2.1 Scanning protocol

Six Barred Plymouth Rock (BPR) hens of approximately 9-10 months old were acclimatized to a LD 16:8 (lights on 6:00am, lights off 10:00pm) period for this Trial. Hens were exposed to an *ad libitum* diet and were caged separately in an animal care facility. Hens were handled with care (carried in containers when removed from pens) to minimize stress that could alter folliculogenesis (Wang et al., 2013). Ultrasound scans were conducted in a dark room away from the common housing space. An assistant restrained the hens by placing them on their right side while simultaneously holding their wings. The right wing was kept under the right side and the left one was held against the body with the assistant's other free hand. After a couple sessions, birds habituated to this position relatively quickly and appeared calm while being scanned. The left leg was raised to expose the natural bald area underneath the thigh; the size of this area was increased if needed, by gently plucking feathers. EcoGel 200 ultrasound coupling gel (Eco-Med Pharmaceutical Inc., Mississauga, ON, Canada) was applied to the exposed skin and the scan was conducted. At first, the ovary and surrounding follicles were quickly located and scans were conducted ventral to dorsal and anterior to posterior, sweeping over the ovary multiple times to ensure that every

follicle had been identified. Afterwards, each follicle was drawn in relation to the ovary and other follicles. Only the optimal diameter was measured and recorded, this was when the follicle was the largest on the ultrasound screen, denoting the centre of the follicle. Each scan took approximately 5-10 mins depending on the number of follicles and the difficulty associated with imaging. Scans began at 8:00am (taking 5-10 mins per bird) and were repeated every two hours until 6:00pm, and then 8:00am the following morning, allowing for 24 hours of follicular growth.

6.3.2.2 Follicle statistical analysis

For each follicle observed on the initial scanning session (8:00am) from the six BPR hens, the proportional change was calculated over the subsequent scanning sessions, represented by equation 1, and plotted against time from the initial scan. Where $DM_{initial}$ is the follicle diameter on the initial scanning session, and DM_x is the diameter on the subsequent scanning sessions. These values were then converted to percentiles and graphed with means ± SEM.

Equation (1): Follicle diameter proportional change = $(DM_x - DM_{initial}) / DM_{initial}$

A Univariate ANOVA test was used to determine if there was a significant ($P<0.05$) difference in the proportional change in follicle diameter over time. Follicle diameter proportional change rates (means ± SEM) were then coded and grouped based on time point (2-24hrs) and on individual follicle ID (1 to 19). Follicle ID was analyzed as a random factor, we expected follicle ID to play a significant role in growth rates, and so this was not evaluated further. Time was analyzed as a fixed factor as this was a repeated measure test. After it was determined if proportional change in follicle diameter over time was significant a Post hoc (Tukey) test was run to determine which time points were significant ($P<0.05$). All data was analyzed using SPSS (IBM SPSS Statistics, Version 22, Armonk, NY).

6.3.3 Determining ovarian folliculogenesis in Barred Plymouth Rock hens over an 11-day period

6.3.3.1 Scanning protocol

For this Trial, 10 BPR hens were used. These hens were different than the ones used in the first Trial and were approximately 14 months old. They had been acclimatized to a LD 14:10 (lights on 4:00am, lights off 6:00pm) period. The hens were given an *ad libitum* diet and were caged separately. The handling and scanning procedure was the same as for section 6.3.2.1, with the following amendments: each follicle was mapped and the diameter (width and height) was recorded. Scans began at 3:00pm (taking 5-10 mins per bird) and were repeated the following morning at 5:00am; this was continued for 10½ days, allowing for 21 serial scans per bird.

6.3.3.2 Validation of ultrasound method

After the last scan, three birds were randomly selected for euthanasia and dissection. Hens were euthanized by cervical dislocation. Their body cavities were carefully opened and their ovaries were removed and photographed. The F1-F5 follicles were detached from the ovary and were measured using a water bath to retain the spherical shape. The width and height of each follicle was recorded after which they were photographed alongside a ruler.

6.3.3.3 Follicle mapping and statistical analysis

To validate the ultrasound method, the areas determined by the last ultrasound scans were compared side by side graphically to the areas for the same follicles determined by measuring them in the water bath after being removed from the body cavity. The total number of follicles detected via ultrasonography was divided by the total counted at post-mortem to provide a detection rate.

The follicle/ovary diagrams combined with the measurements taken were used to construct a follicle progression graph for each bird (App. C.1). The measurements were used to calculate the area of an ellipse (Area=$\pi \times H \times W$), where H and W were the radiuses height and width. This represented the area of the plane at which the follicle appeared the largest on the ultrasound screen, deemed to be the centre. By using the area and follicle/ovary diagram, which took into consideration the position of the follicle, each individual progression of each follicle was mapped from emergence to final ovulation. This was visualized by plotting time 0 – 254 hours against area (cm^2) for each follicle. This follicle graph showed the folliculogenesis for each individual hen over the entire scanning period.

In the first analysis, the goal was to determine the relationship between follicle size and time before ovulation to estimate the growth rate. Three follicles were selected from each of the ten hens (an example of the selection method from two hens can be seen in App. C.1). These follicles were selected based on having the most complete data set from emergence to ovulation. Using the area of the follicle, these values were graphically aligned based on time before ovulation (App. C.2). Also, the three follicles from each hen were combined and run through a curve estimation analysis, which fits a variety of curves (Linear, Quadratic, Cubic, Compound, Exponential, Logarithmic, Inverse) to determine which could explain the follicle growth rate the best, and show that there is a significant ($P<0.05$) relationship. After which, the 95% confidence interval was calculated.

The second analysis was conducted to determine if factors such as follicle type, (F1-F5) time period, (day/night) and follicle ID had an effect on growth rate of follicles. Growth rate was determined for each interval (FIGR) between datum points using equation (2); x was the datum point being analysed and x-1 was the previous datum value, either for area or time.

Equation (2): Follicle Interval Growth Rate (FIGR) = $(Area_x - Area_{x-1}) / (Time_x - Time_{x-1})$

Follicle interval growth rates (means ± SEM) were then coded and grouped based on whether they came from F1-F5 follicles (1-5), day and night periods (1 or 2), or based on individual follicle ID (1 to 99). A hundred different groups of growth rates were analyzed from these three factors. These groups were then analysed using a Univariate ANOVA test, to determine if any of these factors or interactions between factors were significant ($P<0.05$). Day/night and follicle types were entered as fixed factors with follicle ID being entered as a random factor, as this was a repeated measure test. We expected follicle ID to come back not significant, and wasn't analysed further. If any of the fixed factors were significant ($P<0.05$), a post-hoc (Tukey) or a T-test was conducted to determine which averages were higher or lower than other groups ($P<0.05$). All data was analyzed using SPSS (IBM SPSS Statistics, Version 22, Armonk, NY).

6.4 Results

6.4.1 Determining the optimal period between ultrasound scanning sessions in chickens

There was an increase ($P<0.001$) in follicle diameter between the 24-hour scan period and all the other scanning periods (Fig. 6.1). Scans 10 hours apart in the daytime did not miss any significant growth. Scans every 24 hours would result in a significant difference in the growth of follicles. Scans occurring twice a day (morning and evening) was shown to be the most suitable to capture follicle growth in a day.

6.4.2 Determining ovarian folliculogenesis in Barred Plymouth Rock hens over an 11-day period

Ultrasound scans on three hens on the last day detected 12 follicles, after euthanization and dissection 13 follicles were retrieved, giving a 92.3% detection rate of F1 to F5 follicles. By graphical comparison there was little difference between the two methods of determining follicle area (Fig. 6.2).

The curve estimation analyses determined that the equation that represented the follicle area growth the best (R^2=0.907, P<0.001) was a cubic curve (*solid line*), $y = -1.75\times10^{-6}(x)^3 + -3.98\times10^{-4}(x)^2 + 0.04(x) + 9.46$ (Fig. 6.3), y = follicle area (cm^2) and x = time before ovulation (hours). Only two follicles fall below the 95% confidence interval (*dotted line*) these two follicles were from the same hen #9.

There was a difference in growth rates for both day/night and follicle type (F1-F5), respectively P=0.008 and P<0.001 (Fig. 6.4). The interacting term between these two factors was not significant (P=0.122). By t-test the growth of follicles at night (5.95 ± 0.21) was higher (P=0.018) than daytime (4.98 ± 0.33) growth [averages not shown graphically]. The increase in follicle area for types F2 (5.83 ± 0.44) and F3 (6.91 ± 0.37) was higher (P<0.05) than F1 (4.29 ± 0.48) and F5 (2.67 ± 0.38). This increase was almost two-fold higher than F5 follicles. For F4 follicles, their increase in follicle area (5.50 ± 0.36) was not significantly different than any of the aforementioned follicle types (Fig. 6.4).

6.5 Figures and Tables

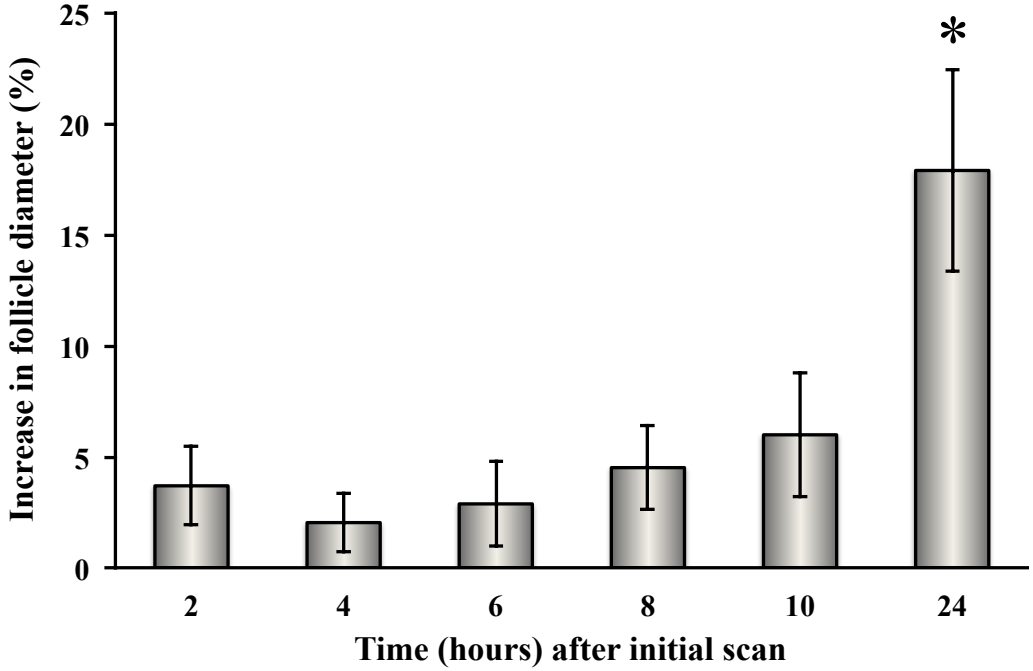

Figure 6.1 Proportional change (Mean ± SEM) in follicular diameter compared to initial scan measurements for a 24-hour period from six Barred Plymouth Rock hens. Barred Plymouth Rock hens were acclimatized to a LD 16:8 photoperiod, (lights: on 6:00am, off 10:00pm). Scans were performed every 2hrs (starting at 8:00am) for 10 hrs, and at 24 hrs. *P<0.001.

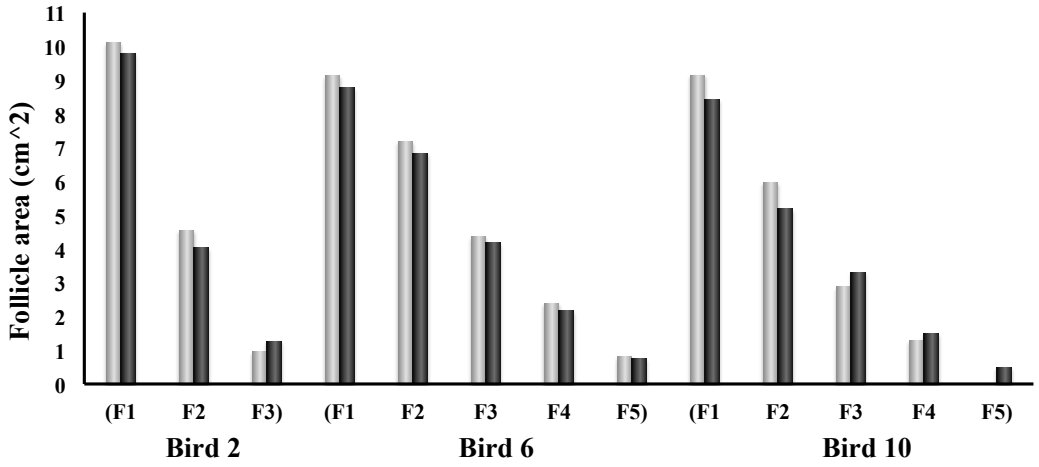

Figure 6.2 Comparison of F1-F5 follicle areas determined by ultrasonography [grey bars] or by post-mortem analysis [black bars] from Barred Plymouth Rock hens (2, 6 and 10). Only one follicle (Bird 10, F5) was not detected through ultrasonography giving a detection rate of (12/13) 92.3%.

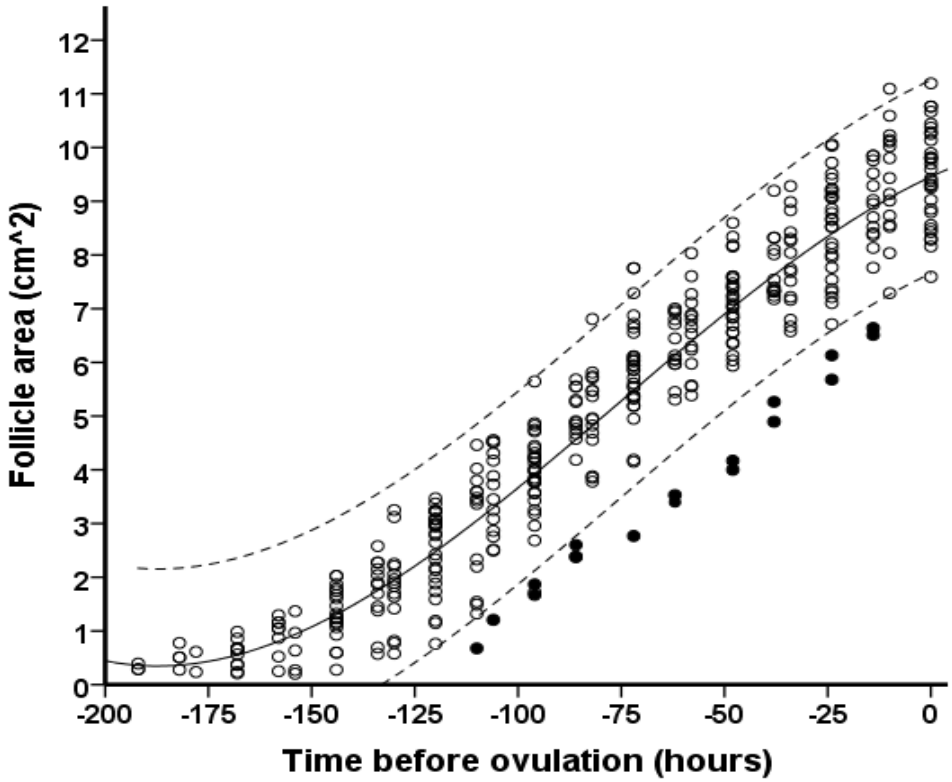

Figure 6.3 Curve estimation analyses for thirty follicles from 10 Barred Plymouth Rock hens. Three follicles were used from each hen (App C.2). Follicles were tracked from visual detection to eventual F1 follicle status and ovulation. Follicle area was plotted against last observation of follicles in proximity to the ovary (follicles are assumed to have ovulated soon after). The equation that best described (R^2=0.907, P<0.001) the relation between follicle area (x) and time before ovulation (y) was a cubic equation (*Solid line*), $y = -1.75\times10^{-6}(x)^3 + -3.98\times10^{-4}(x)^2 + 0.04(x) + 9.46$; the 95% confidence limits (*Dotted lines*) are also shown. **Black data points** represent two follicles from hen (#9), which progressed outside the 95% confidence interval for a majority of their growth.

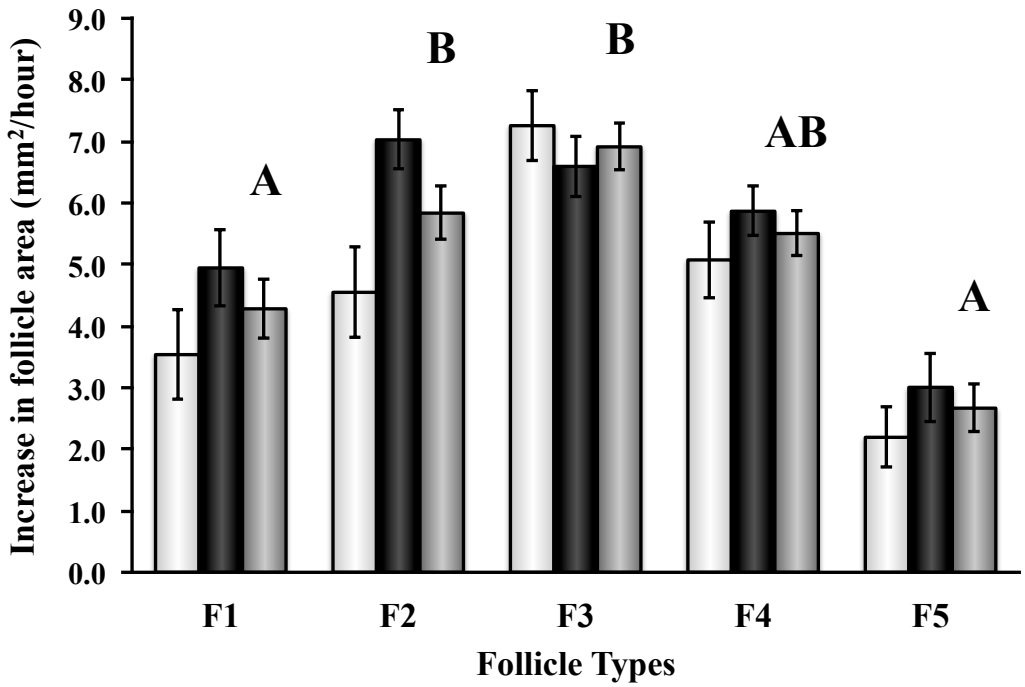

Figure 6.4 Comparison of F1 to F5 follicles increase in area (Mean ± SEM) over time, from 10 Barred Plymouth Rock hens monitored over a 254hr time period. Barred Plymouth Rock hens were acclimatized to a LD 14:10 photoperiod, (lights: on 4:00am, off 6:00pm), based on day growth [white], night growth [black], and combined [grey]. Growth rate at night was significantly higher (P=0.009) for follicles when compared to day. Growth rate was also significantly different between follicle types (F1-F5), different characters (A,B) are significant (P<0.05).

6.6 Discussion

The goal of this study was to track individual follicles to determine the overall rate of growth from F5 to ovulation, and if certain factors (day/night or type) had a significant effect on follicle area growth rates in chickens. It was shown that ultrasound scans performed at an interval rate of 24hrs would encounter a significant change in follicle size, leading to the recommendation of serial scans twice a day instead. Based on the high detection rate (Fig. 6.2) ultrasound scans were shown to be very accurate when observing follicles in BPR. The curve estimation analysis of follicles from F5 to ovulation had a cubic growth rate, showing that there was a significant difference between growth rates for different follicle types (F1-F5). It was also shown that growth at night for follicles was significantly higher than the growth rate in the daytime. This work demonstrates that it is possible to predict the follicular growth in chicken using a non-invasive method.

In chickens preovulatory follicles have a rapid increase in size and can have up to a ten fold (4 – 40mm, diameter) increase (Marza and Marza, 1935) in a short space of time, 7-11 days (Gilbert, 1972). To track follicles increasing in size at such a rapid rate, it is crucial to perform serial scans at an appropriate frequency as to be able to track this drastic change. It was shown that the proportional change in follicle diameter over a 10-hour period in the daytime did not have a significant change in follicle size, although a 24-hour period did see a dramatic increase in follicle size (Fig. 6.1). This means that scans can occur 10 hours apart, without missing a significant proportional change in Barred Plymouth Rock. The recommendation here is to scan BPR hens, which have 3-6 large yellow preovulatory follicles, twice a day morning and afternoon to accurately monitor follicle growth.

It is crucial next to validate this technique, by comparing detection of follicles by ultrasound with the same post-mortem detection. Our validation of technique, on three birds euthanized after the final scanning session showed a high level of detection at 92.3%, with only one F5 follicle being missed (Fig.

6.2). This is a much higher accuracy than previously reported for ostriches, which was 58% (Bronneberg and Taverne, 2003). This is most likely due to the number of rapid growth follicles (9-15) in ostriches, which are between 3.1-9.0 cm in diameter, when compared to BPR, which had on average 3-6.

Here through curve estimation analysis we have shown that the relationship between time before ovulation and the follicle area for BPR hens was best described by a cubic equation (Fig. 6.3). Meaning that the growth rate (slopes) of follicles at the beginning and end of the curve, are lower than the middle portion. Due to the apparent void in the literature with respect to folliculogenesis in BPR hens a comparison to other poultry and wild birds was necessary. There has been a variety of growth rates already reported in the literature for different types of birds (Etches et al., 1983; Astheimer and Grau, 1989; Jensen and Durrant, 2006). Although a cubic growth rate hasn't been reported to date. A similar curve pattern has already been shown for White Leghorn chicken. Whose follicle growth rate was best described by the quadratic equation: follicle diameter = $-6.36 \times 10^{-5}(\text{time}^2) + 2.47 \times 10^{-2}(\text{time}) + 1.11$ (Etches et al., 1983). The Etches equation only differs from our own by one term (x^3) this is most likely a result of the time windows used between groups. Our work analysed follicles 192 hours before ovulation, whereas the Etched group analysed follicles 120 hours before ovulation, a shorter time window (Etches et al., 1983). Although both groups follicles at the 120 hr time point and ovulation were similar, which means we can reject the idea that the follicles grew faster for Leghorn than the BPR ones. For seabirds and kiwi's, follicle growth was shown to fit a positive quadratic and exponential equation respectively (Astheimer and Grau, 1989; Jensen and Durrant, 2006). Chickens are required to produce a large quantity of follicles in a year, whereas wild birds are only required to produce one or two clutches per season. Seabirds must react quickly to optimum breeding periods, which is possible one explanation why their follicle growth rates are higher (Astheimer and Grau, 1989). Kiwis apart from being a wild bird are also one of the only species, which only produce one rapidly growing follicle at a time (Jensen and Durrant, 2006). This allows all the energy directed toward reproduction to be directed to one follicle, possibly allowing for this exponential follicle growth. It is unclear if domestication of chickens has lead to a

change in follicle growth patterns. Folliculogenesis studies on distant relatives would answer this question.

Another application of deriving a growth curve for follicles would be to determine outlying follicle growth curves, especially subpar growth curves. Two follicles from BPR hen #9 had low growth rates, outside the 95% confidence interval, showing this subpar growth rate (Fig. 6.3). This subpar growth rate from BPR #9 would be a reason to eliminate this bird if it was part of a high performance laying line and could be removed from the genetic pool to improve efficiency and uniformity.

In the case of Kiwi's mentioned previously follicle growth occurs one at a time (Jensen and Durrant, 2006), in other species the follicles grow in defined clutch's, with a specific preovulatory hierarchy (F1-F5). For this study, it was shown (Fig. 6.4), that the growth rate of follicles in the night was higher ($P=0.009$), than that of the follicle growth in the day. This is in agreement with yolk ring studies which also showed increased growth at night verses the day for coturnix quail and cackling goose, but not to such a drastic degree in domestic chickens (Grau, 1976). As yolk production has a large energetic cost associated with it (Astheimer and Grau, 1989), and as most wild birds become inactive at night, it seems that the energetic cost of yolk depositing is most compatible with the night resting period. It is unclear if this means that nutrients consumed in the evening are more likely to be incorporated into the yolk.

When the follicle types were looked at separately, the growth rate between F1-F5 follicles (Fig. 6.4) was shown not to be constant among all types. Follicle growth by type showed a two-fold increase from F5 to F3 follicles and then a subsequent drop back down with the F1 follicles (Fig. 6.4). The lower value of growth for F1 follicles could be influenced by the rest phase of follicles before eventual ovulation (Etches et al., 1983). The F5 to F3 growth rate increase shows the acceleration of the rapid growth phase in yolk depositing. The acceleration of yolk growth would come from the ability of the follicle to allow the

hepatic yolk precursors to come into contact with the developing ovum or the ability of the ovum to allow these precursors to transverse across the plasma membrane.

Ultrasonography of chickens to measure folliculogenesis could be implemented by the poultry industry to determine the growth rate of follicles between different individuals within a specific poultry line. On the other hand if the goal of the producer were to look solely at egg production then collecting of eggs would be a sufficient technique. Ultrasonography gives the industry a tool to measure follicle growth rates, and allows for the elimination of birds with subpar follicle growth rates. These eliminations could strength the commercial poultry lines, by discarding the non-fit animals. This all can be done in a non-invasive humane manner, and allows for repeated measure of the same individuals. Also superior breeding stocks can be analyzed without having to reduce the population size. In conclusion, scanning of layer hens via ultrasonography as a way to determine folliculogenesis growth rates and patterns has now been demonstrated as a valid technique, for the first time.

CHAPTER 7: DISCUSSION

The overall goal of this thesis was to show that turkey gonads could be cryogenically stored and upon retrieval be transplanted into a recipient line, which would allow the immature gonads to develop normally. This would allow for the entire genome of turkeys to be cryopreserved and would be a way to preserve the genetic diversity still present in turkey breeds for future generations. In addition, I also wanted to show that ultrasonography is a valid tool in monitoring folliculogenesis in chickens from emergence of follicles all the way to ovulation. From the ultrasound data, I determined the growth rates and patterns of follicles. This study will give the poultry industry another method of producing a more uniformed flock with respect to follicle growth patterns. Based on our vitrification results from chapter 4, I have shown for the first time that turkey ovaries can be cryopreserved and upon warming be deemed viable, through a morphological grading scheme. For testes, I have shown that the standard protocol used on quail gonads is not adequate. Although, I have improved this vitrification protocol for testes, it appears that the protocol still needs further optimizations and improvements. Here it was demonstrated for the first time that turkey poults at 1 to 7 days of age could survive gonad transplantation (Chapter 5), with the future recommendation to use day old poults. After transplantation, grafts from fresh or vitrified tissue did not develop normally or at all, suggesting an immune suppression issue. Here it was demonstrated for the first time that folliculogenesis in chickens could be monitored by ultrasonography, to track follicles from emergence to eventual ovulation (Chapter 6). I was able to show that in BPR hen's follicle growth was best represented by a cubic equation. Also, follicles grew more in the night than compared to the day, with F2 and F3 follicles having the highest growth rates.

Throughout this research, it has become clear that the vitrification and subsequent transplantation of gonadal tissue to produce donor-derided offspring will not be a standard procedure among all poultry species. This technique will inevitably require troubleshooting and fine-tuning for each new application. The cryobiological properties of different cell lines from a variety of species and taxa will require species-

specific protocols. The first step must be to demonstrate that vitrification of gonads can provide viable tissue post-warming and this can be determined by using cell and tissue viability tests. For day old turkey gonads, we showed that the time spent in cryopreservation solutions needs to be increased for gonads that are left whole. This increase of time could plausibly allow a proper saturation of cryoprotectants to occur. It appears that further troubleshooting needs to be conducted on testes, as their tissue viability scores for centre seminiferous cords are still not below the acceptable threshold (Score ≤ 1). This troubleshooting could potentially look at four areas *(1) membrane permeability to water and cryoprotectants; (2) cryoprotectant toxicity; (3) tolerance to osmotic changes; and (4) resistance to cooling and freezing temperatures* (Comizzoli et al., 2012). Staying with vitrification as the cooling method and by-passing the tolerance to osmotic changes, it would seem appropriate to focus on points (1) and (2) with respect to permeability of the membranes and cryoprotectant toxicity (Gandolfi et al., 2006; Curaba et al., 2011; Shaluei et al., 2014). Biochemical manipulation of the membranes would be an extreme measure. Instead, mechanical modification by tearing the tunica vaginalis (testes) or germinal epithelium (ovary) of the organ to allow for cryoprotectants to come into contact with the plasma membranes of cells is easier, more appropriate, and standard practice. This tearing either keeps the tissue below whole and only disrupts the external membranes, or separates all layers leaving the tissue in smaller more appropriate sizes. For ovarian cortex, the dimensions of sections appropriate for cryopreservation have been determined at approximately 0.5-1mm^3 for human and a wide variety for species (Gandolfi et al., 2006; Jewgenow et al., 2011; Comizzoli et al., 2012). For immature testicular tissue, the appropriate dimensions are slightly larger at 3-4 mm^3 for vitrification and subsequent viability post-warming (Curaba et al., 2011; Honaramooz, 2012; Baert et al., 2013). For day old turkey gonads, it seems applicable to section the gonads into smaller pieces and to repeat the vitrification procedure and viability tests.

The toxicity from cryoprotectant agents' (CPA's) is challenging to tease apart from the overall cryopreservation damage, from the freezing process itself. One way to test the toxicity of CPA's without being masked by damage from cryopreservation would be to analysis tissue that had been treated with

CPA's but was not vitrified. This type of control was not performed here. A variety of permeable and non-permeable CPA's must be screened to determine which facilitates the highest viability post-warming. Permeable CPA's that are commonly citied in the literature are: glycerol (Gly), dimethyl sulfoxide (DMSO), methanol (MeOH), propylene glycol (PG) and ethylene glycol (EG) (Gandolfi et al., 2006; Curaba et al., 2011; Honaramooz, 2012; Shaluei et al., 2014). On the non-permeable CPA's side, the common ones are: glucose, sucrose, fructose, trehalose and polyvinylpyrrolidone (PVP) (Curaba et al., 2011; Honaramooz, 2012; Shaluei et al., 2014). For further trouble-shooting we can eliminate glycerol based on its contraceptive qualities with avian sperm, plus DMSO, EG and sucrose as they have already been tested, we are left with a reduced field of CPA's (MeOH, PG, glucose, fructose, trehalose and PVP) to troubleshoot.

The second step is to determine the most appropriate age group of the poults on which to perform this challenging surgery and the best type of method to secure the donor gonads (Chapter 5). The former was achieved by monitoring the vital rates of the chicks throughout surgery, by comparing the survival rates of different age groups, and by observing the graphs within the first couple of days post-surgery. Poults can withstand this procedure at one day of age, like chicks. However, they require a combination of drugs to remain in a constant plane of anaesthesia. Although previous literature has shown that securing of the grafts orthotopicly is not necessary for chicken, quail and ducks (Song and Silversides, 2006; 2008a; Song et al., 2012), placing the gonads underneath the peritoneum membrane orthotopicly appears to allow for a more stationary position, which could facilitate proper vascularization.

In the final step, grafts were recovered post-surgery from poults (chapter 5). This allowed for visualization of the tissue morphology by light microscopy to determine if proper growth and maturation occurred. At the beginning of this monitoring, it is unavoidable that birds are sacrificed for graft retrieval and analysis. At later time points, post-surgery the testes can be observed by ultrasonography when they reach a substantial size. For females when folliculogenesis reaches the rapid growth development stage,

the status and activity of the ovaries can also be monitored using ultrasonography. For turkeys, it is apparent that grafted testes lost normal structure (Fig. 5.1) and did not develop naturally within the first 4-weeks post-surgery. This corroborates the previous evidence of improper cryopreservation technique, from chapter 4, as one possible factor, which led to failure of the grafts to develop. It was apparent that the immunosuppression of the poults was inadequate as fresh grafts also failed to grow. Although Mycophenolate Mofetil (MMF) has been shown to be an effective immunosuppressant with respect to mammals with the prevention of transplant rejection (Wu, 1994). It appears in current literature to be relatively untested on avian species. Previous work on quail showed that there was a statistical difference between the non-immunosuppressed (NIS) groups versus the immunosuppressed (IS) groups in their ability to produce donor-derive offspring only (Song and Silversides, 2008a). With only 25% of the NIS quails producing donor-derived offspring compared to the IS group which had a 100% success rate. In chicken the NIS and IS groups produced donor-derived offspring 25% and 33% of the time respectively (Song and Silversides, 2007a). In our work we did not compare NIS to IS groups, as we only had IS groups. For the quail work, it was not determined if white blood cell counts were actually lowered in IS verses NIS groups; instead success of the immunosuppression therapy was inferred by a higher production of donor-derived offspring. From this inferring strategy, it would appear that MMF works on quail but does not have an effect on chickens as an immunosuppressant. If we move our focus away from vitrified grafts in our own work, due to potential improper cryopreservation technique, and instead focus on fresh grafts of which there were six. We would have expected based on the chicken and quail results from Song and Silversides (2007a; 2008a) to have somewhere around a 25% percent success rate, in retrieving mature grafts. Even if MMF had no effect in reducing the T and B cell count in turkeys. It is possible that the immune system of turkeys function in a different way to that of quails and chickens, having a much lower base line for transplantation success. This lowered baseline might be from the turkey having a stronger adaptive immune system, although current evidence shows that domestic turkeys are on the whole more susceptible to infections from viruses (Tumpey et al., 2004; Spackman et al., 2010) suggesting a weaker immune system. In any cases, the options moving forward are to test MMF and its

actual effectiveness on lowering white blood cell count in turkeys, or to change to another immunosuppression method, which has been proven to work on turkeys. One such option would be bursectomy, which removes the Bursa of Fabricius, the organ producing B cells in birds. With its removal, the body is unable to produce novel anti-bodies, severely reducing the body's adaptive immune response (Schlink and Olson, 1987). Ethical questions might arise from conducting gonad transplantations and bursectomies on day old poults. Thus, a different immunosuppressant appears a better solution. Two immunosuppressants that have been proven to be effective in turkeys and chickens are: Cyclosporin A (Bucy et al., 1990) and Cyclophosphamide (Ficken and Barnes, 1988; Al Afaleq and Jones, 1991; Jones et al., 1992). These drugs have also been compared side by side in trials (Loa et al., 2002). Cyclosporin A (CsA) works by inhibiting T cell activation and reduces the ability of these cells to produce cytokines needed in an immune response (Schreiber and Crabtree, 1992). This was demonstrated by a 2 to 3-fold decrease in proliferation of lymphocytes in response to a T cell mitogen (Loa et al., 2002). Cyclophosphamide (CP) works by reducing the number of lymphocytes in the thymus and bursa (Ficken and Barnes, 1988). However, the most noticeable change is the deficiency in B cells indicated by a reduction in antibody production in response to foreign erythrocytes (Loa et al., 2002). A combination of CsA and CP could be a valid alternative given the likely ineffectiveness of MMY to suppress the immune system in turkeys.

Due to the fact that adult turkeys with grafts were not available in the folliculogenesis study, chickens (Barred Plymouth Rock) were substituted and the growth rates and patterns of follicle development were observed. Although the study of folliculogenesis is not a new study, the application of ultrasonography as a means of observing and tracking this biological process from the detection of a follicle to ovulation in chickens is a novel application. This technique has been used on ostriches and kiwis among other birds (Bronneberg and Taverne, 2003; Jensen and Durrant, 2006). The major scientific advantage of studying folliculogenesis by ultrasonography over dichromatic staining of yolk or post-mortem analysis of follicles is the ability to gather accurate data in the context of neighbouring follicles. By using ultrasonography,

follicle growth rates can be determined within the context of follicle type (i.e. F1-F5) and as a follicle progresses through each of these positions in the preovulatory hierarchy. This has not been possible with previous techniques. With post-mortem analysis of follicles, it would be difficult to tell if a follicle was undergoing atresia. With dichromatic staining of yolk atretic follicles would be missed altogether. It is understood that as soon as follicles enter the rapid growth phase, they only rest at the end, just before ovulation (Johnson and Woods, 2007). In our study, we noticed that follicle growth of the F1 follicle was lower than the F2 showing this pause in growth before ovulation (Fig. 6.4). If follicle growth paused at any other position, due to stress or nutritional deficiency of the bird, this ultrasound technique would be able to determine this as well. With respect to the direct application of ultrasonography on gonad transplantation the grafts can be monitored later in development; this monitoring can shed new light on the control mechanism behind follicle development. It could determine whether a transplanted ovary's follicle pattern matches that of the original donor or the new host, or a new pattern could emerge due to an amalgamation between the genetically different brain and ovary. This also allows for the study of transplant recipients that do not produce eggs due to internal ovulation but are still producing follicles.

Ultrasonography detection of follicles can also be used by the poultry industry both on the broiler and layer side. For breeder broiler parental lines, EODES causes a number of disorders that lower productivity, (an increasing issue due to the fact that the industry pushes broilers to reach a market weight in an increasingly shorter time frame). Future work will determine which biological system has the most control over folliculogenesis: the ovary or the recipient's brain, and will lead towards a solution to this industry problem. On the laying side, although maximization of egg production is reaching the upper limit of an egg a day, there may still be room to eliminate birds with subpar folliculogenesis patterns from the pool of primary breeders. For lines with a superior egg laying efficiency, determining the time of day with the greatest yolk growth would allow for better timing of nutrients to be delivered. It is probably best to deliver nutrients before this growth period, if the goal was to deliver these nutrients into the yolk.

CHAPTER 8: FUTURE DIRECTIONS

With respect to day old turkey gonads, it appears that further troubleshooting of the vitrification protocol is required especially in the case of testicular tissue. The next parameters to be tested would be the most appropriate size of the gonadal tissue (1-4mm^3) and the different types of CPA's (MeOH, PG, PVP, glucose, fructose, trehalose) used before vitrification. If the gonads are left whole, then the conditions of the tissue with respect to tearing of the tunica albuginea and tunica vaginalis in the case of testes needs to be tested. Viability can be assessed again using the same methods outlined in this thesis.

The number of fresh tissue transplants in this study limited the amount of possible time points assessed. The result of no fresh grafts being retrieved at the later time points suggests an immunosuppression issue. Even though MMY has been shown to work on quail, it has not been shown to reduce the level of T and B cells in turkeys. This highlights a need to determine if the levels of white blood cells are actually being reduced in turkeys. It is possible that MMY is reducing the level of B and T cells, but administering this immunosuppressant weekly in the older poults is insufficient to reduce the levels over the entire week. Alternatively, MMY does not work on poults and so switching to a combination of CsA and CP is suggested.

With respect to using ultrasonography as a tool to determine the folliculogenesis pattern in poultry species, only one breed at one specific age was explored. By expanding this work and looking at a variety of age groups, breeds, species and commercial or heritage lines, this technique will be validated further and shed light on potential folliculogenesis traits not yet fully exploited by the poultry industry. Comparisons between egg laying lines versus broiler breeders might help in understanding why broiler breeders have issues with multiple follicular waves.

CHAPTER 9: GENERAL CONCLUSIONS

In conclusion, the results of this thesis show that for large white day old turkey gonads left whole, the standard vitrification protocol used on other poultry species gonads is not appropriate for proper cryopreservation. Instead, a longer period of time in cryopreservation solutions is required for ovarian tissue, and while this improved the viability for testes they still appeared to be unviable upon recovery.

This study also showed that bronze day old turkeys could withstand ovariectomy or testectomy with a relatively high survivability. Previously vitrified and fresh gonads transplanted into the aforementioned poults did not develop normally, suggesting both a vitrification and immunosuppression problem.

Finally, it was also shown that ultrasonography was a useful tool in determining folliculogenesis growth rate and patterns in Barred Plymouth Rock hens.

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APPENDICES

B. Supplementary materials from Chapter 5

Appendix B.1 The average (Mean ± SEM) minimum and maximum values for isoflurane, body temperature and respiration count for Large White turkey poults throughout surgery Trial 1. BPM = Breaths Per Minute.

WT (ID#)		Isoflurane Range (%)	Body Temperature Range (^{o}C)	Respiration Count Range (BPM)
2		2.5-3	-	16-60
1		3	-	40-64
3A		1.75-3	-	24-44
3B		1.5-3	-	52-56
3		2-3	-	28-44
4A		2.5-3	32.4-35.9	36-60
4B		1-3	34.1-36.8	32-64
5		1.5-2	38.2-38.7	16-56
6A		3-5	38.2-39.2	60-70
6B		2-3	37.4-39.5	16-48
6		3-4	35.2-38.8	44-80
7A		2-2.75	38.6-39.4	24-60
7B		2-3.5	38.1-39.3	16-60
8		3-3.5	37.3-38.6	68-80
9		3-4.5	37.1-39.2	44-68
10A		2.5	38.0-38.2	16-48
10		3-3.25	34.7-36.0	52-68
11		3-4	36.2-37.3	40-68
12A		3.5-3.75	37.9-38.4	16
Mean ± SEM	**Min**	**2.41 ± 0.16**	**36.7 ± 0.5**	**34 ± 4**
	Max	**3.30 ± 0.16**	**38.2 ± 0.3**	**59 ± 3**

Appendix B.2 The average (Mean ± SEM) minimum and maximum values for isoflurane, body temperature and respiration count for Large White turkey poults throughout surgery Trial 2. BPM = Breaths Per Minute.

WT (ID#)		Isoflurane Range (%)	Body Temperature Range (^{o}C)	Respiration Count Range (BPM)
14		2.5	36.6-38.1	16-28
16		1.5-3	37.6-38.7	52-64
17B		2-3	37.8-38.4	24-72
18		2.5-3	37.3-38.9	20-64
15		2-3	37.6-38.7	28-76
17A		1.5-3.5	37.0-38.1	12-56
19		2.5-3.5	36.6-37.5	16-52
22		2-2.5	38.4-39.0	32-56
24		1.5-2	38.6-39.0	16-32
23		1-2.5	37.5-38.7	52-64
25		1.5-3.5	38.6-39.0	40-68
26		1-4	37.5-38.9	28-56
20		1-2.5	36.7	40
21		1-3	37.9-38.4	54
20A		3	38.0-38.5	40
27A		2.5-4	37.3-39.0	56-64
27		1.5-3.5	37.6-37.8	16-44
28B		3-4	35.0-37.8	40-60
28A		3-5	36.8-38.3	44-72
28		3-5	38.2-38.5	44-68
29A		3.5-5	36.8-37.8	40-60
Mean ± SEM	**Min**	**2.05 ± 0.17**	**37.4 ± 0.2**	**34 ± 3**
	Max	**3.38 ± 0.19**	**38.4 ± 0.1**	**57 ± 3**

Appendix B.3 The average (Mean ± SEM) minimum and maximum values for isoflurane, body temperature and respiration count for male Bronze turkey poults throughout surgery Trial 3. BPM = Breaths Per Minute.

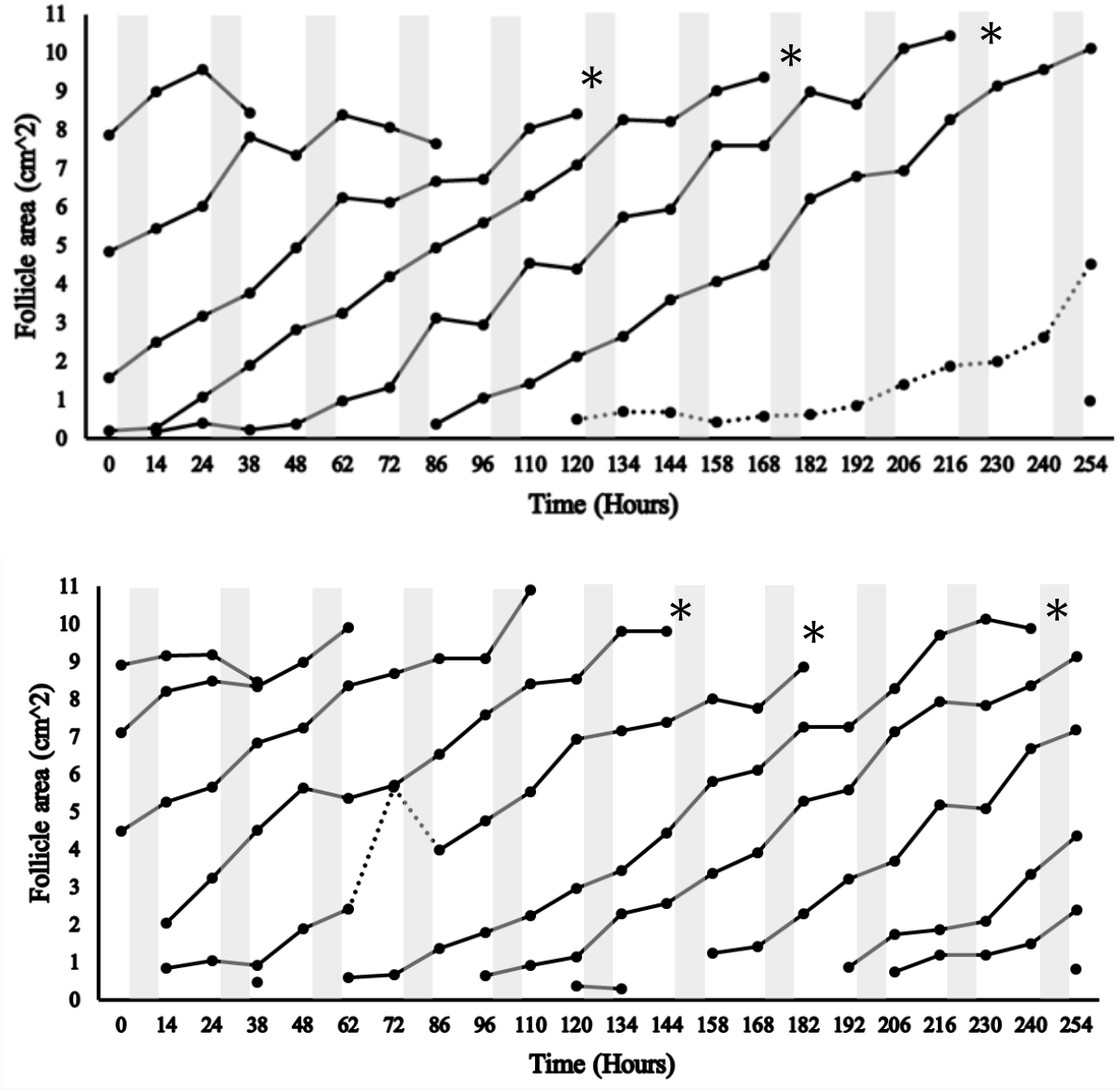
BT (ID#)		Isoflurane Range (%)	Body Temperature Range (^{o}C)	Respiration Count Range (BPM)
30C		2-5	37.1-38.5	52-72
30		2.75-4	38.5-39.4	28-64
32		0.5-1.5	37.9-38.6	12-24
33		0.5-4.5	38.3-39.2	12-32
34		1-3	37.8-38.9	12-16
35		1.5	36.2-38.9	12-16
36		1-2	37.4-39.4	20-30
37		0.5-3	36.8-38.4	20-30
39		0.5-2	38.9-39.5	12-20
40		0.5-1.5	34.9-36.7	12-24
45		1	38.2-39.9	16-24
46		1	35.7-37.6	16-20
53		0.5-2	37.5-38.6	12-28
54		0.5-1	36.0-38.3	8-24
55		0.5-2	36.6-39.3	16-28
58		1-4.5	37.7-39.8	28-56
59		0.5	38.0-39.3	16-24
60		0.5-1	36.9-38.8	12-36
63		0.5-1.5	37.6-39.0	12-20
67		0.5-1.5	35.2-38.3	16-20
68		1.5-2.5	34.9-35.9	60
70		1	35.3-36.6	12-20
72		0.5-1	36.7-38.7	16-20
Mean ± SEM	**Min**	**0.88 ± 0.12**	**37.0 ± 0.2**	**19 ± 3**
	Max	**2.11 ± 0.27**	**38.6 ± 0.2**	**31 ± 3**

Appendix B.4 The average (Mean ± SEM) minimum and maximum values for isoflurane, body temperature and respiration count for female Bronze turkey poults throughout surgery Trial 3. BPM = Breaths Per Minute.

BT (ID#)		Isoflurane Range (%)	Body Temperature Range (^{o}C)	Respiration Count Range (BPM)
30A		2-3	37.3-37.9	16-60
30B		2.5-5	38.1-38.6	16-64
41		1-2.5	35.4-38.3	20-32
42		1-1.5	38.0-39.2	20-32
43		1	37.2-38.7	16
44		1	38.0-39.0	12-20
52A		0.5-2	38.6-39.5	12-24
52		1-1.5	38.1-39.4	12-24
56		0.5-1.5	38.5-39.2	12-24
57A		0.5-2	38.3-39.7	16-44
57		0.5-2.25	39.5-40.0	16-36
61A		0.5-4	37.7-39.0	20-56
61B		0.5-2.5	36.8-38.8	12-20
61		0.25-0.5	36.3-38.4	16-24
62		0.5-3	37.7-39.4	12-28
64		0.5-2	37.4-38.9	12-24
65		0.5-1.5	37.0-38.7	20-32
69		0.5-1	36.5-38.0	40-60
71		0.5	35.6-38.0	12-20
Mean ± SEM	**Min**	**0.80 ± 0.13**	**37.5 ± 0.2**	**16 ± 1**
	Max	**2.01 ± 0.27**	**38.9 ± 0.1**	**34 ± 4**

C. Supplementary materials from Chapter 6

Appendix C.1 Follicular progression from two Barred Plymouth Rock hens over a 254-hour time period, acclimatized to a LD (14:10) photoperiod. Follicle area was plotted against time after the first scanning session. Light grey backgrounds represent the 10hr dark period. Solid black lines, which represent a growth rate, where used to determine which factors: time (day vs. night), type (F1-F5) and bird ID (1-10) played a significant role in follicle area growth rates. Follicles denoted by (*) at there terminal point, were aligned based on time before ovulation (App. C.2). Dotted lines were not analysed and were rejected either due to abnormal follicle growth pattern or due to human error in data collection.



Appendix C.2 Follicular progressions for three follicles from two Barred Plymouth Rock hens selected from (App. C.1). Follicle area was plotted against last observation of follicles in proximity to the ovary (follicles are assumed to have ovulated soon after). Individual follicles are denoted by different line patterns.