Effects of oocyte bisphenol A exposure on aspects of oocyte maturation and early embryo development in *Bos taurus*

by

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The microenvironment of the oocyte can influence oocyte quality, competence, and developmental potential. Alterations in this environment can have negative effects on oocyte maturation and subsequent embryo development. Oocyte quality, which can determine embryonic viability, is easily perturbed, thus factors that can alter normal oocyte maturation are a considerable concern. Bisphenol A (BPA) is an endocrine disrupting chemical that has been found to elicit a variety of reproductive effects. Exposure to BPA is considered to be ubiquitous and it has been found in a variety of human samples, including follicular fluid. BPA has previously been found to disrupt meiosis in mouse and human oocytes, however the embryonic effect in mammals are not as well documented. In the current study, bovine oocytes were matured in vitro under various treatment conditions such as no-treatment control (IVM media), vehicle control (0.1% ethanol), estradiol (2 μg/mL E2), and 15 ng/mL and 30 ng/mL BPA. The mature oocytes or subsequent embryos were collected for various analyses to determine effects on quality and developmental potential. Exposure of groups of oocytes to 15 ng/mL and 30 ng/mL BPA resulted in an average oocyte uptake of 1.69 and 2.48 ng/mL BPA, respectively. Exposure of bovine oocytes in vitro to 30 ng/mL BPA during maturation induced meiotic perturbations as well as poor embryonic outcomes. Meiosis progression was reduced and abnormal spindle morphology and chromosome
alignment were increased. Under the same treatment conditions, resulting embryos exhibited decreased embryonic development rates, increased apoptosis, and a skewed sex ratio. Gene expression in blastocysts were not altered, whereas treatment with 15 ng/mL BPA resulted in increased expression of CDC2, AURKA, DAZL, TRβ and p53 in MII oocytes relative to that of the IVM group. This increased p53 expression resulting from the 15 ng/mL BPA group was also significantly greater than the expression levels resulting from 30 ng/mL BPA. There appeared to be a slight vehicle effect, with the vehicle group (0.1% ethanol) resulting in significantly increased expression of AURKA mRNA and non-significant increases in several other genes analyzed. BPA exposure during oocyte maturation in vitro can therefore, in a dose-dependent way, decrease oocyte and embryo quality and developmental potential.
DECLARATION OF WORK PERFORMED

I declare that with the exception of the items indicated below, all work reported in the body of this thesis was performed by me.

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LIST OF ABBREVIATIONS

AI – anaphase I
ART – assisted reproductive technology
AURKA – aurora kinase A
BPA – bisphenol A
CDK1 – cyclin-dependent kinase 1
CDC2 – see CDK1
COC – cumulus oocyte complex
DAZL – deleted in azoospermia-like
DES – diethylstilbestrol
EDC – endocrine-disrupting chemical
EGA – embryonic genome activation
ERα – estrogen receptor alpha
ERβ – estrogen receptor beta
FSH – follicle stimulating hormone
GLUT1 – glucose transporter 1
GV – germinal vesicle
GVBD – germinal vesicle breakdown
hCG – human chorionic gonadotropin
HPG – hypothalamus-pituitary-gonad axis
HSP – heat shock protein
ICM – inner cell mass
IVC – *in vitro* culture
IVF – *in vitro* fertilization
IVM – *in vitro* maturation
IVP – *in vitro* produced
KIF5b – kinesin family 5b
LH – luteinizing hormone
LOAEL – lowest observed adverse effect level
MBT – mid-blastula transition
MET – maternal to embryonic transition
MI – metaphase I
MII – metaphase II
MTOCs – microtubule organizing centers
MZT – maternal-zygotic transition
N-CoR – nuclear receptor corepressor
OP – octylphenol
PMII – prometaphase II
ProI – prometaphase I
ROS - reactive oxygen species
T₃ – triiodothyronine
T₄ - thyroxine
TCDD – 2,3,7,8-tetrachlorodibenzo-dioxin
TE – trophoblast
THs – thyroid hormones
TI - telophase I
TRα – thyroid receptor alpha

TRβ – thyroid receptor beta

TUBA – alpha tubulin

VEGF – vascular endothelial growth factor (VEGF)
INTRODUCTION

The oocyte and early embryo are particularly sensitive to their immediate surroundings. The composition of follicular fluid greatly influences the developmental potential of oocytes and resulting embryos, and alterations may result in decreased fertility of the mother, or have long-term, permanent effects on the offspring (Eppig et al., 1996; Carabatsos et al., 2000; Garrido et al., 2000; Voronina & Wessel, 2003; Da Broi et al., 2013). Alterations in the oocyte’s microenvironment (follicular fluid or in vitro maturation media) can influence oocyte metabolism and developmental competence, gene expression patterns, and blastocyst quality and implantation as well as pregnancy success (Leroy et al., 2012). Furthermore, evidence suggests that the environment of the oocyte may affect adult health of the resulting offspring, as well as the subsequent generation (Wolstenholme et al., 2012; reviewed by Krisher, 2013). The components of follicular fluid impact oocyte maturation and embryonic development since oocytes rely on external signals from their environment for growth, maturation, fertilization capability, and developmental potential (Zheng et al., 2003; Aardema et al., 2013; Krisher, 2013). Thus the ovarian follicle represents a fragile microenvironment which strongly influences oocyte competence.

Variables in follicular fluid such as hormone levels, temperature, free radicals, fatty acid levels, and glucose levels can alter oocyte maturation and alter embryonic developmental potential (Kreiner et al., 1987; Hodges et al., 2002; Leroy et al., 2006; Marei et al., 2010; Aardema et al 2013; Valckx et al., 2014). Alterations of follicular fluid can also have negative effects on oocyte maturation and early embryo development by activating stress pathways such as is observed in heat stress, oxidative stress, and metabolic stress (Hodges et al., 2002; Robker et al., 2009; Leroy et al., 2012). Since oocyte quality appears to be easily perturbed, a growing concern is how
environmental exposure to chemicals that have been detected in follicular fluid, may affect fertility and early development.

Endocrine disrupting chemicals (EDCs) are a group of chemicals which can disrupt normal endocrine functions by mimicking or antagonizing the actions of hormones (Colborn et al., 1993). EDCs may alter fertility through disruption of the hypothalamus-pituitary-gonad (HPG) feedback loop, or by their presence in the follicular fluid where they can act directly on the oocyte and surrounding cumulus cells (Craig et al., 2011; Fowler et al., 2012; Qin et al., 2013). In general, EDC exposure to oocytes during maturation results in decreased fertilization rate and decreased developmental potential (Petro et al., 2012). Interestingly, some EDCs such as octylphenol (OP) appear to be acting through alternative mechanisms than direct estrogen disruption (Pocar et al., 2003). In recent years it has been recognized that EDCs may alter fertility by acting as environmental stressors (Wang et al., 2012; Krisher, 2013). Thus EDCs may affect oocyte developmental competence through hormonal pathways, alternative pathways such as stress activation, or via multiple mechanisms. This is why it has been difficult to fully elucidate the mechanism of EDCs, such as bisphenol A (BPA), in early development, and why controversy remains in the field.

BPA was first synthesized in 1891 and its estrogenic properties first discovered in the 1930s (Dodds & Lawson, 1936; reviewed by Wolstenholme et al., 2011). BPA would have been used as a pharmaceutical if it were not for the discovery that diethylstilbestrol (DES) was much more potent than BPA (Dodds & Lawson, 1936). BPA’s other properties made it central in the development of polycarbonate plastics and epoxy resins (Ortiz et al., 2009). As such, BPA is now a common component of many consumer products that we come into contact with every day (Vandenberg et al., 2007; Ye et al., 2009). It is not surprising therefore that in one study in 2005
BPA was detected in >95% of an American reference population (Calafat et al., 2005) and >97% detection was recorded in 2011 (Braun et al., 2011). BPA levels have also been detected in follicular fluid of women at an average level of 2.4 ng/mL (Ikezuki et al., 2002). In vitro and in vivo studies have demonstrated BPA exposure to the cumulus oocyte complex (COC) can result in oocyte abnormalities such as aneuploidy and meiotic arrest (Hunt et al., 2003; Can et al., 2005; Susiarjo et al., 2007; Lenie et al., 2008; Machtinger et al., 2013). However, the mechanism by which BPA exposure to oocytes during maturation affects the earliest life stages is not as clear. Early developmental changes that occur as a result of environmental exposures may give an idea of how these chemicals exert their effects.

Oocyte quality and developmental competence are critical in the establishment of a healthy pregnancy (Leroy et al., 2012). Examination of effects at the oocyte and early embryo stages shed light on how maternal exposure to BPA may impact fertility and offspring health. The goal of this thesis is to evaluate the effects of BPA exposure during bovine oocyte maturation in vitro on aspects relating to developmental competency and quality of the oocyte and early embryo. Nuclear maturation of the oocyte, and the integrity of the metaphase II spindle were assessed to determine the effects of BPA on oocyte quality and embryo developmental potential. Secondly, the preimplantation embryo was assessed following oocyte maturation exposures to evaluate embryonic development, by calculating development rates, and embryo quality by evaluating apoptosis, sex ratio, and total cell number of blastocysts. Finally, gene expression was evaluated in both oocytes and blastocysts to further elucidate the actions of BPA and how early developmental changes may be regulated at the transcriptional level.
LITERATURE REVIEW

Mammalian oogenesis

Oocytes provide both the maternal portion of genetic material, as well as cytoplasmic components necessary for development of the oocyte itself and the early embryo. The oocyte pool is established during gestation, and the life-time supply of oocytes is present in the ovaries at birth contained within ovarian primary follicles. The follicle houses the oocyte throughout its development, and the follicular environment in which the oocyte grows and matures is critical to developmental success. Oocyte development involves highly complex processes consisting of signalling between the oocyte and somatic cells of the follicle. These interactions allow for the regulation of developmental processes such as oocyte metabolism, fertilization, and cell cycle progression (reviewed by Li & Albertini, 2013). These events, brought on by cell-cell communication, particularly that between oocytes and granulosa cells, are essential for the initiation and maintenance of early embryo development (reviewed by Li & Albertini, 2013).

Primary oocytes contained within the follicles that are present from birth are arrested at prophase I, and are maintained in this state by inhibitory signals from cumulus cells surrounding the oocyte (Norris et al., 2009). The primary follicles remain dormant until the onset of menarche, the first egg release. Continuation of development is signalled by somatic cells of the follicle (reviewed by Voronina & Wessel, 2003). The oocyte begins to grow within the follicle, but remains meiotically arrested, and is characterized by an enlarged nucleus, the germinal vesicle (GV). During the growth phase, the oocyte accumulates and stores large amounts of maternal mRNAs, proteins, and organelles that are invaluable for the growth and maturation of the oocyte (Hunter & Moor, 1987; Wessel et al., 2001; Krisher, 2013). Following fertilization, these stores function to support and regulate early embryonic development, particularly until the maternal to
embryonic transition (MET), where embryonic transcription takes over (Voronina & Wessel, 2003; Krisher, 2013). Thus the maternal mRNAs and proteins accumulated during the growth phase of oogenesis are critical factors in oocyte competence (Paczkowski et al., 2011; Krisher, 2013). Following the growth phase, the oocyte will begin maturation, another important period of time in the oocyte’s development.

**Oocyte maturation**

The LH surge which leads to ovulation initiates oocyte maturation which is, in part, characterized by resumption of meiosis from the germinal vesicle stage. Oocyte maturation is a critical period of oocyte development that dictates the developmental potential of the oocyte. It is a highly coordinated process comprising of a series of changes in the oocyte’s nucleus and cytoplasm. These changes are referred to as nuclear and cytoplasmic maturation. Both are essential to oocyte quality as these events directly influence the success of fertilization and embryo development (Combelles & Albertini, 2002).

Nuclear maturation is the resumption of meiosis from prophase I to metaphase II (MII). The oocyte is arrested at this phase until fertilization occurs, or it is degraded without having been fertilized. Nuclear maturation includes germinal vesicle breakdown (GVBD), chromosome condensation, assembly of the meiotic spindle, and ultimately the formation of a large secondary oocyte as well as the first polar body. The metaphase I (MI) spindle is positioned cortically so that loss of cytoplasm during the extrusion of the first polar body is minimal (Brunet & Verlhac, 2010). Following extrusion of the first polar body, the chromosomes which remain in the oocyte continue to progress through meiosis until their arrangement on the meiotic spindle at MII (reviewed by Voronina & Wessel, 2003). Nuclear maturation is complete at this stage, however meiosis II is not
complete until fertilization and extrusion of the second polar body has occurred (reviewed by Li & Albertini, 2013).

The resumption of meiosis and the completion of nuclear maturation are linked to developmental competence. Decreased maturation success, or incomplete meiosis progression are indicators of poor quality oocytes. An oocyte unable to resume and complete maturation under proper conditions is of poor quality and has not obtained the necessary factors during growth that allows it to initiate and sustain maturation. Suboptimal conditions can also lead to an oocyte’s inability to resume and complete maturation, and has been associated with a decrease in the proportion of oocytes reaching nuclear maturity. Thus the ability of an oocyte to complete nuclear maturation may be used as an indicator of oocyte quality and/or the quality of environment in which the oocyte is maturing. Oocytes of good competence may be able to withstand environmental perturbations, whereas lower quality oocytes may not be able to survive under such conditions. Furthermore, the competence of oocytes that are able to reach maturity may be compromised, possibly resulting in long-term detrimental effects on the embryo and offspring health. An important marker of oocyte competence of mature oocytes is the MII spindle.

The MI and MII spindles are critical to the completion of meiosis. The spindle is composed of bundles of microtubules that attach to kinetochores of chromatin, and guide the chromosomes during segregation. At MII, the spindle is barrel- or ovoid-shaped with the microtubules extending inwards from two microtubule organizing centers (MTOCs) to the chromosomes aligned equatorially along the metaphase plate. Proper positioning of the spindles helps to minimize the loss of ooplasm during polar body extrusion (Brunet & Verlhac, 2010), and the risk of aneuploidy that results from improper segregation of the chromosomes (Volarcik et al., 1998). Human oocytes have been found to exhibit a relatively high incidence of errors in chromosome segregation, and
these errors have been linked to pregnancy failure, embryonic abnormalities as well as genetic diseases (Hassold & Hunt, 2001; Li & Albertini, 2013).

Nuclear maturation is one of two important components that together comprise oocyte maturation. Cytoplasmic maturation, though less well defined, plays a critical role in preparation of the cell to support fertilization and early embryonic development. Cytoplasmic maturation consists of various cytoplasmic rearrangements. These changes allow the oocyte to support fertilization and commence embryonic development (reviewed by Krisher, 2013). Some important events that occur during cytoplasmic maturation include metabolism of carbohydrates and lipids, rearrangement of the mitochondria, oxygen radical reduction, epigenetic programming, growth factor secretion, and cross-talk between the oocyte and its surrounding cumulus cells (reviewed by Krisher, 2013). Cytoplasmic maturation is thus also an important determining factor for oocyte quality and ideally should be considered when assessing oocyte quality as well as nuclear maturation. However the only current method of evaluating the completion of cytoplasmic maturation is successful fertilization and positive developmental outcome of embryonic development. Thus nuclear maturation is largely assessed in efforts to determine if oocyte maturation has been successful.

Oocyte maturation is a particularly critical phase of the oocyte’s development and the establishment of developmental competency. The environment in which the oocyte matures can have significant and long-term impacts on the resulting embryo’s ability to sustain development (Combelles & Albertini, 2002), as well as the health of resulting offspring. Because of the highly complex signalling that occurs in the follicular environment, any alterations made to that environment that may disrupt proper signalling can have detrimental effects on the maturing oocyte.
Oocyte competence

Prior to fertilization and embryonic development, the oocyte must acquire developmental competency, defined by various cellular and molecular properties, that enable the oocyte to resume and complete meiosis, support fertilization and oocyte activation, initiate mitosis following fertilization, and support embryonic development until the time at which embryonic transcripts take over (First et al., 1988; Albertini et al., 2003; Krisher, 2013). The process through which these competencies are acquired is lengthy and complex, with important events affecting competency occurring late in the growth phase and during oocyte maturation.

Oocyte competence, or quality, is critical to female fertility, having both short- and long-term implications regarding embryonic survival and implantation, fetal development, and adult health of the offspring. The oocyte is particularly vulnerable and oocyte competence can be affected both by internal and external factors. Internal factors such as gene transcription, protein translation, metabolism, and organization of the cytoplasm contribute to the competence of the oocyte, and competence can be further influenced by the external environment to which the oocyte is exposed.

The importance of spindle function with respect to oocyte quality has long been accepted. The MII spindle is therefore often studied as an indicator of oocyte quality due to its role in meiosis completion, polar body extrusion, and segregation of the chromosomes. A bipolar spindle with normal microtubule assembly is necessary for proper chromosome segregation during meiosis, with improper chromosome segregation at this time resulting in aneuploidy which can cause chromosomal disorders such as Down’s syndrome, or errors that cannot be sustained and lead to pregnancy loss (Hassold & Hunt, 2001; Mtango et al., 2008). Chromosomal aneuploidies are common, and the proportion of aneuploid cells within an embryo directly correlates with the
developmental potential of the embryo (Baltaci et al., 2006; Mtango et al., 2008).

In human oocytes, flattening of both spindle poles was associated with increased incidence of misalignment of the chromosomes at the metaphase plate (Coticchio et al., 2013). An association has been found in mammalian models between spindle abnormalities and aneuploidy incidence (Sanfins et al., 2003; Bromfield et al., 2009). For instance, abnormalities in spindle morphology and chromosome alignment at the metaphase plate have been found to be predictors of aneuploidy in aging women (Battaglia et al., 1996). In this study, the spindle of oocytes from older women, in comparison to those from younger women, exhibited a significantly higher incidence of abnormal tubulin placement within the spindle as well as chromosomal displacement from the metaphase plate at MII. Battaglia et al. (1996) speculated that this increase in spindle abnormalities leads to the high prevalence of aneuploidy in oocytes of older women.

**The follicular environment**

The mammalian oocyte grows and matures within a follicle, and the environment of that follicle, which is reflective of the external maternal environment, is extremely important in the developmental fate of the oocyte. The follicle provides nutrients necessary for the growth of the oocyte, and produces signals that regulate oocyte development (Eppig et al., 1996; Carabatsos et al., 2000; Voronina & Wessel, 2003). Follicular growth, as well as oocyte competence, are controlled by complex signalling and communication between theca, granulosa, and cumulus cells within the follicle. Bidirectional signalling in the form of autocrine, paracrine, and endocrine regulation as well as gap junctional interactions, are responsible for regulating growth and development of the oocyte and the follicular cells (Voronina & Wessel, 2003). The oocyte plays a dominant role, secreting factors that regulate follicular development and the function of cumulus
cells (Eppig et al., 2002; Matzuk et al., 2002). Conversely, the cumulus cells help to ensure proper growth and maturation of the oocyte by transporting essential molecules to the oocyte (Eppig, 2001; Gilchrist et al., 2008). Therefore, oocyte competence is acquired both by the oocyte’s ability to produce and secrete the necessary factors, as well as the response of the cumulus cells.

Follicular fluid is vital to the success of oocyte maturation and alterations to the composition of follicular fluid have the potential to affect oocyte maturation and the quality of oocytes, possibly by interfering with these complex signalling interactions. For instance, Da Broi et al. (2013) found that follicular fluid from infertile women with endometriosis, which has been reported to differ in comparison to follicular fluid from fertile women (Garrido et al., 2000), resulted in increased incidence of meiotic abnormalities.

Hormone levels in follicular fluid play an important role in the developmental competency of the oocyte. The levels of E2 (Aardema et al., 2013), progesterone (Aardema et al., 2013), and growth hormone (Modina et al., 2007) can be predictors of blastocyst production and markers of oocyte competence. E2 and progesterone levels in preovulatory follicles were found to be predictors of the developmental potential of oocytes in heifers (Kreiner et al., 1987; Aardema et al., 2013), and were also found to improve developmental competence when added to in vitro maturation (IVM) culture media (Zheng et al., 2003). Furthermore, follicular fluid concentrations of growth hormone (Modina et al., 2007), FSH (Suchanek et al., 1988), hCG (Ellsworth et al., 1984), and LH (Cha et al., 1986) have all been associated with oocyte competence, with levels of FSH, hCG, and LH being positively correlated with oocyte competence in humans, and levels of growth hormone inversely related to oocyte competence in cows.

The complex milieu of factors contained within the follicular environment explains the difficulty assisted reproductive technologies (ARTs) have had in creating successful pregnancies.
from oocytes that have been matured *in vitro* (reviewed by Chian, 2004). In domestic animals, on the other hand, *in vitro* oocyte maturation is more commonly used and has resulted in greater success in the production of offspring from embryos which were derived from *in vitro* matured oocytes (Rodriguez-Martinez, 2012). However, pregnancy rates from *in vitro* matured oocytes remain substantially lower than oocytes which matured *in vivo* (Rizos et al., 2002). Furthermore, environmental conditions of the oocyte, *in vivo* and *in vitro*, can have significant impacts on oocyte quality and developmental potential (reviewed by Krisher, 2013). Thus viability of an oocyte and resulting embryo can be strongly influenced by the environment in which the oocyte matures.

**Environmental influences**

The follicular environment is reflective of the external environment experienced by the female and the maternal environment can have a direct impact on oocyte competence. Environmental factors that can lead to a decrease in developmental competency include oxidative stress, heat stress, maternal hormone status (Hodges et al., 2002), *in vitro* culture conditions (Trounson et al., 2001) and interruptions to normal oocyte-somatic cell signalling (Carabatsos et al., 1998). Of particular relevance to the current work are EDCs.

EDCs are environmental chemicals that are commonly encountered and may impact female fertility; most are man-made chemicals that interfere with endogenous hormone functioning. Certain EDCs may alter endogenous hormone synthesis and secretion, metabolism, transport, and ligand action. Most commonly, EDCs interfere with estrogenic pathways, exhibiting estrogen-like actions by binding to and activating the estrogen receptors. EDCs can interfere with normal reproductive functioning at various stages of the reproductive life cycle. Oocyte, embryonic and fetal developmental period are of particular concern, with exposure during this time potentially
leading to significant reproductive detriments that may be passed on to the subsequent generation (reviewed by Krisher, 2013). The severity of effects as a result of developmental exposure depends on the nature of the specific EDC, the exposure level and frequency to the EDC, and the timing of exposure during development. EDCs may affect oocyte competence in a number of ways. They may act directly on the oocyte, or indirectly either via the follicular cells, or disrupting the hypothalamic-pituitary-gonadal axis to interfere with the secretion of gonadotropins (reviewed by Krisher, 2013).

The impact that the external environment has on the oocyte may also vary depending on the inherent competence of the oocyte. For instance, oocytes with poor inherent competence may be more severely impacted by poor environmental conditions than those oocytes with good inherent competence. The oocyte must withstand environmental insults while retaining its developmental competency in order to successfully perform its reproductive function. Poor quality oocytes may be unable to properly support embryo development, and adverse developmental outcomes that may be passed on to future generations are significantly affected by the quality of the oocyte from which they developed (reviewed by Mtango et al., 2008). There are a number of developmental implications of poor oocyte competence. Maturational effects include a decrease in maturation success, altered gene expression of the cumulus oocyte complex (COC), disruption of the meiotic spindle assembly, changes in distribution of mitochondria, chromosome dispersal at the metaphase plate, and chromosomal abnormalities such as aneuploidy. Poor developmental outcomes such as these can be used to assess the quality of the oocyte from which an embryo developed.
**Early embryo development**

Following fertilization the ovum has become a single diploid cell, referred to as a zygote. The zygote is enclosed within the zona pellucida, an acellular glycoprotein membrane surrounding the embryo, and undergoes its first mitotic cleavage resulting in a 2-cell embryo (Hardy et al., 1989). The early embryo undergoes rapid mitotic division cycles, during which the amount of DNA is exponentially increasing while the amount of cytoplasm in the embryo remains constant eventually becoming a morula of at least 16 cells. At the morula stage the cells are compacted (Hardy et al., 1989), but remain undifferentiated. Cellular differentiation and cavitation of the embryo occur to form the early blastocyst. Embryonic cells differentiate into trophoectoderm cells (TE cells) and the inner cell mass (ICM) that form the blastocyst, and fluid accumulates in a blastocoel cavity (Hardy et al., 1989). The early blastocyst in bovine is reached 7-8 days post fertilization. The blastocyst, although small at first, begins to expand. Eventually the blastocyst will hatch from the protective zona pellucida in preparation for implantation into the uterine endometrium in humans, or attachment in bovine.

During the early cell cycles, the cells of the embryo are undifferentiated and transcriptionally silent (reviewed by Vigneault et al., 2009) and maternally provided proteins and mRNAs regulate development (reviewed by Schier, 2007). The embryonic genome becomes transcriptionally active at later cell cycles during early embryo development. This period of time is referred to as the MET, and has also been called the maternal-zygotic transition (MZT), embryonic genome activation (EGA), and mid-blastula transition (MBT) (reviewed by Schier, 2007). The MET is a critical period of early embryo growth. The maternal products begin to degrade, embryonic transcription is initiated and the embryo becomes dependent on expression of embryonic genes (reviewed by Memili & First, 1999). Prior to the MET, the embryo is largely
dependent on maternal RNAs and proteins synthesized during oogenesis (reviewed by Memili & First, 1999). The timing of the MET varies between species, but similarly it is a transitional period at which time the transcriptional contribution provided by the mother begin to decline, and that of the developing embryo begin to increase (reviewed by Memili & First, 1999). In bovine embryos, genomic activation occurs at the 8- to 16-cell stage (reviewed by Vigneault et al., 2009).

RNAs stored during oogenesis are important in sustaining embryonic development before embryonic transcription takes over (reviewed by Memili & First, 1999). This can thus be a vulnerable period for the embryo if it is not well equipped to take control of its genomic activity. This also sheds light on the importance of oocyte competence. The embryo must be supplied with sufficient transcripts and proteins acquired during oocyte growth and maturation to survive until the embryonic genome takes over. Furthermore, alterations in the gene and protein expression profiles of mature oocytes may affect early embryonic development (Evsikov et al., 2006), possibly resulting in embryonic arrest, cell death, and/or poor developmental outcome.

**Vulnerability of the embryo**

Successful development of an early embryo will largely depend on its environment as well as its innate ability to respond to environmental changes or stressors. Early embryos are void of defense systems such as immune or nervous systems that are present in the adult. Parental defenses are in place to provide some protection to the embryo, however the embryo still remains vulnerable to certain types of stresses in its environment. The embryo has developed its own defense mechanisms which increase its chances of survival. In real world conditions, embryo development is stable and early embryos are equipped with high levels of cellular defenses in preparation for environmental changes (reviewed by Hamdoun & Epel, 2007). These cellular defenses are present
in the egg prior to fertilization. Later on in preimplantation development, the embryo may be able to buffer stress to adapt to the environmental conditions (reviewed by Hamdoun & Epel, 2007). Whether a particular maternal stress affects embryonic survival depends not only on the magnitude of the stress imposed on the embryo, but also on the effectiveness of the embryonic adaptive response to that stress.

*In vivo* embryo development is difficult to duplicate *in vitro*. Sub-optimal culture environments are thought to be responsible for the high frequency of early developmental failure that is associated with *in vitro*-produced embryos (Johnson & Nasr-Esfahani, 1994; Betts & King, 2001). Over recent years much research has gone into improving the *in vitro* fertilization (IVF) system in hopes of improving cleavage, embryo developmental potential, blastocyst development, and ultimately successful pregnancy and live birth. However it is not only *in vitro* systems that can hinder early embryo development. Various conditions of stress *in vivo* can also result in consequences to the embryo including delayed development, apoptosis induction, altered gene expression, and miscarriage (Wilson et al., 1985; Arck, 2004; Dennery et al., 2007; Ornoy, 2007). These stresses include hypoxia, changes in temperature, pathogens, UV radiation, free radicals, environmental toxins, and maternal diet and health (reviewed by Hamdoun & Epel, 2007). The developmental fate of the embryo rests upon the embryo itself and its defense capabilities of withstanding such stressors.

The ability of embryonic developmental programs to withstand stressors that have the potential to disrupt cellular and molecular mechanisms of embryogenesis dictates the severity of the effects caused by the stressor (Hamdoun & Epel, 2007). These programs are evidenced by the survival and maintenance of embryonic viability despite exposure to potential stressors (Hamdoun & Epel, 2007). The evolution of embryonic defenses was shaped by environmental perturbations
expected to be encountered by the embryo, and resulted in embryos with stronger defense systems against commonly encountered stressors (Hamdoun & Epel, 2007). Embryonic robustness, however, can be overwhelmed if the stress exceeds the limit than can be buffered by the embryo (Hamdoun & Epel, 2007). A growing concern is that man-made chemical stressors currently encountered by embryos may be beyond the range of these protective mechanisms, and may lead to poor developmental outcomes.

**Apoptosis in the preimplantation embryo**

Apoptosis is a form of cell death that is critical to normal development. Ineffective apoptotic mechanisms can lead to disorders of the central nervous system, and webbed hands and feet. Apoptosis, or programmed cell death, is a structured series of events that is triggered by the cell and essentially causes it to self-destruct. In doing so, the damaged cell can be destroyed without affecting neighbouring cells. Apoptosis eliminates abnormal or damaged cells as well as cells with abnormal developmental potential (Hardy, 1999; Betts & King, 2001; Paula-Lopes & Hansen, 2002a). Apoptotic cell death results in the removal of these cells without inducing inflammation, thus preserving the cellular integrity of neighbouring cells (Betts & King, 2001; Paula-Lopes & Hansen, 2002b).

In the blastocyst, cell death may be a way to regulate development, eliminating abnormal or damaged cells (Betts & King; 2001; Hansen & Fear, 2011). By removing cells that are damaged, abnormal, or have an inappropriate developmental potential, apoptosis acts as a quality control mechanism and is an important factor in animal development (Jacobson et al., 1997; Betts & King, 2001). The ability of the embryo to induce apoptosis in times of stress may help it to survive suboptimal conditions, however the extent to which apoptosis occurs in the blastocyst plays an
important role in the fate of that embryo (Betts & King, 2001). Where mild induction of apoptosis might help the embryo survive by eliminating damaged cells, severe stress may cause extensive apoptosis that could compromise development and survival of the embryo (Byrne et al., 1999; Betts & King, 2001; Paula-Lopes & Hansen, 2002b; Hansen & Fear, 2011). Thus the successful embryonic development is dependent on the embryo’s ability to protect itself and respond to stressors (Hamdoun & Epel, 2007).

Adverse environmental conditions during preimplantation development can increase the proportion of cells undergoing apoptosis in the early embryo (Paula-Lopes & Hansen, 2002b). Stressors encountered by preimplantation embryos include heat stress, oxidative stress, pathogens, and exposure to toxic or xenobiotic chemicals (Betts & King, 2001; Hamdoun & Epel, 2007). These environmental stressors have been found to induce apoptosis in early embryos under certain conditions and may compromise embryonic viability and survival depending on the severity of the stress, and the time of exposure during development (Paula-Lopes & Hansen, 2002a; Hamdoun & Epel, 2007).

**Embryo sex ratio**

Mendelian genetics assumes a 1:1 sex ratio with equal proportions of males and females born. However, variation in sex ratio is common and deviations from the 1:1 expectation are often recorded in humans and other mammals. The natural sex ratio is thought to be about 1.05, 105 males born for every 100 females, or about 52.5% males. Studies have found that sex ratio can shift as a result of various factors including fertility, nutrition, food availability, hormone status of either parent, the timing of insemination in relation to ovulation, the use of certain ART techniques in both humans (Menezo et al., 1999; Kallen et al., 2005; Luna et al., 2007; Dean et al., 2010) and
non-human mammals (Iwata et al., 2008).

Maternal stress has been found to impact the sex ratio of offspring in some cases. In rodents, females exposed to social stress produced fewer sons in mice (Krackow, 1997), hamsters (Pratt & Lisk, 1989), and rats (Lane & Hyde, 1973; Moriya et al., 1978), and the factors that may result in a decrease in sex ratio (fewer males) includes, but is not limited to, stressors such as pollution (James, 1998) and confinement stress (Krackow, 1997). Fewer males also tend to be conceived in humans under suboptimal conditions, including environmental disasters (Fukuda et al., 1998), pollution (Mocarelli et al., 2000; Weisskopf et al., 2003), and mothers of advanced age (Orvos et al., 2001). This trend indicates that male embryos are more fragile and less likely to survive under stressful conditions than female embryos. These observations comply with the Trivers & Willard (1973) hypothesis that postulates an excess of male offspring is only favoured by natural selection when conditions, and likelihood to survive, is good, and mothers in poor conditions benefit most by the production of daughters (Trivers & Willard, 1973). This may be a result of differential survival rates of male and female embryos during the preimplantation development.

*In vitro*, blastocyst sex ratio has been found in many instances to be altered by environmental conditions such as hyperglycemia, oxidative stress, *in vitro* culture media composition, and exposure to EDCs. Increased glucose levels in *in vitro* culture media appears to favour male embryos and inhibit female embryo development (Gutiérrez-Adán et al., 2001a; Larson et al., 2001), whereas paternal exposure to the dioxin TCDD resulted in skewed sex ratio in favour of female embryos (Ishihara et al., 2007).

These differences in response to environmental conditions are speculated to be a result of metabolic, genetic, and epigenetic differences between male and female embryos that have been
observed *in vitro*. During the preimplantation period, male and female embryos differ only in the content of their sex chromosomes, and differences exhibited by embryos at this time must be a result of transcriptional dimorphism (reviewed by Bermejo-Alvarez et al., 2011). Furthermore, epigenetic differences resulting from the presence of one versus two X-chromosomes may be a driving force in sex differences and how embryos respond to environmental conditions (reviewed by Gutiérrez-Adán et al., 2006).

Expression of genes encoded by the sex chromosomes, which may also affect autosomal gene expression, differs between male and female preimplantation embryos (reviewed by Bermejo-Alvarez et al., 2011). Y-linked genes are only expressed in male embryos, and X-linked genes are expressed doubly in females. X-chromosome inactivation (XCI) compensates for this disparity ensuring X-linked genes (in most cases) are transcribed equally in male and female adult tissues (reviewed by Bermejo-Alvarez et al., 2011). However, during early embryo development XCI is incomplete, or genes are reactivated, and female embryos exhibit higher expression of many X-linked genes (Kobayashi et al., 2006; Bermejo-Alvarez et al., 2010a). This phenomenon is exhibited in mouse (Kobayashi et al., 2006), bovine (Gutiérrez-Adán et al., 2000), and human embryos (Taylor et al., 2001).

These transcriptional alterations are responsible for variations between male and female embryos such as metabolic differences, and dimorphic susceptibilities to suboptimal *in vivo* and *in vitro* conditions (reviewed by Gutiérrez-Adán et al., 2006). Resulting alterations in molecular pathways, such as the pentose-phosphate pathway (PPP) that regulates glucose metabolism, may result in varying susceptibilities of male and female embryos. For instance, male embryos have been reported to metabolize glucose at a higher rate than females (Tiffin et al., 1991) and appear to benefit more than females under hyperglycemic conditions *in vitro*; however the opposite has
also been reported (Jimenez et al., 2003). Conversely, female embryos exhibit increased expression of X-linked genes such as those related to energy metabolism, the regulation of oxygen radicals, and apoptosis inhibition (Gutiérrez-Adán et al., 2000; Jimenez et al., 2003). Since various stressors can lead to embryonic overproduction of reactive oxygen species (ROS), female embryos may be better equipped to survive such a stress due to enhanced ability to buffer the amount of cellular ROS (Perez-Crespo et al., 2005). Furthermore, male embryos have been shown to be more sensitive to oxidative damage induced by heat stress, which may be due to the increased expression of X-linked genes in females that result in an increased ability to buffer environmental stress (Perez-Crespo et al., 2005).

Therefore, whether the embryo possesses one or two X chromosomes may underlie the differences in the early embryo (Gutiérrez-Adán et al., 2006). This may explain observations that male embryos may be more vulnerable to stressors than female embryos under certain environmental conditions. Since male and female embryos evidently respond differently to stress, a skew of the natural sex ratio may be indicative of environmental stress imposed on the embryo. Sex ratios have therefore been widely used as an indicator for reproductive health and the embryonic environment.

**Blastocyst gene expression**

A milestone of preimplantation development is formation of the blastocyst. As discussed earlier, the MET is a period of embryonic genome activation where transcripts from the mother begin to degrade and those originating from the embryo increase and take over development. During this period, the embryonic gene expression program is developed (reviewed by Schultz, 2005). Although early embryo development is autonomous, environmental conditions of the embryo or the oocyte from which it developed can influence preimplantation development and, if
conditions are extreme, lead to abnormal embryonic development (Niemann & Wrenzycki, 2000). Embryonic gene expression can be influenced by environmental factors including *in vitro* culture conditions (Lonergan et al., 2006). Though not as thoroughly studied, the environment in which the oocyte matures has also been found to alter transcript abundance in the mature oocyte (Lonergan et al., 2003a) as well as in the blastocyst from which it develops (Russell et al., 2006).

Changes in mRNA transcript abundance at the blastocyst stage caused by environmental perturbations may indicate an altered quality, or developmental competency of the blastocyst, however the functional significance of variations in transcript abundance can be difficult to interpret (reviewed by Duranthon et al., 2008). El-Sayed et al. (2006) analyzed differences in transcript abundance of genes from embryos resulting in pregnancy versus those that did not. Transcript abundance for genes involved in carbohydrate metabolism, implantation, and placental development, among others, were found to be higher in embryos associated with successful pregnancies whereas those of inflammatory cytokines, transcription factors, glucose metabolism, and implantation inhibition were lower (El-Sayed et al., 2006).

It is evident that alterations to the *in vitro* culture environment of embryos can influence the gene expression of a variety of genes in the blastocyst, however the influence of oocyte stress on resulting blastocyst gene expression is controversial. Studies examining oocyte exposure tend to focus on acute effects in the oocyte whereas blastocyst effects studied are normally a result of environmental exposures during embryo development. Evidence has suggested that the preimplantation period is the most important factor when considering blastocyst quality (reviewed by Lonergan et al., 2006), however the environment of the oocyte has the capability of inducing long term effects including changes in gene expression in the blastocyst (Pocar et al., 2001; Russell et al., 2006). For instance, oocyte exposure during maturation to elevated non-esterified fatty acid
concentrations resulted in changes to embryonic gene expression and phenotype, possibly as a result of altered metabolic strategies (Van Hoeck et al., 2013). Understanding the importance of the environment in which the oocyte matures and embryo develops is critical in formulating a comprehensive picture of how environment affects early development. Analyzing gene expression changes in the blastocyst as a result of environmental perturbations provides an indication of the embryo quality and stress that has been experienced by the embryo.

**Hormone receptors**

Nuclear hormone receptors are transcription factors that are activated when a ligand, such as steroid or thyroid hormones (THs), binds to the receptor forming a complex that will bind to DNA response elements contained within promoter regions of the target genes (Kinyamu & Archer, 2003; reviewed by Rastinejad et al., 2013), and thereby regulate the gene expression of their target genes (Mangelsdorf et al., 1995; Gronemeyer et al., 2004). Nuclear receptors play an important role in the oocyte and early embryo (Beker-van Woudenberg et al., 2004), and are essential for implantation/attachment (reviewed by Vasquez & DeMayo, 2013). Alterations in hormone receptor mRNA may indicate poor developmental programming or improper conditions, and may be predictive of undesirable reproductive outcomes.

E2, a steroid hormone, plays a critical role in fertility, oocyte development, and early embryo development. The hormone receptors to which E2 binds in order to exert its effects, ERα and ERβ, and the levels at which they are expressed can therefore impact the proficiency of these programs and the viability of oocytes and embryos. ERα is expressed in cumulus cells whereas ERβ is expressed both in cumulus cells and oocytes (Beker-van Woudenberg et al., 2004). In the porcine embryo, ER mRNA is present in the early cleavage divisions, but is undetectable around
the 5- to 8- cell stages, and then reappears at the blastocyst stage (Ying et al., 2000; Chingwen et al., 2000). In mice embryos, a similar pattern is observed with expression disappearing at the 8-cell stage and reappearing at the morula and blastocyst stages (Hou & Gorski, 1993; Hiroi et al., 1999). It has been suggested that the reappearance of ER mRNA at the blastocyst stage indicates that E2 at this time may act directly on the embryo (Chingwen et al., 2000); this correlates with the essential role of the hormone in the establishment and maintenance of pregnancy. Changes in ER mRNA levels at blastocyst may therefore lead to reproductive deficits such as pregnancy loss. Disruption of ERα expression in blastocysts did not affect embryo development and implantation in mice (Saito et al., 2014), however Hou et al. (1999) suggested that the lack of natural ER mutations may be evidence that ERs are essential for embryonic survival.

Thyroid hormones, T3 and T4, are required for normal growth and differentiation of most organs in vertebrates (Darras et al., 2011). T3 and T4 regulate metabolism, cardiac function, remodelling of bone and brain development (reviewed by Boas et al., 2012). However, the benefits of THs precede organ development. THs have been found to be present in the female reproductive tract and follicular fluid (Ashkar et al., 2010a), and beneficial to early embryo quality (Ashkar, 2013). THs exert their action by binding to the TH receptors (TRs) TRα and TRβ (reviewed by Darras et al., 2011). TRs are expressed from the 2-cell to the blastocyst stage in bovine embryos, suggesting active transcription of TR mRNA throughout preimplantation development (Ashkar, 2013). TH supplementation to in vitro culture (IVC) media did not alter TR mRNA expression of the blastocyst, however TR expression may be altered by other exposures. For instance, TRβ expression was suppressed as a result of bisphenol A (BPA) exposure in Xenopus embryos (Iwamuro et al., 2003). As will be discussed later, BPA is a chemical with demonstrated anti-thyroid properties, and given the importance of THs during preimplantation development, more
research is needed regarding the disruption of TR expression during early development.

**Embryonic stress, metabolism, and gene expression**

As discussed earlier, stress incurred by the embryo can lead to increased incidence of apoptosis and a skewed sex ratio. There are a number of genes in preimplantation development that are involved in the embryonic response to stress. Two well-characterized examples of embryonic stress include heat and oxidative stress, both of which have been shown to result in a decrease in oocyte competence and embryonic developmental potential in mammals (Al-Katanani et al., 2002; Sartori et al., 2002; Tamura et al., 2008; reviewed by Takahashi, 2012). Genes known to be involved in the embryonic response to these stressors include the heat shock protein, HSP70, the tumour suppressor, p53, and the glucose transporter, GLUT1. Transcription levels of these genes have been shown to vary as a result of embryonic stress, though these responses appear to be dependent on both the level of stress experienced and the stage of development at which the stress occurs.

Heat shock proteins (HSPs) play an important role in oocyte fertilization and early embryo development in mammals (Anderson, 1998; Neuer et al., 1998, 1999). The two major roles of HSPs are that of molecular chaperones, and in the protection against cellular damage caused by stress (Welch, 1992). HSP70 is a major heat shock protein that protects cells against detrimental effects of stress (Welch, 1984; Hendrey & Kola, 1991) by preventing the denaturation of proteins and inhibiting apoptosis (Matwee et al., 2001; Kregel, 2002). HSP70 plays a vital role in early embryo development as evidenced by observations that exposure to HSP70 antibodies resulted in decreased murine and bovine embryo development, and increased DNA fragmentation and apoptosis (Neuer et al., 1998, 1999; Matwee et al., 2001). These results along with avian studies
which have demonstrated a correlation between developmental HSP70 expression and apoptosis resistance during stress indicate that HSP70 plays an important role in apoptosis inhibition during early embryo development (Bloom et al., 1998).

HSP70 transcription is increased in immature oocytes and embryos of *Bos taurus* as a result of heat stress (*in vivo*) (Camargo et al., 2007) or heat shock (*in vitro*), respectively (Kawarsky & King, 2001). Additionally, other stressors such as inadequate culture conditions of *in vitro* produced (IVP) bovine embryos (Wrenzycki et al., 1999), and exposure of zebrafish (*Danio rerio*) embryos to heavy metals and pesticides (Scheil et al., 2010) resulted in increased HSP70 mRNA and protein expression, respectively. This protective capacity of HSP70, however, appears to be developmentally acquired with embryos of at least the 8-cell stage exhibiting protective increases in HSP70 expression (Kawarsky & King, 2001). HSP70 expression, though vital for normal embryo development, appears to become increasingly critical in the face of adverse environmental conditions experienced by the embryo. Expression of HSP70 mRNA is therefore often used as a marker of stress in preimplantation embryos (Wrenzycki et al., 2001).

HSP70 interacts with the tumour suppressor protein p53, which is recognized for its role in cell cycle arrest and apoptosis signalling (Ko & Prives, 1996). P53 acts as a transcription factor for genes involved in apoptosis and cell cycle arrest, thereby nuclear translocation of the protein is essential for its function (Ko & Prives, 1996). Under normal circumstances, p53 has been observed in the cytoplasm of bovine blastocysts (Matwee et al., 2000), and its translocation to the nucleus may be regulated by HSPs. In the preimplantation embryo, p53 is expressed at low levels (Li et al., 2005). Stress experienced by the preimplantation embryo may result in an upregulation of p53 as well as nuclear localization of its protein (Li et al., 2005).

The role of p53 during embryonic development is controversial. P53 may not play a role
in embryonic developmental arrest (Favetta et al., 2004), and apoptosis under non-stressed conditions has been suggested to occur independently of p53 (Matwee et al., 2000). However, the role of p53 appears to be critical in the embryonic response to environmental stress (Lichnovsky et al., 1998; Hu et al., 2011), though it was not upregulated in response to oxidative stress in bovine embryos (Favetta et al., 2007). In times of stress, p53 induces apoptosis in embryos thus ridding the embryo of cells containing DNA damage (Stewart & Pietenpol, 2001), and p53 null embryos exhibited low apoptosis and a high proportion of developmental abnormalities (Nicol et al., 1995; Norimura et al., 1996). Furthermore, it appears that the levels of p53 in the early embryo may be essential for proper development (reviewed by Choi & Donehower, 1999). Over- or under-expression of p53 can lead to increased incidence of malformations or embryo death (reviewed by Choi & Donehower, 1999). Levels of p53 mRNA in the blastocyst may therefore indicate whether the embryo is experiencing undue stress and give insight into its developmental fate.

Another well documented stress commonly encountered in embryos produced in vitro is oxidative stress. Oxidative stress results in lower developmental competence and blastocyst quality in IVP embryos (Batt et al., 1991; Farrell & Foote, 1995; Thompson et al., 1990; Rho et al., 2007). Concurrent with this decrease in embryo development rate and quality, is an altered expression of GLUT1, VEGF, Bax, and Bcl-2 among others as a result of varying levels of oxygen tension in vitro (Rho et al., 2007). For instance, GLUT1 expression decreased with increasing oxygen tension, with in vivo embryos exhibiting the highest expression of GLUT1 (Batt et al., 1991). Furthermore, downregulation of GLUT1 can lead to decreased blastocyst cell number (Balasubramanian et al., 2007). However, higher levels of GLUT1 expression as a result of oxidative stress were observed in a similar study (Rho et al., 2007). It was suggested that this difference may be due to altered embryonic metabolism as a result of oxidative stress experienced
by the IVP embryos (Rho et al., 2007).

GLUT1 is expressed at all embryonic developmental stages (Lequarre et al., 1997; Bertolini et al., 2002) and becomes increasingly important following compaction, when the embryo begins to use glucose as its primary energy source (reviewed by Pantaleon et al., 2001; Harvey et al., 2004). The embryonic cellular uptake of glucose is mediated by GLUT1, and expression has been observed to increase as a result of cellular stress caused by glucose deprivation (Baldwin, 1993). Similarly, culture environments can affect the levels of GLUT1 gene expression in bovine embryos (Wrenzycki et al., 1999, 2001; Lazzari et al., 2002) indicating that embryos may respond to environmental conditions by altering GLUT1 expression levels (Harvey et al., 2004).

It is therefore apparent that genes that are involved in physiological processes such as apoptosis and metabolism during early embryo development can be affected by the conditions to which the embryo is exposed. However, the effects that conditions during oocyte maturation have on blastocyst gene expression is controversial. Some reports have suggested that blastocyst gene expression may be altered due to maturation conditions (Silva et al., 2013), whereas others have not observed such effects (Knijn et al., 2000). Heat and oxidative stress are common stress pathways studied in the early embryo, and knowledge of these pathways is important in understanding how exogenous stresses may impact embryo development.

**Bisphenol A**

Bisphenol A (BPA; 4,4’isopropylidenediphenol) has garnered much attention over the last few decades due to its endocrine-disrupting properties, particularly for its effects as an estrogen agonist (Nishikawa et al., 2010). BPA is a synthetic chemical that is polymerized in order to
produce polycarbonate plastics, epoxy resins, and flame retardants (Pastva et al., 2001; Lahnsteiner et al., 2005; Nishikawa et al., 2010). BPA is one of the highest volume synthetic chemicals produced worldwide (Welshons et al., 2006; Pearce et al., 2009; Vandenberg et al., 2009), and the yearly production of BPA continues to increase and is expected to exceed 5.4 million tons in 2015 (Merchant Research & Consulting, 2014).

A wide range of plastic products contain BPA, including toys, water pipes, electronics, baby bottles, medical equipment, tubing, and dental sealants (Shelby, 2008; Aghajanova & Giudice, 2011). Incomplete polymerization results in the leaching of BPA from epoxy resins that are used to line the inner surface of metallic food cans (Vandenberg et al., 2009). BPA has also been found to leach from polycarbonate plastics such as baby bottles and reusable water bottles, resulting in ingestion of BPA (Vandenberg et al., 2009). Ingestion is the primary route of BPA contamination, although there is also evidence for dermal and inhalation exposure (Mørck et al., 2010). BPA levels in various populations around the globe have been tested and BPA has been found to be present in 92.6% (Wetherhill et al., 2007) of Americans, and about 90% of Canadians (Bushnik et al., 2010) and has been detected in human urine, blood, serum, follicular fluid, and tissue samples (Ikezuki et al., 2002; Schönfelder et al., 2002; Calafat et al., 2005; Vandenberg et al., 2007). Thus it is imperative that we understand how this chemical acts and what risks it poses to us and future generations.

**Metabolism and Concentration**

There are many controversies regarding BPA exposure and the study of BPA. One of these involves its metabolism. A large amount of BPA is metabolised by the liver, and to a lesser extent, the intestine. BPA is primarily glucuronidated to BPA-G with the help of cytochrome p450. The
next most common conjugation of BPA is sulfonation to BPA-S. Conjugated forms of BPA are considered to be biologically inactive, having lost their estrogenic potential (Matthews et al., 2001), however there is evidence that the toxicity of certain BPA metabolites may be even more potent than unconjugated BPA (Yoshiihara et al., 2004; Ishibashi et al., 2005; Baker & Chandsawangbhuwana, 2012).

BPA is not metabolised to the same extent in all individuals, species, and tissues. BPA metabolism is thought to be significantly influenced by an individual's genotype. Furthermore, there are sex and age differences in the ability to metabolise BPA, with that of infant and young children being relatively inefficient (Beydoun et al., 2014; Takeuchi et al., 2004; Doerge et al., 2011; Yang et al., 2013). The varied responses that different species exhibit to BPA also make it difficult to study. For instance, the rate of metabolism in humans differs from that in mice and rats. Some studies have suggested that BPA is metabolized more quickly in rats (Elsby et al., 2001) whereas Pritchett et al. (2002) predicted that when metabolic levels, which were slowest in human hepatocytes, were extrapolated to the whole liver, humans had a greater capacity for BPA metabolism than rats and mice.

Differences have also been found to occur at the tissue level. Much of BPA is conjugated in the liver, and there is evidence of kidney and intestinal glucuronidation, however other tissues are inefficient or unable to metabolise BPA (Trdan Lušin et al., 2012). The lungs, for instance, are unable to metabolise BPA, making inhalation exposure of particular concern. There is also evidence that some tissues are able to deconjugate BPA-G and BPA-S back into free BPA (reviewed by Vandenberg et al., 2009). For instance, intestinal tissue can deconjugate BPA in the presence of glucuronidases and sulfatases, releasing the bioactive form of BPA back into the tissue (Zalko et al., 2003). Perhaps equally important as the metabolism is the presence of bound and free
BPA in human serum as well as in a wide variety of tissues (Calafat et al., 2005, 2008).

The concentration of BPA used in research is an additional factor contributing to the complexity of its study. BPA has been found in a wide variety of serum and tissue samples however controversy remains as to how much is actually in the tissue, and whether or not it is enough to pose a risk to humans. The concentration of BPA detected in humans varies based on a person's age, sex, race/ethnicity, household income or geographical location (Calafat et al., 2005, 2008). Levels of free BPA in human serum has been found at levels ranging from 0.2 to 20 ng/mL with an averages in the 1-3 ng/mL range in adults (Ikezuki et al., 2002; Vandenberg et al., 2007; Calafat et al., 2008). Most relevant to the current study is the amount of free BPA found in follicular fluid. Less data exists regarding follicular fluid content of BPA, but it has been found to average around 2.4 ng/mL in women undergoing IVF treatment (Ikezuki et al., 2002).

Since BPA can still be found in tissues and follicular fluid despite its relatively short half-life, it is thought that humans are exposed to significant amounts of BPA by frequent, low-dose exposure through various sources (reviewed by Lenie et al., 2008). The concentrations measured in human samples are much lower than the lowest-observed-adverse-effect-level (LOAEL) which is considered to be 50 μg/mL as well as the in vitro culture equivalent which is suggested to be 50 ng/mL (Welshons et al., 2006; Wetherill et al., 2007). Of notable concern is that environmentally relevant concentrations (low nanomolar range) have been found in many instances to result in detrimental reproductive effects.

In addition to individual and tissue variability, exposure to BPA is not static, and these concentrations will fluctuate in individuals depending on their eating habits and environmental exposure levels. In a study by Carwile et al. (2011), it was found that ingestion of canned soup, in comparison to fresh soup, resulted in an increase in urinary BPA levels in all volunteers tested.
Bpa has also been found to bind to human serum proteins (Csanády et al., 2002), including human serum albumin (Yang et al., 2015). Therefore low concentrations of exposure may result in only a small fraction of unbound BPA in plasma (Csanády et al., 2002). Thus, although exposure is ubiquitous and constant, the actual concentrations found in humans are likely to vary. There are many factors to consider when studying BPA, especially when using an animal model, and these factors must be taken into account when interpreting results obtained in any study hoping to extrapolate results to human exposure risk.

**Known effects of BPA**

BPA is an EDC best known for its ability to mimic estrogen, though it has been cited to have many effects in various physiological systems. BPA toxicity is linked to the interference of hormone regulation and to the disruption of the immune, reproductive, and neurological systems (Rubin et al., 2001; Moriyama et al., 2002; MacLusky et al., 2005; Palanza et al., 2008; Avissar-Whiting et al., 2010; Nishikawa et al., 2010). Of greatest relevance to the current work are the effects BPA incurs on reproduction, particularly that of the oocyte and early embryo. The reproductive effects of BPA published to date are vast. Altered hormone secretion (Vandenberg et al., 2009), decreased implantation success (Berger et al., 2010; Ehrlich et al., 2012a), and altered ovarian morphology (Suzuki et al., 2002) are a few of the many observed reproductive effects. Its effects are also evident during early pregnancy with reports including altered early embryonic development (Takai et al., 2001) and recurrent miscarriage (Kwintkiewicz et al., 2010).
**BPA and the oocyte**

BPA has been documented to affect both the prenatal and adult follicle and oocyte. Both *in vitro* and *in vivo* studies have demonstrated BPA’s effects on the developing ovary. The onset of meiosis, as well as GVBD and follicle formation have been demonstrated to be altered by exposure to BPA (reviewed by Peretz et al., 2014). Some studies have found that the onset of meiosis is affected by BPA exposure, resulting in meiotic nondisjunction in the fetal ovary without evidence of aneuploidy (reviewed by Richter et al., 2007); however, aneuploidy has been observed in other studies as a result of BPA exposure during early development. For instance, gestational exposure during ovary development resulted in meiosis disruption, with fetal oocytes displaying gross aberrations, leading to increased aneuploidy in oocytes and embryos of the mature female (Susiarjo et al., 2007). Thus BPA may disrupt oogenesis in the developing ovary as a result of maternal exposure. Similar effects have been observed as a result of BPA exposure during oocyte maturation in the adult female, and these effects have been observed in humans and mice via *in vitro* and *in vivo* studies.

Negative associations have been found between BPA serum levels and the number of oocytes retrieved, peak serum E2 levels, the number of mature oocytes retrieved, oocyte fertilizability, and oocyte developmental potential in women undergoing IVF (Mok-Lin et al., 2010; Ehrlich et al., 2012b; Bloom et al., 2011a; Fujimoto et al., 2011). BPA exposure during oocyte maturation in mammalian studies has resulted in meiotic abnormalities such as delayed cell cycle progression (Can et al., 2005), spindle aberrations (Can et al., 2005; Eichenlaub-Ritter et al., 2008), misalignment of chromosomes (Hunt et al., 2003; Eichenlaub-Ritter et al., 2008), centrosomal alterations (Can et al., 2005) and increased aneuploidy (Hunt et al., 2003; Susiarjo et al., 2007), all of which are indicators of poor oocyte quality. These effects have been shown to
vary with different exposure levels. Higher doses of BPA are linked to decreases in meiotic progression, and lower doses resulting in increased abnormalities of MII oocytes (Lenie et al., 2008; Machtinger et al., 2013). These data provide strong evidence that BPA at certain relevant exposure levels may detrimentally affect oocyte maturation.

**BPA and the embryo**

Despite the many reported effects of BPA on female fertility and reproduction, the possible effects of BPA on preimplantation development in mammals is not as well documented. There is evidence that gestational or perinatal exposure can result in short- and long-term effects with the possibility of a grand-maternal effect. Developmental exposure to BPA has resulted in poor pregnancy outcomes such as pre-term birth (Cantonwine et al., 2010), predisposition to the development of metabolic syndrome (Wei et al., 2011) as well as alterations in genes and behaviour of offspring that may be passed on to the subsequent generation (Wolstenholme et al., 2012). However, fewer studies have analyzed the effects of early exposure to BPA on blastocyst development and quality in mammals. Existing studies have found detrimental outcomes as a result of preimplantation BPA exposure with evidence suggesting that embryo development may be altered as a result of BPA exposure.

In humans, urinary BPA concentration in women undergoing IVF was negatively associated with blastocyst formation, but not embryo quality (Ehrlich et al., 2012b). Additionally, male but not female urine BPA concentrations were found to decrease embryo quality in a prospective cohort study of couples undergoing IVF (Bloom et al., 2011b). *In vivo* animal models have demonstrated varying maternal BPA exposure doses can alter embryo development (Tsutsui et al., 1998; Xiao et al., 2011). Despite delayed development, there was no alteration in sex ratio
of pups at weaning (Xiao et al., 2011). Conversely, Yan et al. (2013) found no differences in development rate or total blastocyst number, but decreased hatching rates and increased proportion of apoptotic cells were observed.

*In vitro* studies examining BPA exposure during preimplantation development have exhibited similar results, and the same variability, as *in vivo* studies. Takai et al. (2000, 2001) observed opposite effects of low (1-3 nM) and high (100 μM) doses of BPA on murine embryos. Two-cell embryos exposed to 100 μM resulted in fewer embryos reaching the blastocyst stage, whereas treatment with 1nM BPA resulted in a great number of embryos reaching blast. Embryo quality and sex ratio did not differ between treatment groups and the control (Takai et al., 2000). Additionally, both the high and low treatments led to pups that were significantly heavier than controls at weaning (Takai et al., 2001). Thus BPA exposure during the preimplantation period has the potential to affect blastocyst development (Xiao et al., 2011), as well as induce postnatal effects. More information is needed regarding early exposure to BPA and developmental effects on the early embryo as well as blastocyst quality in mammals.

**BPA and male reproductive effects**

Human studies regarding the male effects of BPA on sperm quality are limited but generally agree that higher urinary BPA levels in infertile, but not fertile, men are associated with decreased sperm count and motility (Mendiola et al., 2010; Li et al., 2011). Male urinary BPA concentrations have also been associated with low embryo quality in IVF produced embryos (Bloom et al., 2011b). Furthermore, prenatal or early postnatal exposure had resulted in adverse effects in adult spermatogenesis and sperm quality of rodents (reviewed by Richter et al., 2007; reviewed by Peretz et al., 2014). Exposure to low-dose BPA during gestation has been reported to
decrease sperm count in rats (Salian et al., 2009) as well as the number of elongated spermatids in seminiferous tubules of pubertal mice (Okada & Kai, 2008). In addition to developmental exposures, adult exposure has resulted in decreased sperm counts and increased apoptosis in rats (Jin et al., 2013; Tiwari & Vanage, 2013). Additionally, sperm motility in rats and mice has been impaired as a result of low-dose BPA exposure through various exposure routes as well as with various developmental, and adult, exposures (Salian et al. 2009; Minamiyama et al. 2010; Dobrzynska and Radzikowska 2013). Thus although the current thesis does not examine the effects of male exposure to BPA, implications of male exposure on embryo development are important to consider when interpolating in vitro results into real world significance.

**BPA and gene expression**

Gene expression analyses are commonly utilized to determine differences between conditions and treatments of embryos developed in vitro, and is an excellent tool to examine the mechanistic action of BPA. BPA has been reported to alter the expression of a variety of genes in different cell and tissue types. Hormone receptors (Rubin, 2011), cell cycle regulators (Peretz et al., 2012), apoptotic genes (Peretz et al., 2012), genes related to the stress response (Tabuchi et al., 2002), and those related to the onset of meiosis, chromatin modification, remodeling, and chromosome condensation (Lawson et al., 2011), among others have been altered as a result of BPA exposure. Furthermore, BPA has been shown to exhibit nongenomic effects (reviewed by Vandenbergh et al., 2009), decrease methylation (Dolinoy et al., 2007), and alter imprinted gene expression (Susiarjo et al., 2013). Analysis of how BPA affects gene expression, as well as epigenomic responses, is important in the determination of how BPA exerts its action as a result of early exposure.
Nuclear receptors

BPA has been documented to bind both the ERα and ERβ, though its influence on gene transcription and its reproductive effects in the literature varies (Matthews et al., 2001; Kang et al., 2006; Takao et al., 2003; Levy et al., 2004; Lahnsteiner et al., 2005). As mentioned, the primary ligand of the ERs is E2. BPA competes with E2 to bind with the ERs (Levy et al., 2004), and it is thought that some of BPA’s effects are elicited by its binding to these receptors (Welshons et al., 2006; Chapin et al., 2008; Aghajanova & Giudice, 2011). BPA has been found to alter ERα and ERβ under different experimental conditions, however BPA has higher affinity for ERβ than for ERα (Matthews et al., 2001; Vandenberg et al., 2009). Additionally, BPA’s affinity for the ERs in relation to E2 is weak, and reports of BPA’s estrogenicity in the literature vary (reviewed by Berger et al., 2010).

Nonetheless, BPA has been found to alter gene and protein expression of ERs in a number of reproductive (and non-reproductive) tissues. BPA exposure has resulted in altered ER expression in vivo and in vitro. Observed effects include upregulation of ERα in uterine epithelium (Markey et al., 2005), increased ERα and ERβ in brain tissue (Ramos et al., 2003), and increased ER protein expression in uterine cells following a non-monotonic dose response (Berger et al., 2010). In vitro BPA exposure has resulted in downregulation of ERα in sertoli cells (Tabuchi et al., 2002) and endometrial stromal cells (Aghajanova & Giudice, 2011). However, as the ER antagonist, ICI, has no effect on endometrial stromal cell gene expression, the effects of BPA may not be mediated by the ERs (Aghajanova & Giudice, 2011).

Susiarjo et al. (2007) observed that meiotic defects as a result of maternal BPA exposure resembled that of ERβ knockout mice. Additionally, ERβ knockout mice did not exhibit any further BPA effects, suggesting both that BPA acts via the ERβ to exert its effects on fetal oocytes,
and fetal oocytes are sensitive to estrogentic actions. Preimplanation development can also be influenced by BPA exposure via the ER (Takai et al., 2000). Culture of 2-cell mouse embryos in vitro exposed to BPA resulted in dose-dependent effects on embryo developmental rates and success (Takai et al., 2000). Exposure to 1 nM and 3 nM BPA resulted in increased development rate whereas 100 μM BPA decreased the proportion of embryos developing to blastocyst. These effects were inhibited by co-exposure to the anti-estrogen tamoxifen, suggesting BPA may be acting via the ERs.

Although much attention has been paid to the estrogentic actions of BPA, interactions are also known to exist between BPA and the TRs, antagonizing TH actions both in vitro and in vivo (Moriyama et al., 2002; Iwamuro et al., 2003; Zoeller et al., 2005; Vandenberg et al., 2009; Meeker & Ferguson, 2011). Activation of the TR by T₃ is inhibited by BPA, resulting in antagonistic effects on TR-response genes (Moriyama et al., 2002). BPA has been found to inhibit T₃ action by reducing T₃ binding to the TR, suppressing transcriptional activities that are mediated by the TRs, and recruiting nuclear receptor corepressor (N-CoR) to the TR promoter, resulting in transcriptional inhibition (Moriyama et al., 2002). BPA is able to suppress TR-mediated transcription by inhibiting positively regulated genes and by activating negatively regulated genes (Moriyama et al., 2002; Heimeier et al., 2009). Although T₃ is studied more widely due to its prevalence compared to T₄, BPA has also been found to exhibit an inverse relationship with T₄ (Meeker & Ferguson, 2011).

Due to the importance of THs to preimplantation development, the effects of chemicals such as BPA which have been reported to disrupt thyroid signalling, should be assessed in the early embryo. In fish models low level BPA exposure during early development accelerated embryonic development and time to hatch (Ramakrishnan & Wayne, 2008) and decreased growth
(Ramakrishnan & Wayne, 2008; Aluru et al., 2010). Admiodarone, a TR-antagonist blocked the alterations in body growth and time to hatch in medaka fish (*Oryzias latipes*) (Ramakrishnan & Wayne, 2008), suggesting that BPA may act through a thyroid pathway to disrupt early development. However, it has been suggested that thyroid effects of BPA may require higher doses of BPA than is required to produce estrogenic or antiestrogenic actions (Welshons et al., 2003).

**Stress and metabolism**

In addition to disruption of hormone pathways, BPA has been reported to alter gene expression related to stress pathways and metabolism. For instance, genes which have been observed to be overexpressed as a result of stress (such as oxidative or endoplasmic reticulum stress) in mammalian cells were also upregulated following BPA exposure (Tabuchi et al., 2002). These results suggest that BPA can induce cellular stress (Tabuchi et al., 2002). Additionally, BPA has been found to decrease antioxidant enzymes in rat (Chitra et al., 2003) and mouse tissues (Kabuto et al., 2003).

Apoptosis, which can be used to evaluate cellular stress, has been found to be induced by BPA in various *in vitro* and *in vivo* models. It has been postulated that BPA may induce apoptosis in cells by upregulating the proapoptotic genes Bax and p53 in rat embryonic midbrain cells *in vitro* (Liu et al., 2013), as well as in murine antral follicles following maternal exposure to BPA (Peretz et al., 2013). As discussed earlier, p53 can regulate apoptosis induction by activating transcription of various proapoptotic genes (Amaral et al., 2010), thus increased p53 expression as a result of BPA exposure may indicate a mechanism through which BPA exerts cellular stress. Furthermore, BPA has been shown to upregulate hsp70 indicating increased stress experienced in species of worm (Schirling et al., 2006), dinoflagellate (Guo et al., 2012), insect larvae (Planelló
et al., 2008), and crab (Park & Kwak, 2013). Further information on whether BPA influences hsp70 transcription in mammalian embryos is required.

The metabolic effects of BPA during development have been studied in terms of rate of development, and the relationship between prenatal BPA exposure and metabolic disorders later in life. As mentioned, exposure of 2-cell embryos resulted in accelerated development at low exposure levels, and delayed development at higher exposure levels, and these results were reversed by tamoxifen supplementation (Takai et al., 2000). Furthermore, embryonic exposure to various levels of BPA resulted in significantly increased weight of pups at weaning (Howdeshell et al., 1999; Takai et al., 2000, 2001; Rubin et al., 2001; Markey et al., 2003; Akingbemi et al., 2004). That developmental BPA exposure can result in altered metabolism has been observed in various analyses. Perinatal exposure to BPA at the LOAEL dose led to increased body weight, elevated serum insulin, impaired glucose tolerance, and decreased insulin sensitivity, all of which were exacerbated by a high fat diet leading to metabolic syndrome (Alonso-Magdalena et al., 2010; Wei et al., 2011).

At the molecular level, BPA exposure of adipocytes in vitro resulted in an upregulation of mRNA of glucose transporters GLUT1 in human (Valentino et al., 2013) and GLUT4 in mouse (Sakurai et al., 2004) as well as glucose uptake and utilization in both cases. Information regarding BPA’s effects on gene expression, as well as the stress and metabolic responses in the early embryo is limited. Considering the existing links between BPA and altered gene expression relating to these pathways, the important roles of these genes in stress and metabolism, and the critical nature of the embryonic response to stress, possible effects of BPA exposure on preimplantation stress and metabolism is warranted.
RATIONALE, HYPOTHESIS, AND OBJECTIVES

Rationale

It is evident that BPA's influence is complicated and multifaceted, and it is clear why reaching a consensus in regards to its mode of action has proven to be so difficult. Early exposure to BPA can lead to a variety of detrimental alterations in development, throughout life, and, as recent evidence suggests, onto future generations. Reproductive ability is compromised by early BPA exposure by decreased gonad viability and fertility, and the ability of the embryo to implant in the uterus. Those embryos that do successfully implant are exposed directly to BPA through placental transfer, which can have serious implications on development by altering serum levels, mRNA expression, and synthesis of various hormones and cytokines. This may explain BPA's relationship to a plethora of diseases related to disruption of the reproductive, immunological, and/or neurological systems.

Although the biological effects of BPA have been widely studied in recent years, studies concerning embryo development in mammals are sparse and consist largely of embryonic exposures. We know that BPA is present in the follicular fluid, and thus must also examine the potential effects of BPA exposure at the oocyte stage on oocyte competence and quality of the resulting blastocyst. A full picture of exposure during both the oocyte and embryo stages is required, but first exposure during each of these stages separately must be assessed to get a greater idea of how exposure at various steps affects early development. Studies that have examined oocyte exposure have looked at effects on the oocyte itself but not later embryonic effects. The purpose of the current research is to determine the effects of BPA exposure during oocyte maturation on both the oocyte and embryos arising from these oocytes.
Hypothesis

The hypothesis of this study is as follows: exposure of bovine oocytes during *in vitro* oocyte maturation to BPA will disrupt oocyte maturation, decreasing oocyte quality and developmental potential, and compromising preimplantation embryonic development.

Objectives

To test this hypothesis, the following objectives were addressed:

Objective 1: To determine the effects of BPA exposure on oocyte maturation and resulting quality.

Objective 2: To determine the effects of BPA exposure during oocyte maturation on early embryo development.

Objective 3: To identify the effects of BPA exposure during oocyte maturation on mRNA levels of key target genes in MII oocytes and blastocysts.
CHAPTER ONE*

Bisphenol A exposure during oocyte maturation in vitro results in spindle abnormalities and chromosome misalignment in Bos taurus

*A portion of the material in this chapter has been published in Cytogenetics and Genome Research (Ferris et al., 2015), and is reproduced with the permission of S. Karger AG, Basel, Switzerland.
INTRODUCTION

Bisphenol A (BPA) is an endocrine-disrupting chemical (EDC) used in the manufacturing of many products consisting of polycarbonate plastics and epoxy resins. It is perhaps best studied for its estrogenic actions and its ability to interact with estrogen receptor beta (ERβ) (reviewed by Mtango et al., 2008). Many reproductive effects of BPA have been cited, yet controversy remains concerning its reproductive toxicity. BPA has been detected in blood, serum, urine, and tissue samples, and has been found in follicular fluid at an average concentration of 2.4 ng/mL in women (Ikezuki et al., 2002). Humans are thought to be exposed to significant amounts of BPA by frequent, low-dose exposure through various sources (reviewed by Lenie et al., 2008). Exposure to BPA primarily occurs as a result of incomplete polymerization leading to the leaching of BPA from epoxy resins that are used to line the inner surface of metallic food cans as well as from polycarbonate plastics such as baby bottles and reusable water bottles (Vandenberg et al., 2009). Ingestion is the primary route of BPA contamination, although there is also evidence for dermal and inhalation exposure (reviewed in Mørck et al., 2010).

Negative associations have been found between BPA levels in urine or serum and number of oocytes retrieved, peak E2 levels, normal oocyte fertilization and oocyte developmental potential in women undergoing in vitro fertilization (IVF) (Mok-Lin et al., 2010; Fujimoto et al., 2011). BPA exposure during oocyte maturation in mammalian studies has resulted in meiotic abnormalities such as delayed cell cycle progression (Can et al., 2005), spindle aberrations (Can et al., 2005; Eichenlaub-Ritter et al., 2008), misalignment of chromosomes (Hunt et al., 2003; Eichenlaub-Ritter et al., 2008), centrosomal alterations (Can et al., 2005) and increased aneuploidy (Hunt et al., 2003; Susiarjo et al., 2007), all of which are indicators of poor oocyte quality. These
data provide strong evidence that BPA at certain relevant exposure levels may detrimentally affect oocyte maturation.

Oocyte maturation is a critical period of oocyte development that can determine the developmental potential of the oocyte. Meiosis progression, spindle morphology, and chromosome alignment at the metaphase plate are used as indicators of oocyte quality due to their importance regarding further developmental success. The metaphase II (MII) spindle is often studied as an indicator of oocyte quality due to its importance in the completion of meiosis, polar body extrusion and segregation of the chromosomes. An association has been found in mammalian models between spindle abnormalities and aneuploidy incidence which may result in pregnancy loss or genetic diseases (Rama Raju et al., 2007; Ye et al., 2007; Tomari et al., 2011).

Poor quality oocytes are unable to properly support embryo development, and developmental outcomes are significantly affected by the quality of the oocyte from which they developed (reviewed by Mtango et al., 2008). External factors that have the potential to interfere with normal oocyte maturation must therefore be thoroughly assessed in order to understand the potential risk. The current study was designed to evaluate the effects of BPA during bovine in vitro oocyte maturation on meiosis progression, as well as spindle formation and chromosome alignment in MII oocytes. Based on findings in the literature, we hypothesized that exposing bovine oocytes to BPA during oocyte maturation would result in decreased meiosis progression and increased spindle abnormalities, thereby decreasing oocyte quality.
MATERIALS AND METHODS

Experimental design

Oocytes were matured in vitro (described below) in one of five treatment groups: (1) no-treatment control (IVM), (2) vehicle control (0.1% ethanol), (3) E2 (2 μg/mL), (4) 15 ng/mL BPA (65 nM), and (5) 30 ng/mL BPA (130 nM). The concentrations of BPA used in this study are within the range that have previously been shown to induce MII abnormalities in mouse (Lenie et al., 2008) and human (Machtinger et al., 2013) studies. Both concentrations fall below the estimated lowest observed adverse effect level (LOAEL) exposure dose for in vitro cell studies (Wetherill et al., 2007). These doses are higher than what has been measured in vivo since we expect some BPA to bind to the plastic dish, be absorbed by the oil surrounding the IVM droplets, and bind to bovine serum albumin present in the IVM media.

Chemicals

All chemicals were obtained from Sigma Life Sciences, Oakville, ON unless otherwise stated.

Oocyte collection and in vitro oocyte maturation

Ovaries from domestic cattle (Bos taurus) were collected from a government inspected abattoir (Cargill Meat Solutions, Guelph, ON, Canada). Cumulus oocyte complexes (COCs) were aspirated from visible ovarian follicles for collection into HEPES-buffered Ham's F-10 plus 2% steer serum (Cansera; Rexdale, ON, Canada). COCs containing several layers of cumulus cells were collected and randomly assigned into one of five treatment groups. Groups of 10-15 COCs were matured in 80 μL drops for in vitro maturation under silicone oil (Paisley Products, Toronto, ON, Canada) for 24 hours at 38.5°C in 5% CO₂ in air. The IVM drops consist of TCM199 medium + 2% steer serum supplemented with 1 μg/mL of E2, 0.5 μg/mL of bFSH and 1 μg/mL of bLH.
(NIH, Washington, DC, USA). The treatment groups were composed of IVM media supplemented with 0.1% ethanol, +1 μg/mL (to a total of 2 μg/mL) E2, 15 ng/mL BPA, or 30 ng/mL BPA.

Mature oocytes were stripped of their cumulus cells by gentle pipette-vortexing in hyaluronidase (2 mg/mL in HEPES/Sperm TALP). Stripped oocytes were then washed 2 times in PBS containing 0.1% polyvinyl alcohol (PBS-PVA) and either processed for ELISA (described below) or fixed in 4% paraformaldehyde for 20 minutes at room temperature for immunocytochemistry. Fixed oocytes were stored in PBS-PVA for no longer than 2 weeks at 4°C prior to staining.

*Enzyme-linked Immunosorbent Assay (ELISA)*

BPA concentrations were measured using Bisphenol A ELISA Kits (Creative Diagnostics, DEIA12664). This kit can measure BPA levels in various fluids and cells using the procedures described below. It is a competitive ELISA kit by which BPA can be measured in samples through a limited number of binding sites from the anti-BPA antibody which coat the bottom of the wells of the ELISA plate. BPA epitope in the samples compete with BPA-HRP conjugate which is added to all wells with the exception of the “blank” wells. Therefore the amount of BPA conjugate bound to each of the wells is inversely proportional to the BPA concentration in the sample, and is determined by the amount of color obtained when TMB, which reacts with the unbound HRP in the well, is added. Sulfuric acid is used as a stop solution converting the blue coloured product obtained with the addition of TMB to a yellow coloured product which can then be read on a plate reader at 450 nm. The specificity of the BPA ELISA as reported by the manufacturer with regards to BPA and related chemicals is as follows: BPA: 100%; BPS: <0.01%; Reversatrol: <0.01%.
IVM media used for all groups were processed immediately (T0), and at 24 hours following incubation with maturing oocytes (T24+). Mature oocytes were removed from media samples prior to processing the T24+ group. Acetic acid (Fisher Scientific Canada, Ottawa, ON, Canada) was added to 1 mL media to a pH of 4. The following was repeated 3 times to yield 3 mL ethyl acetate product. Equal parts ethyl acetate (Caledon Laboratories Ltd., Georgetown, ON, Canada) was added to the media sample which was then vortexed for 20 seconds and centrifuged at 2000 RPM for 10 minutes at room temperature for phase separation. The top (ethyl acetate) phase was removed and placed in a glass culture tube. The bottom phase was removed with a glass pipette and placed in a new tube for the extraction process to be repeated. Following three extractions, the ethyl acetate was evaporated under N2 gas. Dried samples were then sealed and stored at -20°C until use.

Oocytes for BPA concentration analysis were collected as follows: COCs were aspirated from ovaries, washed in IVM without addition of hormones, and matured in treatment groups containing 0.1% ethanol, 15 ng/mL BPA, or 30 ng/mL BPA, as described, for 24 hours. Immature or mature oocytes were placed in 2 mg/mL hyaluronidase solution and gently agitated to remove cumulus cells. Denuded oocytes were pooled into 3 biological replicates of 50 oocytes each and placed in 0.5 mL DI water in a 1.5 mL eppendorf tube. Oocytes were sonicated for 15 seconds at output power 4 using a Microson Ultrasonic Cell Disruptor (Misonix Inc., Farmingdale, NY, USA). Acetic acid was added to a pH of 4. The following was repeated 3 times to yield 1.5 mL ethyl acetate product. A volume of 0.5 mL ethyl acetate was added to each sample which were vortexed for 1 min followed by centrifugation at 10,000 RPM for 5 minutes at room temperature. Following centrifugation, two phases were visible. The top layer was removed and placed in a
glass culture tube, and the bottom was used to repeat the extraction procedure. Following the three extractions, ethyl acetate was evaporated under N\textsubscript{2} gas and stored sealed at -20°C.

ELISAs were performed according to the manufacturer’s instructions (Creative Diagnostics, DEIA12664). Briefly, samples were thawed, reconstituted with 10 μL ethanol, and diluted with 500 μL sample dilution buffer. Following addition of samples, standards, and HRP conjugate, the ELISA plate was incubated at room temperature for 2 hours. The plate was washed 3x with wash buffer and dried by blotting on paper towel. TMB (provided with kit) was added to each well (200 μL) and the plate was incubated 30 minutes at room temperature in the dark. Sulfuric acid was added to each well (50 μL) as a stop solution and the plate was read with Bio-Tek EL800 Universal Microplate Reader (Bio-tek Instruments Inc., Winooski, VT, USA) at 450 nm.

Immunocytochemistry and Imaging

Oocytes were stained to visualize microtubules and chromatin. Briefly, fixed oocytes (described above) were permeabilized in 0.5% Triton-X in TBST for 90 minutes followed by 3 10-minute washes. Oocytes were blocked in 1% goat serum (Millipore Canada, Etobicoke, ON, Canada) for 1 hour and incubated overnight at 4°C in 1:500 monoclonal anti-α-tubulin produced in mouse (Sigma-Aldrich, St. Louis, MO, USA). The following day, oocytes were washed four times (2x 30min, 2x 15min) in TBST and incubated in the dark overnight at 4°C in 1:500 secondary antibody (Alexa-Fluor 488 Goat Anti-Mouse, Life Technologies, Burlington, ON, Canada). Following the incubation, oocytes were washed three times for 30 minutes each in TBST and transferred to a PBS wash.

Oocytes were mounted onto slides and PBS was evaporated by air drying to allow adhesion to the slide. Vectashield containing DAPI (1.5 μg/mL) was placed on top of oocytes as a
chromosome stain. A syringe was used to place drops of Vaseline on the slide to avoid flattening of the oocytes when covered with a coverslip. Slides were sealed with nail polish and stored in the dark at 4°C for no longer than 2 weeks. Oocytes were imaged at 60x under oil using an Olympus FV1200 Confocal Microscope with laser wavelengths of 405 nm for DAPI and 488 nm for Alexa-Fluor 488 using Fluoview software.

**Analysis of Oocytes**

Oocytes in all treatment groups were analysed for meiotic stage. Representative images are shown in Figure 3. To calculate maturation success, the proportion of oocytes to reach MII versus the proportion of oocytes that did not reach maturation was compared between each of the treatment groups (Fisher’s exact; two-tailed).

**Spindle formation and chromosome alignment analyses**

Oocytes exhibiting a polar body and MII spindle were analyzed for spindle formation and chromosome alignment. Seven oocytes were excluded from the spindle formation analysis due to poor spindle view. Spindles were categorized as displaying a normal or abnormal spindle formation. Spindles were considered normal when there were two poles without flattening, equidistant from the metaphase plate (see Fig. 5A for example). Spindles were considered abnormal when one or both poles were flattened, there was no focussed polar region, and a flattening of the spindle led to a reduction in size (see Fig. 5B for example).

Chromosome alignment at the metaphase plate was analyzed and oocytes were categorized as having chromosomes that were aligned or dispersed at the metaphase plate. Aligned chromosomes showed little to no deviation from the metaphase plate (see Fig. 5A for example), whereas dispersed chromosomes were identified as having one or more chromosomes out of alignment with substantial deviation from the metaphase plate (see Fig. 5C for example).
Statistical analyses

ELISA results were interpreted using Graphpad Prism 6 software. Differences in concentrations of BPA in the media and oocyte analyses were compared between samples using one-way ANOVA and Tukey’s multiple comparison test. A p value of <0.05 was used to establish statistical significance.

Two-tailed Fisher’s exact test was used to calculate differences between the treatment groups of the proportion of oocytes to reach maturation versus those that did not, as well as the proportion of MII oocytes displaying a normal vs. abnormal spindle and those displaying aligned vs. dispersed chromosomes. A p value of <0.05 was used to establish statistical significance. The proportion of oocytes to arrest in the earlier stages of meiosis were analyzed with one-way ANOVA, and a p value of <0.05 was used to establish statistical significance.
RESULTS

Oocyte uptake of BPA and IVM media concentrations

The concentration of BPA measured in MII oocytes are shown in Figure 1. Results are presented as the measured BPA concentration (ng/mL) per oocyte. MII oocytes matured with IVM supplemented with the ethanol vehicle had an average uptake of 1.41 ng/mL BPA, and those matured with IVM supplemented with 15 ng/mL and 30 ng/mL BPA had an average uptake of 1.69 ng/mL and 2.48 ng/mL respectively. The concentration of BPA measured in oocytes exposed to 30 ng/mL was significantly higher than the other groups (p<0.01).

BPA concentrations in treatment group media are presented in Figure 2. BPA concentration in the 0.1% ethanol group did not differ from the IVM group. Media of the 30 ng/mL BPA group had significantly higher levels of BPA, as expected, than the other groups analyzed (p<0.05). Both BPA groups exhibited a significant decrease in BPA following the 24 hour incubation with COCs (p<0.05).

Meiosis progression and MII spindle abnormalities

Representative images of meiotic stages in bovine oocytes are displayed in Figure 3. The proportion of oocytes to reach the MII stage in each of the treatment groups is summarized in Figure 4. The 0.1% ethanol group did not differ from the IVM group. A lower proportion of oocytes exposed to 30 ng/mL BPA during oocyte maturation reached MII than oocytes in the IVM group (57.4% (62/108) and 72.4% (71/98), respectively; (p<0.05)). The proportion of oocytes in the 30 ng/mL BPA group that arrested in germinal vesicle breakdown, prometaphase I, or metaphase I was almost twice that of the no-treatment control group (IVM), however this was not statistically significant (26.9% (29/108) and 15.3% (15/98), respectively).
Figure 1. Mean BPA concentrations of oocytes following IVM in their respective treatment groups. Concentration of BPA (mean ± SEM) in oocytes at 24 hours following oocyte maturation in IVM media supplemented with 0.1% ethanol (n=150), 15 ng/mL BPA (n=150), or 30 ng/mL BPA (n=150). Analysis of variance (ANOVA) and Tukey’s multiple comparison test, **p<0.01.
Figure 2. Mean BPA concentrations of IVM media before and after incubation with oocytes. Concentration of BPA (mean ± SEM) in IVM media with no supplementation (IVM), or supplemented with 0.1% ethanol, 15 ng/mL BPA, or 30 ng/mL BPA prior to (T0) or at 24 hours following oocyte maturation (T24+). Oocytes were removed prior to processing of T24+ media. White asterisk = differences between T0 media samples of different treatment. Black asterisks = differences between T0 and T24+ media samples of the same treatment. (Analysis of variance (ANOVA) and Tukey’s multiple comparison test, *p<0.05).
Figure 3. Representative images of meiotic stages during bovine oocyte maturation. Representative images of bovine oocytes during maturation with α-tubulin in green and chromatin in blue. Stages include (A) germinal vesicle; (B) germinal vesicle breakdown; (C) prometaphase I; (D) metaphase I; (E) anaphase I; (F) telophase I; (G) prometaphase II; (H) metaphase II with polar body (arrow) visible. Scale bar: 20 μm.
Figure 4. Proportion of oocytes to reach MII following IVM in their respective treatment groups. Proportion of oocytes (mean ± SEM) to reach the MII stage following 24 hour incubation in IVM media without (IVM; n=98) or with 0.1% ethanol (n=97), 15 ng/mL BPA (n=110), or 30 ng/mL BPA (n=108) supplementation. Fisher’s exact, *p<0.05.
MII oocytes in all groups were analyzed for spindle morphology and chromosome alignment. Representative images are displayed in Figure 5. Figure 5B and C indicate spindle abnormalities observed in oocytes exposed to 30 ng/mL BPA during maturation. Abnormal spindle morphology and chromosome misalignment were frequently observed in oocytes which were matured with 30 ng/mL BPA supplementation, with abnormal spindle morphology slightly more prevalent than chromosome misalignment (Figs. 6 and 7). The spindle morphological abnormality most frequently observed was of a compressed spindle and loss of focussed polar regions. In some cases only one half of the spindle exhibited this abnormality, however most cases of abnormal morphology consisted of significant microtubule compression at both spindle ends.

Figure 6 shows the proportion of MII oocytes that displayed abnormal spindle morphology. Exposure to 30 ng/mL BPA during oocyte maturation resulted in a significantly higher proportion of oocytes displaying abnormal spindle morphology compared to all of the other treatment groups (67.9% (19/28); p<0.05). There were no significant differences between the remaining treatment groups (IVM = 28% (7/25); 0.1% ethanol = 19% (4/21), 2 μg/mL E2 = 35.5% (11/31), 15 ng/mL BPA = 31.3% (10/32)).

The proportion of oocytes displaying chromosomal dispersal is shown in Figure 7. A higher proportion of oocytes assigned to the 30 ng/mL BPA treatment group exhibited chromosome dispersal at the metaphase plate compared to all of the other treatment groups (IVM = 19.2% (5/26), 0.1% ethanol = 29% (4/21), 2 μg/mL E2 = 25.8% (8/31), 15 ng/mL BPA = 21.2 (7/33), 30 ng/mL BPA = 60% (18/31); p<0.01). There were no significant differences between the other treatment groups, including the lower dose of BPA.
Figure 5. Representative classifications of MII oocytes for spindle morphology and chromosome alignment. Representative images of bovine MII oocytes with α-tubulin in green (left panels), chromatin in blue (middle panels) and merged images (right panels). Oocytes with normal spindle morphology and chromosomes alignment (A), abnormal spindle morphology (B), and chromosome dispersal (C) are displayed. Rows B and C represent spindle abnormalities observed in oocytes treated with 30 ng/mL BPA. Visible polar body is marked with an arrow. Scale bar = 20 μm.
Figure 6. Proportion of MII oocytes displaying normal and abnormal spindle morphology following IVM in their respective treatment groups. Proportion of oocytes in each of the treatment groups displaying abnormal spindle morphology following 24 hour incubation in IVM media without (IVM; n=25) or with 0.1% ethanol (n=21), 15 ng/mL BPA (n=32), or 30 ng/mL BPA (n=28) supplementation. Fisher’s exact, *p<0.05.
Figure 7. Proportion of MII oocytes displaying dispersed chromosomes at the metaphase plate following IVM in their respective treatment groups. Proportion of oocytes in each of the treatment groups displaying dispersed chromosomes at the metaphase plate following 24 hour incubation in IVM media without (IVM; n=26) or with 0.1% ethanol (n=21), 15 ng/mL BPA (n=33), or 30 ng/mL BPA (n=31) supplementation. Fisher’s exact, **p<0.01.
DISCUSSION

In this study we have observed that exposure to 30 ng/mL BPA during bovine oocyte maturation in vitro results in an average oocyte uptake of 2.48 ng/mL BPA, as well as decreased meiosis progression and increased incidence of spindle abnormalities, specifically abnormal spindle morphology and chromosome alignment. Other studies have suggested that exposure to BPA during oocyte maturation may induce meiotic abnormalities in mouse and human oocytes. This was first shown by Hunt et al. (2003) when mice were inadvertently exposed to BPA via damaged water bottles and cages, resulting in meiotic disturbances in mouse oocytes. This correlation was then confirmed experimentally with oral administration of BPA (Hunt et al., 2003). The link between BPA and meiotic disruption has since been observed in mice (Can et al., 2005; Susiarjo et al., 2007; Lenie et al., 2008) and humans (Machtinter et al., 2013) with varying results and experimental parameters. For instance, meiotic abnormalities were observed in mice and humans as a result of BPA supplementation of culture media in which COCs were matured in vitro (Can et al., 2005; Machtinter et al., 2013). In the case of the human oocytes, clinically discarded oocytes with partial disruption to the cumulus cells were utilized (Machtinter et al., 2013). Culture of individual follicles in vitro supplemented with varying concentrations of BPA have also induced meiotic abnormalities (Lenie et al., 2008). Additionally, the onset of meiosis during fetal ovarian development was disrupted, as was oocyte maturation in the adult, following in vivo exposure of pregnant mice via implantation of BPA pellets (Susiargo et al., 2007).

The current study was designed to examine meiosis progression and MII spindle integrity following exposure to BPA during in vitro maturation of bovine oocytes, with the intent of examining the link between oocyte exposure to BPA and the quality of oocytes and resulting embryos. The current study employs a bovine in vitro maturation model due to the high
physiological similarities existing between the bovine and human reproductive systems. Bovine in vitro oocyte maturation is an excellent model to evaluate female reproductive toxicology due to similarities in follicular dynamics and endocrine control between bovine and human (Beker van Woudenberg et al., 2012). Presently, we examined the effects of BPA exposure during oocyte maturation on bovine oocyte quality.

**Oocyte uptake of BPA and IVM media concentrations**

The amount of BPA taken up by the oocytes was much lower than the initial exposure levels. Oocytes exposed to 15 ng/mL took up an average of 1.69 ng/mL BPA whereas those exposed to 30 ng/mL BPA took up an average of 2.48 ng/mL BPA (Fig. 1). These levels are similar to those that have been found in follicular fluid samples in women. Women undergoing IVF were found to have 2.4 ± 0.8 ng/mL BPA in follicular fluid samples (Ikezuki et al., 2002). The average 1.69 ng/mL BPA taken up by oocytes exposed to 15 ng/mL is similar to the lower range found in these women, whereas the 2.48 ng/mL BPA taken up by oocytes exposed to 30 ng/mL is similar to the average exposure level in the follicular fluid of women analyzed (Ikezuki et al., 2002). The exposures used in this study are of environmental significance, and the concentrations taken up by the oocytes are similar to levels of BPA that have been measured in follicular fluid of women.

The oocyte analysis of BPA was conducted using pools of oocytes to ensure a detectable level of BPA. The values obtained were then divided to obtain an average concentration per oocyte. Therefore the values presented in Figure 1 are only an average of what is being taken up by the oocytes and do not necessarily represent the amount of BPA taken up by each individual oocyte. There is likely variability between individual oocytes regarding the amount of BPA taken up within each biological replicate. This could partly explain how all oocytes exposed to the same levels of BPA do not respond identically. The amount of BPA taken up by an oocyte is likely a
determining factor in the results observed. We therefore consider the amount of BPA taken up by
the oocyte to be of greater importance than the initial exposure dose. Thus BPA concentration in
follicular fluid as well as that of experimental supplementation in IVM media does not directly
correlate with the amount of BPA taken up by the oocyte. This may be a result of uptake of BPA
by the surrounding cumulus cells and/or granulosa cells within the follicle, as well as varying
uptake rates of oocytes. The specific mechanism of BPA uptake into the oocyte needs further
analysis to determine how BPA is taken up by the oocyte as well as what factors contribute to
differences in BPA uptake among oocytes.

Although BPA has been quantified in follicular fluid, to our knowledge the measurement
of BPA in oocytes themselves has only been reported once previously. Consistent with the current
results, Aluru et al., (2010) observed that only a small proportion (1.2% and 4.5%) of BPA was
detected in rainbow trout (Oncorhyncus mykiss) oocytes following 3 hours of exposure in
comparison to the t0 measurements of 32 and 417 ng/oocyte respectively. Interestingly, the lack
of cumulus cells in the fish model suggests the amount of BPA taken up by the oocyte is limited
by the rate at which this uptake occurs.

The number of cumulus cells surrounding the oocyte and the initial quality of the oocyte
prior to IVM may affect the amount of BPA that is taken up by the oocyte. Further studies could
analyze if these factors have an influence on the oocyte’s susceptibility to BPA exposure by
evaluating the amount of BPA taken up by oocytes which have been stripped of their cumulus cells
versus those that haven’t, as well as oocytes of higher versus lower initial quality which can be
estimated based on their cytoplasmic appearance (Wood & Wildte, 1997). Determining whether
compromised oocytes take up different levels of BPA than non-compromised oocytes would
provide important information on oocyte susceptibility to BPA. Oocytes of suboptimal quality that
may survive under normal conditions may be unable to withstand the effects of BPA exposure at concentrations that good quality oocytes may be able to endure. Thus this information would be valuable in the future to further evaluate the effects of BPA on oocyte maturation and assess the risk of human exposure.

**Meiosis progression and MII spindle abnormalities**

Meiosis progression, spindle morphology and chromosome alignment of MII oocytes are related to oocyte quality and developmental potential (Rama Raju et al., 2007; Ye et al., 2007; Tomari et al., 2011). Abnormalities in spindle morphology and chromosome alignment at the metaphase plate have been found to lead to embryonic aneuploidy as a result of maternal factors such as advanced maternal age and obesity (Battaglia et al., 1996; Luzzo et al., 2012). External factors such as inappropriate culture conditions have also been shown to induce meiotic abnormalities, resulting in decreased oocyte quality (Wang et al., 2002). The external environmental factor currently discussed, BPA, has been shown under certain conditions to lead to spindle aberrations, misalignment of chromosomes, and aneuploidy as a result of oocyte exposure (Hunt et al., 2003; Can et al., 2005; Eichenlaub-Ritter et al., 2008; Lenie et al., 2008; Machtinger et al., 2013). However, these trends are not observed under all conditions, thus we aimed to identify meiotic and spindle effects in the current experimental model.

We have shown that exposure to 30 ng/mL BPA during maturation which results in an average oocyte concentration of 2.48 ng/mL BPA leads to reduced oocyte maturation success, and increased incidence of spindle abnormalities. We observed an 8-15% reduction in the proportion of oocytes reaching maturity in this group compared to the other treatment groups (Fig. 4). The 15 ng/mL BPA treatment resulting in an average oocyte concentration of 1.69 ng/mL BPA did not show a similar decrease, suggesting that a higher level of oocyte BPA uptake is required to
decrease maturation success. These results are in agreement with studies conducted in mouse (Can et al., 2005; Lenie et al., 2008) and human (Machtinger et al., 2013). In dose-dependent studies, meiotic arrest was largely only seen in the higher doses examined (Can et al., 2005; Lenie et al., 2008; Machtinger et al., 2013), however the oocyte concentration of BPA in these studies is not known. Furthermore, meiosis progression in the 30 ng/mL BPA group was only significantly decreased in comparison to the IVM group but not the 0.1% ethanol group, indicating there is a slight but non-significant effect of the vehicle that is exacerbated by the higher dose of BPA. Ethanol at higher concentrations than currently used has previously been shown to elicit a stress response in porcine COCs (Lee et al., 2014a), resulting in increased expression of pro-apoptotic genes in oocytes and cumulus cells. Thus it is possible that the ethanol vehicle currently used is causing slight but not significant alterations in maturation of the oocyte and must be considered when interpreting results. However effects observed in the spindle and chromosome analysis did differ significantly between the 30 ng/mL BPA group and the 0.1% ethanol group.

In the current analysis, MII oocytes with a BPA concentration of 2.48 ng/mL exhibited a 32.4 – 48.9% increase in the occurrence of abnormal spindle morphology and a 31 – 40.8% increase in the occurrence of chromosome dispersal compared to the other groups (Figs. 6 and 7, respectively). These results are in agreement with studies conducted in mouse (Hunt et al., 2003; Lenie et al., 2008) and human (Machtinger et al., 2013), which have found MII abnormalities as a result of BPA exposure during oocyte maturation. However, the exposure levels of BPA, and likely the resulting oocyte concentration of BPA, are critical to the results obtained, with exposure levels and responses varying between studies. For instance, MII spindle abnormalities were observed following exposure levels of 3 nM (0.685 ng/mL) to 3 μM (685 ng/mL), but not 30 μM (6.85 μg/mL), during mouse in vitro follicular development (Lenie et al., 2008). Additionally, a
significant increase in MII abnormalities were observed following BPA exposure to 20 ng/mL but not 200 ng/mL during in vitro maturation of human oocytes (Machtinger et al., 2013). Furthermore, Lenie et al. (2008) reported most MII abnormalities were due to misalignment of chromosomes at the metaphase plate with relatively few abnormalities as a result of spindle malformations, whereas in the current study, incidence of spindle malformation and chromosome misalignment were similar, but there was a higher incidence of the oocytes containing an average of 2.48 ng/mL BPA displaying both malformations (43.5%) rather than one or the other, compared to the other groups (9.1-23.1%).

Characteristics of the abnormalities also differ between studies. For instance, Hunt et al. (2003) exposed mice in vivo to varying doses of BPA during folliculogenesis, which resulted in spindle malformations, but the typical malformation was of an elongated spindle, in contrast to the shortened spindle observed in our model. Chromosome dispersal observed in other studies was also much more severe than found in the current analysis, with chromosomes in those studies sometimes dispersed throughout the entire length of the spindle as has been observed in mouse (Hunt et al., 2003; Lenie et al., 2008) and human oocytes (Machtinger et al., 2013). These differences may be explained due to varying exposure lengths and concentrations, the time of oocyte development at which exposure occurred, differences occurring between in vitro and in vivo studies, the amount of BPA being taken up by the oocytes, as well as species specific differences.

BPA has been shown to act in an estrogenic and estrogen-independent manner (reviewed by Wetherill et al., 2007). The E2 group was included to compare results obtained following additional E2 supplementation to IVM media with that of the two BPA treatments. E2 has previously been shown to impair meiotic progression and spindle morphology in vitro (Beker et
al., 2002; Beker-van Woudenberg et al., 2004). In the current chapter, treatment with additional E2 did not result in meiotic or spindle perturbations compared to the control groups. However, as E2 is included in standard IVM protocols in our laboratory, the E2 group included additional E2 supplementation to a total of 2 μg/mL as has been done previously (Pocar et al., 2003), whereas in the aforementioned studies, the control groups were void of E2 (Beker et al., 2002; Beker-van Woudenberg et al., 2004). Thus it is possible that E2 is currently inducing meiotic abnormalities, but the incidence of abnormalities do not differ between the additional E2 supplementation in the E2 group, and the standard E2 treatment in the control groups. Additionally, Beker-van Woudenberg et al. (2004) analyzed spindle perturbations throughout meiosis whereas we only assessed the MII spindle for abnormalities. Additional analyses are required to determine the mechanism(s) by which BPA is exerting its effects, and whether these may be through estrogenic pathways. Interestingly, Beker-van Woudenberg et al. (2004) reported that the meiotic abnormalities induced by E2 were not due to membrane receptor interaction.

The mechanism by which the meiotic spindle can be disrupted by BPA, however, is not clear. It has been suggested that BPA may disrupt meiosis by direct interaction with the microtubules and associated proteins during maturation (Pfeiffer et al., 1997; Can et al., 2005). Selective interference with centrosome and microtubule organization by BPA may result in meiotic disturbances observed (Can et al., 2005). BPA may induce errors in cell cycle progression and microtubule assembly and function by targeting microtubule-associated motor proteins, disrupting protein transport, or by interfering with protein interactions during oocyte maturation (Takahashi et al., 2000; Can et al., 2005). Analyses of factors important to meiosis and spindle assembly during oocyte meiosis is required to gain a greater understanding of how BPA may be eliciting these effects.
As has been shown in studies linking decreased maturation success and developmental outcome, meiosis progression and MII spindle morphology are indicators of oocyte quality. Thus the main findings of this research suggest that oocyte quality is compromised following exposure to 30 ng/mL BPA during maturation, resulting in an average oocyte concentration of 2.48 ng/mL BPA. This is evidenced by decreased meiosis progression and increased spindle abnormalities and chromosome dispersal at the metaphase plate. Considering the link between these indicators of oocyte quality and future development, BPA exposure during oocyte maturation resulting in a significant increase in oocyte concentration of BPA could have the potential to disrupt future development of the oocyte. Further analysis is required to determine the factors influencing the amount of BPA taken up by the oocyte, and potential embryonic effects of oocyte exposure to BPA during maturation.

Exposure to 15 ng/mL BPA during in vitro maturation of bovine oocytes resulted in an average uptake of 1.69 ng/mL BPA and showed no significant meiotic or MII effects against the controls in the current analysis. However, the 30 ng/mL exposure level resulted in an average uptake of 2.48 ng/mL BPA and did show significant effects in all parameters analyzed. Following this exposure, fewer oocytes reached maturity, and a higher proportion of MII oocytes displayed abnormal spindle morphology and chromosome dispersal at the metaphase plate. These factors are important to oocyte quality and developmental potential, thus BPA’s ability to influence oocyte maturation suggests that exposure during this time may affect embryonic viability.
CHAPTER TWO

Exposure to bisphenol A during \textit{in vitro} oocyte maturation results in decreased embryo development, skewed sex ratio, and increased apoptosis in blastocysts of \textit{Bos taurus}
INTRODUCTION

Successful development of an early embryo will largely depend on its environment as well as its innate ability to respond to environmental changes or stressors. Various conditions of stress can result in consequences to the embryo including delayed or abnormal development, apoptosis induction, altered gene expression, and miscarriage (Wilson et al., 1985; Niemann & Wrenzycki, 2000; Arck, 2004; Dennery et al., 2007; Ornoy, 2007). These stressors include hypoxia, changes in temperature, pathogens, UV radiation, free radicals, environmental toxins, and poor maternal diet or health (Betts & King, 2001; Paula-Lopes & Hansen, 2002b; reviewed by Hamdoun & Epel, 2007). The developmental fate of the embryo rests upon the embryo itself and its defense capabilities of withstanding such stressors.

Apoptosis, or programmed cell death, eliminates abnormal or damaged cells as well as cells with abnormal developmental potential (Hardy, 1999; Betts & King, 2001; Paula-Lopes & Hansen, 2002a). In the blastocyst, cell death may be a way to regulate development, eliminating abnormal or damaged cells (Betts & King; 2001, Hansen & Fear, 2011). By removing cells that are damaged, abnormal, or have an inappropriate developmental potential, apoptosis acts as a quality control mechanism and is an important factor in animal development (Jacobson et al., 1997; Betts & King, 2001). The ability of the embryo to induce apoptosis in times of stress may help it to survive suboptimal conditions, however the extent to which apoptosis occurs in the blastocyst plays an important role in the fate of that embryo (Betts & King, 2001). Where mild induction of apoptosis might help the embryo survive by eliminating damaged cells, severe stress may cause extensive apoptosis that could compromise development and survival of the embryo (Byrne et al., 1999; Betts & King, 2001; Paula-Lopes & Hansen, 2002b; Hansen & Fear, 2011).
Adverse environmental exposures during preimplantation development can increase the proportion of cells undergoing apoptosis in the early embryo (Paula-Lopes & Hansen, 2002b). Stresses encountered by preimplantation embryos include temperature change, heat stress, oxidative stress, pathogens, and exposure to toxic or xenobiotic chemicals (Betts & King, 2001; Hamdoun & Epel, 2007). These environmental stressors have been found to induce apoptosis in early embryos under certain conditions and may compromise embryonic viability and survival depending on the severity of the stress, and the time at which exposure occurs during development (Paula-Lopes & Hansen, 2002a; Hamdoun & Epel, 2007). Therefore the fate of the embryo may depend on the type of stress, degree of apoptotic response, and the time at which the embryo is exposed to stress.

Another possible outcome of preimplantation stress is a skewed sex ratio. Maternal stress has been found to impact the sex ratio of offspring in some cases. In rodents, females exposed to social stress produced fewer sons in mice (Krackow, 1997), hamsters (Pratt & Lisk, 1989), and rats (Lane & Hyde, 1973; Moriya et al., 1978), and the type of stress that may result in a decrease in sex ratio (fewer males) includes but is not limited to stressors such as pollution (James, 1998) and confinement stress (Krackow, 1997). Hyperglycemia-induced stress in murine embryos resulted in both an increase in apoptosis as well as a skewed sex ratio in favour of females (Jiménez et al., 2003), providing an explanation for the trend that more daughters are born to diabetic mothers (Rjasanowski et al., 1998). Fewer males also tend to be conceived in humans under suboptimal conditions, including environmental disasters (Fukuda et al., 1998), pollution (Mocarelli et al., 2000; Weisskopf et al., 2003), and mothers of advanced age (Orvos et al., 2001). In the current analysis, we assess indicators of stress and blastocyst quality as a result of BPA exposure during in vitro bovine oocyte maturation.
BPA is an EDC best known for its ability to mimic estrogen, though it has been cited to have many effects in various physiological systems. The reproductive effects of BPA published to date are vast. Altered hormone secretion (Vandenberg et al., 2009), decreased implantation success (Berger et al., 2010; Ehrlick et al., 2012a), altered ovarian morphology (Suzuki et al., 2002), abnormal early embryonic development (Takai et al., 2001) and recurrent miscarriage (Kwintkiewicz et al., 2010) are some of the many observed reproductive effects of BPA exposure. BPA has been measured at about 2.4 ng/mL in follicular fluid of women undergoing IVF (Ikezuki et al., 2002), and studies examining the effects of BPA at the oocyte maturation phase have found a variety of effects including delayed cell cycle progression (Can et al., 2005), spindle aberrations (Can et al., 2005; Eichenlaub-Ritter et al., 2008), misalignment of chromosomes (Hunt et al., 2003; Eichenlaub-Ritter et al., 2008), centrosomal alterations (Can et al., 2005) and increased aneuploidy (Hunt et al., 2003; Susiarjo et al., 2007).

Despite the many reported effects of BPA on female fertility and reproduction, the possible effects of BPA on preimplantation development in mammals are not as well documented. There is evidence that gestational or perinatal exposure can result in short- and long-term effects with the possibility of a grand-maternal effect. Developmental exposure to BPA has resulted in poor pregnancy outcomes such as pre-term birth (Cantonwine et al., 2010), predisposition to the development of metabolic syndrome (Wei et al., 2011) as well as alterations in gene expression and behaviour of offspring that may be passed on to the subsequent generation (Wolstenholme et al., 2012). However, fewer studies have analyzed the effects of early exposure to BPA on blastocyst development and quality in mammals. Existing studies have found detrimental outcomes as a result of preimplantation BPA exposure with evidence suggesting that embryo
development may be altered as a result of BPA exposure during the preimplantation period (Tsutsui et al., 1998; Takai et al., 2000, 2001; Xiao et al., 2011; Yan et al., 2013).

As reported in Chapter 1, exposure of bovine oocytes to 30 ng/mL during \textit{in vitro} maturation results in decreased maturation success, and increased incidence of MII abnormalities including a smaller, compressed spindle, and misalignment of the chromosomes at the metaphase plate. These parameters have been linked to poor developmental competency of the oocyte and resulting embryos (Rama Raju et al., 2007; Ye et al., 2007; Tomari et al., 2011). Thus, the current study was designed to evaluate the effects of exposure to BPA during oocyte maturation \textit{in vitro} on early embryo development and blastocyst quality. Cleavage and blastocyst rates, sex ratio of embryos, total cell number, and apoptosis are examined as indicators of embryo quality as well as stress experienced by the embryo during development. Based on findings in the literature, we hypothesized that BPA exposure during oocyte maturation would result in decreased developmental rates, a skewed sex ratio, lower total cell numbers, and a higher proportion of apoptotic cells, thereby indicating decreased blastocyst quality.
MATERIALS AND METHODS

Experimental design

Oocytes were matured *in vitro* in one of five treatment groups as described in Chapter 1.

Chemicals

All chemicals were obtained from Sigma Life Science, Oakville, ON unless otherwise stated.

Oocyte collection and *in vitro* embryo production

Procedures for collection and maturation of bovine cumulus-oocyte complexes (COCs) were performed as described in Chapter 1.

Frozen-thawed *Bos taurus* semen (EastGen, Guelph, Ontario, Canada) with known *in vitro* fertility was used for IVF. Semen was prepared by swim-up in 1.5 mL of sperm-HEPES TALP for 45 minutes at 38.5°C in 5% CO2 in air. Subsequently, swim up product was centrifuged at 2000 rpm for 7 min to obtain a pellet. Mature COCs were washed twice in HEPES/Sperm TALP, twice in fertilization medium IVF-TALP supplemented with 20 μg/mL heparin, and transferred to 80 μL droplets containing IVF-TALP under silicone oil (Paisley Products, Scarborough, ON, Canada). The mature oocytes were co-incubated with a final concentration of 1 x 10^6 of motile sperm for 20 hours at 38.5°C in 5% CO2 in air.

Subsequently, presumptive zygotes were collected and transferred into a 15 mL conical tube containing 2 mL sperm-HEPES TALP. Zygotes were vortexed for 120 seconds to remove the cumulus cells. Stripped zygotes were washed twice in sperm-HEPES TALP and twice in synthetic oviductal fluid (SOF) media supplemented with sodium pyruvate, non-essential and essential amino acids, gentamicin, 15% BSA in SOF, and CanSera. Zygotes were then transferred to 30 μL
droplets of SOF media under silicone oil (Paisley Products, Scarborough, ON, Canada) and incubated at 38.5°C and 5% O₂.

Calculation of development rates

Cleavage rates were calculated at 48 hours post fertilization by comparing the number of zygotes that had cleaved to the number of zygotes originally placed into culture. Blastocyst rates were calculated at 8 days post fertilization by comparing the number of blastocysts that had developed to the number of zygotes originally placed into culture.

Embryo sexing by PCR

A total number of 233 blastocysts were collected on Day 8 post fertilization and sexing analysis was carried out as described by Hamilton et al. (2012). Blastocysts were removed from culture and transferred into 0.2% pronase solution (Sigma-Aldrich, St. Louis, Missouri) for 1-2 minutes to dissolve the zona pellucidae in order to avoid sperm residue between the zona pellucida and oocyte membrane. Blastocysts were then washed three times in PBS with 0.1% PVA, and transferred individually to 0.2 mL PCR tubes with minimal PBS-PVA, submersed in liquid nitrogen and stored at -80°C.

Frozen embryos were thawed and lysed with a lysis solution containing proteinase K using an MJ Research PTC-200 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) for one hour at 37°C, and 15 minutes at 95°C, holding at 4°C at the end. The lysis product was then split into two, half of which was used to amplify testis specific protein Y-encode (TSPY) and the other half was used to amplify glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a reference gene. Primers used in the sexing analysis are displayed in Table 1. The amplifications were performed using the following program by real time polymerase chain reaction (qPCR):
denaturation at 95°C for 10 seconds, annealing at 65°C for 10 seconds, elongation at 72°C for 10 seconds, and acquisition of fluorescence for 10 seconds.

Table 1 – qPCR primers for sexing analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genbank accession number</th>
<th>Source</th>
<th>Primer sequence (5’-3’)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>NM_001034034.1</td>
<td>Hamilton et al., 2011</td>
<td>5’-ttcctggtacgacaatgaatttg-3’</td>
<td>153</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5’-ggagatggggcaggactc-3’</td>
<td></td>
</tr>
<tr>
<td>TSPY</td>
<td>X74028</td>
<td>Caudle, 2013 - MSc thesis</td>
<td>5’-tgettcaggaagacatcg-3’</td>
<td>210</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5’-cctctctgaggtgcctgc-3’</td>
<td></td>
</tr>
</tbody>
</table>

**TUNEL analysis of apoptosis in blastocysts**

Apoptosis in all treatment groups was assessed with the use of terminal deoxynucleotidyl transferase mediated dUTP nick-end labelling (TUNEL) assay (Roche Diagnostics, Indianapolis, IN, USA) as previously described (Matwee et al., 2000; Ashkar et al., 2010b). Blastocysts were collected on day 8 post fertilization and washed three times in PBS containing 0.1% PVA and fixed for one hour in 4% paraformaldehyde. Fixed blastocysts were stored in 1% paraformaldehyde at 4°C for a period no longer than two weeks. Subsequently, fixed blastocysts were permeabilized in 0.5% Triton X-100 supplemented with 0.5% sodium citrate for 1 hour. Positive control embryos were incubated for 1 hour in a humidity chamber at 38.5°C in a DNase solution containing 6.5 μL DNase buffer and 5.0 μL DNase I (400 IU/mL; ZyGEM, New Zealand).

Embryos were exposed to fluorescein isothiocyanate (FITC)-conjugated dUTP and terminal deoxynucleotidyl transferase (TdT) enzyme in a 9:1 ratio for 1 hour in a humidity
chamber at 38.5°C. Negative control embryos randomly selected from each treatment group were incubated with only FITC. Examples of a test embryo, positive control, and negative control are shown in Figure 8. Following incubation, all groups were treated with 50 μg/mL RNase (Roche Diagnostics, Indianapolis, IN, USA) for 1 hour in a humidity chamber at 38.5°C. The embryos were counterstained with 40 μg/mL propidium iodide (PI) for 45 minutes in a humidity chamber at 38.5°C. Embryos were washed three times and placed onto slides individually. VECTASHIELD anti-fade solution (Vector Laboratories, California) was placed on top of each embryo. Blastocysts with TUNEL and PI staining were visualized using an Olympus FV1200 Confocal Microscope with laser wavelengths of 488 nm for FITC and 543 nm for PI at 20x magnification. Z-stacks were taken for each embryo at a depth of 1.0 – 5.0 μM. A total of 35, 20, 28, 33, and 32 embryos were analyzed in the IVM, 0.1% ethanol, 2 μg/mL E2, 15 ng/mL BPA, and 30 ng/mL BPA treatment groups, respectively.

Cell number and apoptosis rates were analyzed using ImageJ software. Image sequences were imported and the Cell Counter plugin was used to count blastocyst nuclei by hand. Since different cell pathways share common biochemical features (Darzynkiewicz et al., 2001; Leist & Jaattela, 2001), only nuclei displaying both biochemical and morphological features of apoptosis were classified as apoptotic, as previously described (Favetta et al., 2004) thereby providing a conservative estimate of apoptosis. This method of categorization of nuclei was adapted from Gjørret et al. (2003) and nuclei were assigned to one of four categories as follows: (1) Normal: non-condensed and TUNEL-negative; (2) Condensed: condensed and TUNEL-negative; (3) DNA-damaged: non-condensed and TUNEL-positive; (4) Apoptotic: condensed and TUNEL-positive. Total cell number was calculated as the sum of all four cell types within a single blastocyst.
Condensed, DNA-damaged, and apoptotic nuclei rates were calculated as the number of cells categorized as such divided by the total cell number of the embryo.

Figure 8. Representative images of test samples (S), and positive (+) and negative (-) controls. Scale bar = 50 μM.
**Statistical analyses**

Two-tailed Fisher’s exact test was used to analyze the differences in cleavage and blastocyst formation rate by comparing the proportion of embryos to cleave or reach blast versus those that did not. The impact of the treatment group on the resulting sex ratio was analyzed by linear regression analysis, and the correlation between blastocyst rates and sex ratios were completed using simple linear regression (Graphpad Prism 6). Furthermore, odds ratios were performed to determine if the sex ratios in each treatment group differed significantly from the expected 1:1 ratio (MedCalc statistical software). A \( p \) value of <0.05 was used to establish statistical significance.

Total cell number, apoptosis rates, as well as rates of other classified nuclei were interpreted using Graphpad Prism 6 software. Differences were compared between treatment groups with one-way ANOVA, and a \( p \) value of <0.05 was used to establish statistical significance. When significance was indicated, Tukey’s multiple comparison test was used to determine differences between individual treatment groups.
RESULTS

Developmental rates, sex ratio, and total cell number of blastocysts

Cleavage and blastocyst rates, sex ratio and total cell number of blastocysts are presented in Table 1. The cleavage rate of the 30 μg/mL BPA treatment group (64.4%) was significantly lower compared to both the no-treatment (IVM) and vehicle controls (0.1% ethanol) (79.7% (p=0.0036) and 77.6% (p=0.0095), respectively). The cleavage rates of the two BPA treatment groups also differed, with a significantly lower proportion of zygotes cleaving following exposure to 30 ng/mL during oocyte maturation compared to 15 ng/mL (64.4% vs. 78%, respectively; p=0.0024). Cleavage rates of the 30 ng/mL BPA treatment group was also lower than the E2 group, however these results were not statistically significant (64.4% vs. 73%, respectively; p=0.0587). The proportion of cultured embryos to reach blastocyst was significantly lower in the 30 ng/mL BPA-exposed group (21.4%) compared to the IVM and 0.1% ethanol groups (36.9% (p=0.0005) and 29.7% (p=0.0077), respectively). Blastocyst rates in the lower dose of BPA (24.1%) was significantly lower than that of the IVM group (36.9%, p=0.0381), but not the 0.1% ethanol group (29.7%) (p=0.2236).

The sex ratio of single blastocysts were calculated for all groups and presented as the proportion of blastocysts tested that were identified as male (Table 1). The proportion of male blastocysts arising from oocytes in both BPA groups were lower than controls, with the 30 ng/mL BPA group exhibiting a significant decrease in the male: female sex ratio compared to the expected 1:1 ratio (p=0.0326), and the 15 ng/mL BPA group showing a non-significant decrease (p=0.0882). Furthermore, all groups were subject to linear regression analysis to determine the likelihood that the changes in sex ratio were a result of the treatment administered (p=0.0103). A strong but non-significant correlation was also found between the resulting blastocyst rates and sex ratios in each
treatment group (p=0.053). Total cell number of blastocysts were calculated and are displayed in Table 1. The mean blastocyst cell number did not differ between any of the treatment groups and ranged from 114.3 ± 9.5 in the 30 ng/mL BPA group to 131.7 ± 7.0 in the E2 group (p=0.67).

*Nuclear condensation, DNA damage, and apoptosis*

Blastocyst nuclei were characterized by their morphological and biochemical characteristics through TUNEL analysis. Nuclei that were TUNEL-negative, but morphologically condensed were characterized as condensed nuclei. Nuclei staining positive for TUNEL, exhibiting the biochemical characteristics of apoptosis, but not condensed were characterized as DNA-damaged. Finally nuclei exhibiting both the biochemical (TUNEL-positive stain) and morphological (condensation and/or fragmentation) characteristics of apoptosis were categorized as apoptotic. Representative images of condensed, DNA-damaged, and apoptotic nuclei are exhibited in Figures 9, 10, and 11 respectively.

The mean proportion of condensed nuclei did not differ between the treatment groups. Examples of condensed nuclei that were not apoptotic as well as the mean proportion of condensed nuclei per blastocyst in all treatment groups are displayed in Figure 9. Representative images as well as the mean proportion of DNA-damaged nuclei are displayed in Figure 10. Blastocysts that developed from oocytes exposed to 30 ng/mL BPA during maturation contained a significantly higher proportion of DNA-damaged nuclei (0.7%) than all of the other groups (0.06% – 0.22%; p<0.05), though incidence was rare in all treatment groups.

Apoptotic nuclei were stained positive for TUNEL and exhibited morphological characteristics of apoptosis. Example images as well as the mean proportion of apoptotic nuclei in blastocysts are displayed in Figure 11. Exposure to 30 ng/mL BPA during oocyte maturation
resulted in blastocysts with a significantly higher proportion of apoptotic cells (13.6\%) in comparison to all of the other groups (7 – 9.3\% p<0.01).
Table 2 – Effect of IVM treatments on development, sex ratio, and total cell number of bovine embryos

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Zygotes</th>
<th>Cleaved Percentage of zygotes</th>
<th>Day 8 blastocysts Percentage of zygotes</th>
<th>Sex Ratio Percentage Male</th>
<th>Blastocyst cell number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$n$</td>
<td>$n$</td>
<td>$n$</td>
<td>$n$</td>
<td>$n$</td>
</tr>
<tr>
<td>IVM</td>
<td>255</td>
<td>207</td>
<td>79.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>94</td>
<td>36.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.1% ethanol</td>
<td>295</td>
<td>235</td>
<td>77.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>92</td>
<td>29.7&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>2 μg/mL E2</td>
<td>259</td>
<td>201</td>
<td>73.0&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>79</td>
<td>27.4&lt;sup&gt;abc&lt;/sup&gt;</td>
</tr>
<tr>
<td>15 ng/mL BPA</td>
<td>321</td>
<td>260</td>
<td>78.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>91</td>
<td>24.1&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>30 ng/mL BPA</td>
<td>323</td>
<td>228</td>
<td>64.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>81</td>
<td>21.4&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>abc</sup> Values in the same column with different superscripts are significantly different (p<0.05). 
(*) indicates significant variation in sex ratio from the expected 1:1 ratio; *p<0.05
Figure 9. Condensed nuclei (white arrows) in blastocysts. Representative images (A) and mean proportions (B) of condensed nuclei without TUNEL staining in blastocysts in each of the treatment groups. Mean ± SEM. Scale bars = 50 μM.
Figure 10. DNA-damaged nuclei (white arrow) in blastocysts. Representative images (A) and mean proportions (B) of TUNEL-positive, non-condensed nuclei in blastocysts in each of the treatment groups. Mean ± SEM. Analysis of variance (ANOVA) and Tukey’s multiple comparison test, *p<0.05. Scale bars = 50 μM.
Figure 11. Apoptotic nuclei (white arrows) in blastocysts. Representative images (A) and mean proportions (B) of TUNEL-positive, condensed nuclei in blastocysts in each of the treatment groups. Mean ± SEM. Analysis of variance (ANOVA) and Tukey’s multiple comparison test, **p<0.01. Scale bars = 50 μM.
DISCUSSION

The results presented in this study indicate that exposure of bovine oocytes to 30 ng/mL BPA during in vitro oocyte maturation decreases embryonic cleavage and blastocyst rates, skews sex ratio towards femaleness, and increases apoptosis, as well as the proportion of non-apoptotic cells undergoing DNA damage, in blastocysts. However, the total cell number of blastocysts did not differ between any of the treatment groups. The lower dose of BPA (15 ng/mL), resulted in a decrease in blastocyst rate, but only in comparison to the no-treatment control, and a skewed sex ratio in comparison to the expected 1:1 ratio, but this did not reach statistical significance. No other indicators of blastocyst quality were altered in the 15 ng/mL BPA group. Taken together these findings suggest that oocyte exposure to low levels of BPA during oocyte maturation can decrease developmental potential and quality of the resulting blastocyst. Other studies have suggested that BPA has the potential to affect early embryo development, however results between studies are variable (Tsutsui et al., 1998; Takai et al 2000, 2001; Bloom et al., 2011b; Xiao et al., 2011; Ehrlich et al., 2012b; Yan et al., 2013).

In vitro studies examining BPA exposure during preimplantation development have exhibited similar results, and the same variability, as in vivo studies. Takai et al. (2000, 2001) observed opposite effects of low (1-3 nM; <1 ng/mL) and high (100 μM; 22.83 μg/mL) doses of BPA on murine embryos. Two-cell embryos exposed to 100 μM resulted in fewer embryos reaching the blastocyst stage, whereas treatment with 1nM BPA resulted in a greater number of embryos reaching blast. Blastocyst morphology, total cell number, and sex ratio did not differ between treatment groups and the control (Takai et al., 2000). Both the high and low treatments led to pups that were significantly heavier than controls at weaning (Takai et al., 2001). Thus BPA exposure during the preimplantation period has the potential to affect blastocyst development, as
well as induce postnatal effects. However this is the first study, to our knowledge, to investigate the effects of BPA exposure during *in vitro* maturation of oocytes on embryo development and blastocyst quality in the bovine species.

Previously we have shown that BPA exposure during oocyte maturation under the same conditions as were used currently, results in only a small proportion of BPA being taken up by the oocyte (Chapter 1). Oocytes exposed to 30 ng/mL during maturation resulted in an average uptake level of 2.48 ng/mL per oocyte, whereas the lower dose, 15 ng/mL resulted in an oocyte concentration of 1.69 ng/mL. Since the amount of BPA taken up by an oocyte is likely of greater importance than the initial exposure dose, it is essential to consider uptake of BPA when assessing developmental effects. Additionally, we observed that maturational exposure decreased the success of oocytes reaching MII, and an increased incidence of MII spindle abnormalities including altered spindle morphology and misalignment of the chromosomes at the metaphase plate (Chapter 1). Thus the effects observed in this study are likely the result of alterations made during oocyte maturation, and differ from similar studies in which exposure occurs during embryo development.

*Embryo development, sex ratio, and blastocyst cell number*

As mentioned, blastocyst development was delayed as a result of embryonic exposure to 100 μM BPA *in vitro* (Takai et al., 2000), and urinary BPA concentrations have been associated with decreased embryo development (Ehrlich et al., 2012b). The results presented here suggests that exposure to BPA during oocyte maturation decreases the embryo’s ability to cleave following fertilization, and develop to the blastocyst stage. Total cell number and sex of embryos that did reach blastocyst were determined to further analyze blastocyst quality. The sex ratio was significantly affected by oocyte maturational exposure to 30 ng/mL BPA, with a 15.8 - 20%
decrease in sex ratio, meaning 34.1% of blastocysts in the 30 ng/mL group were male compared to 49.9% in the IVM group and 54.1% in the 0.1% ethanol group. We found no differences in total cell number of blastocysts between all of the treatment groups.

Sex ratio was analyzed as it is considered to be an indicator of embryo quality and can give an idea of the severity of stress experienced by the embryo. For instance, sex ratio has been altered as a result of maternal stress in rodents (Lane & Hyde, 1973; Moriya et al., 1978; Pratt & Lisk, 1989; Krackow, 1997; James, 1998) and humans (Fukuda et al., 1998; Mocarelli et al., 2000; Orvos et al., 2001; Weisskopf et al., 2003). In vitro, blastocyst sex ratio has been found in many instances to be altered by environmental conditions such as hyperglycemia, oxidative stress, in vitro culture media composition, and exposure to EDCs (Mocarelli et al., 2000; Weisskopf et al., 2003; Gutiérrez-Adán et al. 2001a; Larson et al., 2001; Ishihara et al., 2007). Increased glucose levels in in vitro culture media appears to favour male embryos and inhibit female embryo development (Gutiérrez-Adán et al. 2001a; Larson et al., 2001), whereas paternal exposure to the dioxin TCDD resulted in skewed sex ratio in favour of female embryos (Ishihara et al., 2007).

Studies from our lab have previously indicated a skew in sex ratio as a result of environmental conditions. In vitro maturation of bovine oocytes in an open-well glassware system with ethanol-supplemented media resulted in a sex skew in favour of females (34% males) (Macaulay et al., 2011). This skew in sex ratio was accompanied by a decrease in cleavage and blastocyst rates versus the standard control IVM system, indicating that alterations in IVM protocols and culture environment of oocytes during maturation can have developmental consequences such as decreased developmental potential and embryo quality (Macaulay et al., 2011), which is in line with the results currently obtained. Furthermore, our lab has previously described a positive correlation between blastocyst formation rates and the percentage of male
embryos, indicating that a decrease in blastocyst rates was accompanied by a decrease in the proportion of male embryos (Macaulay et al., 2013).

These trends indicate that male embryos are not as resilient as female embryos and may be less likely to survive under stressful conditions and support the Trivers and Willard hypothesis that postulates that an excess of male offspring are favoured by natural selection when conditions, and likelihood to survive, are good, and mothers in poor conditions benefit most by the production of daughters (Trivers & Willard, 1973). This may be a result of differential survival rates of male and female embryos during preimplantation development. Similar to the above-mentioned studies, the male:female sex ratio in the current analysis was decreased, meaning there was a higher proportion of female blastocysts following exposure to 30 ng/mL BPA during oocyte maturation than the control groups. The skew in sex ratio may indicate embryonic stress, to which male embryos are less likely to survive than female embryos. Determination of the sex of arrested embryos would provide valuable information on whether male embryos are arresting at a higher rate than female embryos.

*Nuclear condensation, apoptosis, and DNA-damage*

Apoptosis in the blastocyst is common, and is an important mechanism by which the embryo can remove damaged cells or cells with abnormal developmental potential without affecting neighbouring cells (Jacobson et al., 1997; Betts & King, 2001; Hansen & Fear, 2011). An increased incidence of apoptosis may indicate an embryonic response to stress. The ability of the embryo to induce apoptosis in times of stress may help it to survive suboptimal conditions, however the extent to which apoptosis occurs in the blastocyst plays an important role in the fate of that embryo (Byrne et al 1999; Betts & King, 2001; Paula-Lopes & Hansen, 2002b; Hansen & Fear, 2011).
In the current study, exposure of oocytes during maturation to 30 ng/mL BPA resulted in a significantly higher proportion of cells undergoing apoptosis at the blastocyst stage. BPA has previously been found to increase apoptosis in embryonic midbrain cells in vitro (Liu et al., 2013), and murine antral follicles following maternal exposure to BPA (Peretz et al., 2013). Furthermore, apoptosis in the blastocyst has been induced by stressors such as heat stress and exposure to xenobiotic chemicals (Betts & King, 2001; Hamdoun & Epel, 2007). The increased incidence of apoptosis currently observed may indicate a decreased developmental potential, possibly caused by embryonic stress and/or cellular abnormalities as a result of exposure to BPA during oocyte maturation. Further analysis is required to assess the pathway through which apoptosis is being induced by BPA exposure.

Morphologically, apoptotic cell death differs from other forms of cell death, such as necrosis, by the presence of chromatin condensation and nuclear fragmentation (Majno & Joris, 1995). It is important to distinguish between nuclei which display both the biochemical and morphological characteristics of apoptosis, from those which display one or the other (Gjørret et al., 2003). Observation of more than one of the characteristics of apoptosis is essential for proper identification (Darzynkiewicz et al., 2001). Nuclei displaying morphological characteristics of apoptosis are commonly observed in embryos after the 8-cell stage (Gjørret et al., 2003). Nuclear condensation may be a result of early apoptosis in which DNA fragmentation is yet to occur (Collins et al., 1997), or a misinterpretation of prophase nuclei due to the higher chromatin content than interphase nuclei (Gjørret et al., 2003). Alternatively, nuclei may condense and become fragmented by alternative mechanisms such as abnormal mitotic chromosome segregation, which can result in micronuclei-like structures (Gjørret et al., 2003). Currently, the proportion of non-apoptotic condensed nuclei did not differ between treatment groups.
TUNEL allows for detection of apoptotic cells *in situ* by labelling DNA fragmentation. However, DNA fragmentation is not exclusive to apoptotic cell death and all nuclei undergoing DNA fragmentation, not just apoptotic nuclei, will stain positive for TUNEL. Thus it is important to distinguish apoptotic cells from non-apoptotic cells that contain DNA fragmentation. Nuclei displaying the biochemical features of apoptosis in absence of morphological features were therefore not considered to be apoptotic. The proportion of cells displaying normal morphology with DNA damage (TUNEL-positive) presented here was significantly higher in the 30 ng/mL BPA group than all of the other groups. Therefore in addition to the increased apoptosis rate in the 30 ng/mL BPA group, there was also evidence of increased non-apoptotic DNA fragmentation. Further analysis is required to determine the cause of this DNA damage and whether it represents an alternative form of cell death.

Taken together, the findings presented indicate that exposure to low levels of BPA during *in vitro* oocyte maturation has the potential to result in decreased embryonic developmental potential and quality as evidenced by decreased development rates, skewed sex-ratio, and increased proportion of cells exhibiting apoptosis and non-apoptotic DNA damage. The maturing oocyte is vulnerable to environmental perturbations and can influence further development of the embryo. In Chapter 1, we observed that the same experimental conditions resulted in low levels of BPA taken up by the oocyte. Exposure to 15 ng/mL BPA resulted in an average uptake of 1.69 ng/mL and exposure to 30 ng/mL resulted in an average uptake of 2.48 ng/mL BPA. As we have previously observed meiotic aberrations under the same conditions (Chapter 1), it is possible that irregularities resulting from an abnormal spindle and chromosome misalignment could result in aneuploid blastomeres, which could be removed from the blastocyst via apoptosis. There are various causes of stress in the preimplantation embryos and these stressors can result in increased
apoptosis and/or a skewed sex ratio. In some cases activation of stress pathways, as has been observed in heat and oxidative stress, may induce apoptosis by altering gene expression in the preimplantation embryo (Kawarsky & King, 2001; Camargo et al., 2007; Rho et al., 2007). Mechanistic analyses are essential to determine how BPA exposure during oocyte maturation is disrupting the development and quality of the early embryo.
CHAPTER THREE

Gene expression of developmentally important genes in *Bos taurus* MII oocytes and blastocysts following bisphenol A exposure during *in vitro* oocyte maturation.
INTRODUCTION

Integrity of early embryonic development is influenced by the environmental conditions surrounding the embryos or the oocyte from which it developed. Alterations in the oocyte’s microenvironment can influence not only oocyte metabolism and developmental competence, but also embryo quality and gene expression patterns (Guérin et al., 2001; Leroy et al., 2012). Environmental conditions, such as in vitro culture conditions, temperature change, oxygen tension change, and toxic exposures can cause abnormalities in oocyte maturation, embryonic development, lead to poor developmental outcomes, and alter gene expression of the early embryo (Niemann & Wrenzycki, 2000; Li et al., 2005; Lonergan et al., 2006; Camargo et al., 2008). Furthermore, changes in gene expression of the mature oocyte may be indicative of a disruption to developmental programs (Paczowski et al., 2011; Yuan et al., 2011).

Although many aspects of oocyte maturation remain unknown, it has been well established that a complex variety of factors are involved in meiotic progression, and spindle assembly, integrity, and localization. Meiosis is largely regulated by the cell cycle regulator maturation-promoting factor (MPF). MPF is one of the main signalling pathways of oocyte maturation and one of the key regulators of cell cycle progression and oocyte maturation (reviewed by Voronina & Wessel, 2003). It consists of two subunits, the catalytic cyclin-dependent kinase 1 (CDK1) (aka CDC2, p34cdc2), an important cell cycle regulator, and the regulatory cyclin B (Dunphy et al., 1988; Gautier et al., 1988, Pines et al., 1989, Gautier et al., 1990). Additionally, genes important to spindle assembly, function, and/or translational regulation include aurora kinase A (AURKA) (Uzbekova et al., 2008), deleted in azoospermia-like (DAZL) (Chen et al., 2011), and kinesin family member 5b (KIF5b) (Brevini et al., 2007; Kidane et al., 2013). KIF5b is a motor molecular and is critical to germinal vesicle breakdown (GVBD), kinetochore assembly, chromosome
stability, polar body extrusion, integrity and polarity of the mitotic spindle, as well as metaphase alignment (Kidane et al., 2013). AURKA has been found to be essential for centrosome and spindle assembly, chromosome attachment, and chromosome alignment (Marumoto et al., 2005; Uzbekova et al., 2008). DAZL is a microtubule regulator and directs translation of various genes important to cell cycle regulation, chromatin remodeling, spindle function, and centrosome and spindle assembly (Chen et al., 2011). Alterations in these genes during oocyte maturation has resulted in impaired embryonic development (KIF5b) (Tanaka et al., 1998; Takamiya et al., 2004), meiotic arrest and increased aneuploidy in oocytes (AURKA) (Uzbekova et al., 2008; Lane et al., 2010), and disruption to meiotic spindle assembly (DAZL) (Chen et al., 2011).

Alterations in expression of stress- and metabolism- related genes in the MII stage may leave the resulting embryo unequipped for early embryonic development (Brevini et al., 2002; Percell & Moley, 2009). In the embryo, stress can result in alterations of stress-responsive genes including heat shock protein 70 (HSP70), tumour protein p53, and glucose transporter 1 (GLUT1) (Batt et al., 1991; Kawarsky & King, 2001; Camargo et al., 2007; Rho et al., 2007; Hu et al., 2011). Expression levels of these genes have been shown to vary as a result of embryonic stress, and these responses appear to depend on both the level of stress experienced and the stage of development at which the stress occurs (Wrenzycki et al., 1998; Niemann & Wrenzycki, 2000; Kawarsky & King, 2001; Peretz et al., 2012). In addition to mRNA expression alterations as a result of stress experienced during embryonic development, the environment in which the oocyte matures has also been found to influence gene expression in the mature oocyte and the resulting embryo (Russel et al., 2006; Sagirkaya et al., 2007; Wells & Patrizio, 2008; reviewed by Virant-Klun et al., 2013).

Among the markers that allow us to evaluate embryonic viability, the expression of hormone receptors is of significant importance at different stages of development and especially
at the oocyte and blastocyst stages (Beker-van Woudenberg et al., 2004; Vasquez & DeMayo, 2013). Alterations in hormone receptor mRNA synthesis may indicate poor developmental programming or improper conditions, and may be predictive of undesirable reproductive outcomes. Nuclear receptors play an important role in the oocyte and early embryo (Beker-van Woudenberg et al., 2004), and are essential for implantation or attachment (reviewed by Vasques & DeMayo, 2013). The steroid hormone, estradiol (E2) plays a critical role in fertility, oocyte development, and early embryonic development. E2 primarily exerts its actions by binding to its receptors ERα and ERβ. Expression of ERs by the embryo may be critical to its survival (Hou & Gorski, 1993). Thyroid hormones (THs), which activate the TH receptors TRα and TRβ, play a beneficial role in preimplantation development including increased blastocyst and hatching rates (Ashkar et al., 2010b, 2013). Both the ERs and TRs have been found to be altered transcriptionally by exposure to the endocrine disruptor bisphenol A (BPA) (Heimeier et al., 2009; Aghajanova & Giudice, 2011), but the influence of BPA on mammalian embryo development on ER and TR expression, as well as that at the oocyte maturation stage, is not fully understood.

In the current study, oocyte and blastocyst gene expression were evaluated under various treatment conditions occurring solely during oocyte maturation. Genes involved in oocyte maturation and spindle assembly (CDC2, AURKA, DAZL, KIF5b, alpha tubulin [TUBA]), response to stress and/or metabolism (HSP70, p53, GLUT1), and hormone receptor genes (ERβ, TRβ), were quantified in germinal vesicle (GV) stage and metaphase II (MII) oocytes. Blastocysts resulting from oocytes under the same treatment conditions were further analyzed for variations in gene expression of the stress, metabolism, and hormone receptor genes. As increased transcript degradation due to poor environmental conditions may lead to a decrease in mRNA in MII oocytes, we hypothesized that transcripts in the oocyte would be decreased after BPA treatment during
maturation, with the exception of TUBA, which we speculated would not be altered as it has previously been used as a reference gene in BPA analyses (Gentilcore et al., 2012). Furthermore, due to existing reports of both embryonic response to stress and BPA action on these genes in alternate tissues (Niemann & Wrenzycki, 2000; Wrenzycki et al., 2001; Matthews et al., 2001; Kang et al., 2002; Moriyama et al., 2002; Takao et al., 2003; Levy et al., 2004; Lahnsteiner et al., 2005; Schirling et al., 2006; Planelló et al., 2008; Heimeier et al., 2009; Vandenberg et al., 2009; Guo et al., 2012; Liu et al., 2013; Park & Kwak, 2013; Peretz et al., 2013), we have hypothesized that in the blastocyst, p53, HSP70, and ERβ would be increased as a result of BPA exposure during oocyte maturation *in vitro*, whereas GLUT1 and TRβ would be decreased.
MATERIALS AND METHODS

Experimental design

Oocytes were matured in vitro in one of five treatment groups as described in Chapter 1.

Chemicals

All chemicals were obtained from Sigma Life Sciences, Oakville, ON unless otherwise stated.

Oocyte collection and in vitro embryo production

Procedures for oocyte collection and in vitro embryo production are described in Chapters 1 and 2.

RNA extraction

Oocytes from three biological replicates were collected in pools of 40 at GV and at MII following the various treatments. Embryos from three groups were collected in pools of 5 at the blastocyst stage. Oocytes were stripped, as described in Chapter 1, and oocytes and blastocysts were washed three times in PBS-PVA 0.1%, snap-frozen in liquid nitrogen and stored at -80°C. RNA was isolated from pooled embryos using the AllPrep™ DNA/RNA Micro Kit (QIAGEN, Inc., Burlington, ON), following the manufacturer’s instructions. In brief, Buffer RLT plus was added to lyse the cells. Samples were vortexed for homogenization and transferred to an AllPrep DNA spin column to bind genomic DNA. Flow through was mixed with 70% ethanol and run through an RNeasy MinElute spin column to bind total RNA. The column was then washed and dried, followed by RNA elution in RNase-free water. RNA samples were reverse transcribed immediately following extraction using the one-step protocol with qScript™ cDNA SuperMix (Quanta Biosciences, Canada) following the manufacturer’s instructions. cDNA samples were stored at -20°C until needed.
Gene expression analysis

Quantitative real-time PCR (qPCR) was used to measure mRNA expression profiles of selected genes in the five treatment groups and in the GV oocyte group. Each analysis was performed on three biological replicates with three technical replicates each. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and peptidylprolyl isomerase A (PPIA) were used as reference genes for all qPCR analyses. Relative quantity of target genes were log-transformed and normalized to relative quantity of the reference genes, GAPDH and PPIA, across samples (ΔΔCq) (Bio-Rad CFX Manger 3.1). Therefore values presented in figures in the current chapter are relative normalized gene expression levels.

The primer sequences are listed in Table 3. The expression of all genes were previously detected in domestic cattle as confirmed by the references provided in Table 3. qPCR was carried out using the Bio-Rad CFX96 Real-Time PCR System and products were detected with SsoFast TMEvaGreen® Supermix (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer’s instructions. Each reaction contained 5 μL of the SsoFastTM EvaGreen® Supermix reaction mix, 1 μL of a mix of the forward and reverse primers (at a 0.1 μM concentration for each set of primers, except the ERβ primers, used at a concentration of 0.5 μM) and 2 μL cDNA for all genes with the exception of ERβ (3μL). The final volume was adjusted to 10 μL using RNase-free water (Ref Ambion PCR-grade water). A standard curve was established for each primer set using ovarian tissue cDNA template in five serial dilutions and primers efficiencies were calculated and used in the analysis. The amplification program was as follows: preincubation for EvaGreen® Supermix polymerase activation at 95°C for 10 minutes, followed by 50 amplification cycles of denaturation at 95°C for 10 seconds, annealing at 65°C for 10 seconds, elongation at 72°C for 10 seconds, and acquisition of fluorescence for 10 seconds. After the last
cycle, fluorescence acquisition was begun at 72°C, and measurements were taken every 0.5°C until 95°C to generate the melting curve.

**Statistical analysis**

Expression levels were calculated relative to those of internal reference genes GAPDH and PPIA in Bio-Rad CFX Manager 3.1. Differences in MII and blastocyst gene expression levels between treatment groups were analyzed with One-way analysis of variance (ANOVA), and when statistical significance was observed (p < 0.05), Tukey’s multiple comparison tests were performed to evaluate differences between specific treatment groups within a single gene (Graphpad Prism 6.0). Differences in expression levels between GV and MII stages were analyzed for each gene using unpaired student’s t-test (Graphpad Prism 6.0). Differences at p < 0.05 were considered statistically significant.
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<th>Gene</th>
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<th>Source</th>
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RESULTS

mRNA expression in GV and MII oocytes

Expression levels in GV and MII oocytes of the various genes analyzed are shown in Figure 12. The genes studied showed variability in mRNA levels before and after maturation. There were no significant differences observed between GV and MII stages in ERβ (Figure 12 A), GLUT1 (Figure 12 B), and HSP70 (Figure 12 C) mRNA expression levels. mRNA levels for CDC2 (Figure 12 D), TRβ (Figure 12 E), and TUBA (Figure 12 F) were lower in MII oocytes (p < 0.05), whereas those for DAZL (Figure 12 G), p53 (Figure 12 H), KIF5b (Figure 12 I), and AURKA (Figure 12 J), were increased in MII oocytes compared to GV (p<0.05), however the increase in KIF5b was not statistically significant.

Genes involved in oocyte maturation and spindle assembly

The mRNA expression levels of genes involved in meiosis progression, spindle assembly, integrity, and chromosome stability and alignment following IVM in the various treatment groups are shown in Figure 13. Significant differences between groups were observed in the CDC2, AURKA, and DAZL analyses. MII oocytes matured with 15 ng/mL BPA exhibited significantly higher expression of CDC2 and DAZL mRNA than those matured under standard conditions (IVM) (Figure 13 A and D, respectively). A similar trend was observed in the KIF5b group, however these results were not significant. A vehicle effect was observed in several of the genes studied in the oocyte, though this effect was only significantly different between the no-treatment and vehicle controls in the AURKA analysis (Figure 13 B). No significant differences were observed in the TUBA mRNA expression analysis, though there appears to be a non-significant vehicle effect.
**Hormone receptor gene expression**

Differences in mRNA levels of ERβ (A) and TRβ (B) in MII oocytes following maturation under various treatments are shown in Figure 14. There were no significant differences in ERβ mRNA expression in MII oocytes, however changes were observed in TRβ mRNA expression (p < 0.05). Similar to results observed regarding CDC2 and DAZL mRNA expression, TRβ expression in MII oocytes following maturational exposure to 15 ng/mL BPA was increased compared to the no-treatment control (Figure 14 B).

Relative mRNA abundance of ERβ and TRβ in blastocysts resulting from oocyte matured under various treatments is presented in Figure 15. There were no significant differences in mRNA expression of ERβ or TRβ between the different treatment groups at the blastocyst stage. Although there appears to be an elevation of ERβ mRNA expression in the 15 ng/mL BPA group, this did not reach statistical significance (Figure 15 A).

**Genes involved in Stress and Metabolism**

The mRNA expression profiles of HSP70 (A), p53 (B), and GLUT1 (C) in MII oocytes are shown in Figure 16. Significant differences in p53 mRNA expression between treatment groups were observed (p < 0.05). Expression of p53 mRNA was significantly increased in the 15 ng/mL BPA group relative to the IVM and the 30 ng/mL BPA groups (Figure 16 B). There were no significant differences in HSP70 and GLUT1 mRNA expression between treatment group (Figure 16 A and C, respectively). Relative mRNA expression in blastocysts of HSP70, p53, and GLUT1 transcripts is shown in Figure 17. There were no differences in the relative expression levels of these transcripts at the blastocyst stage between treatment groups.
Figure 12. mRNA expression profiles of GV and MII oocytes in ERβ (A), GLUT1 (B), HSP70 (C), CDC2 (D), TRβ (E), TUBA (F), DAZL (G), p53 (H), KIF5b (I), and AURKA (J). Unpaired t-test; (*) indicates statistical significance, *p<0.05; **p<0.01. Experiments were conducted on three different pools of 40 oocytes (n = 3) and replicated three times (r = 3) for each sample.
Figure 13. mRNA expression levels (Mean ± SEM) of CDC2 (A), AURKA (B), KIF5b (C), DAZL (D), and TUBA (E) in MII oocytes following IVM in respective treatment groups. One-way ANOVA and Tukey’s multiple comparison test. Bars with different letters indicate statistical significance (p<0.05; **p<0.01). Experiments were conducted on three different pools of 40 oocytes (n = 3) and replicated three times (r = 3) for each sample.
**Figure 14.** mRNA expression levels of ERβ (A) and TRβ (B) in MII oocytes following IVM in respective treatment groups. One-way ANOVA and Tukey’s multiple comparison test. Bars with different letters indicate statistical significance (p<0.05). Experiments were conducted on three different pools of 40 oocytes (n = 3) and replicated three times (r = 3) for each sample.
Figure 15. mRNA expression levels of ERβ (A) and TRβ (B) in blastocysts arising from oocytes matured in vitro in respective treatment groups. One-way ANOVA and Tukey’s multiple comparison test. Bars with different letters indicate statistical significance (p<0.05). Experiments were conducted on three different pools of 40 oocytes (n = 3) and replicated three times (r = 3) for each sample.
**Figure 16.** mRNA expression levels of HSP70 (A), p53 (B), and GLUT1 (C) in MII oocytes following IVM in respective treatment groups. One-way ANOVA and Tukey’s multiple comparison test. Bars with different letters indicate statistical significance (p<0.05). Experiments were conducted on three different pools of 40 oocytes (n = 3) and replicated three times (r = 3) for each sample.
Figure 17. mRNA expression profiles of HSP70 (A), p53 (B), and GLUT1 (C) in blastocysts arising from oocytes matured *in vitro* in respective treatment groups. One-way ANOVA and Tukey’s multiple comparison test. Bars with different letters indicate statistical significance (p<0.05). Experiments were conducted on three different pools of 40 oocytes (n = 3) and replicated three times (r = 3) for each sample.
DISCUSSION

The results presented in this chapter showed that there were little to no effects of 30 ng/mL BPA treatment during oocyte maturation on gene expression in MII oocytes and blastocysts. Treatment with 15 ng/mL BPA resulted in increased expression of CDC2, AURKA, DAZL, TRβ and p53 in MII oocytes relative to that of the IVM group. This increased p53 expression resulting from the 15 ng/mL BPA group was also significantly greater than the expression levels resulting from 30 ng/mL BPA. There appeared to be a slight vehicle effect, with the vehicle group (0.1% ethanol) resulting in significantly increased expression of AURKA mRNA and non-significant increases in several other genes analyzed. There were no significant differences observed in blastocyst mRNA expression levels of the genes analyzed.

The results currently presented indicate that BPA exposure during oocyte maturation may alter transcript levels of developmentally important genes in MII oocytes, and the nature of these alterations is dictated by exposure dose. A number of the genes analyzed showed a trend exhibiting decreased mRNA expression in MII oocytes matured with 30 ng/mL BPA compared to the vehicle control, however these results were not statistically significant and require further investigation to reach definitive conclusions. As mentioned, a vehicle effect was observed and increased expression as a result of 15 ng/mL BPA was partly due to the ethanol vehicle. Increased mRNA expression as a result of ethanol exposure has previously been observed in bovine MII oocytes (Gomez et al., 2004) and porcine COCs (Lee et al., 2014a). The concentrations used were much higher in these studies than that used in the current study, and we did not observe developmental effects similar to those that were associated with the higher doses (1% and 3%) (Lee et al., 2014a). Since we did not observe developmental effects as a result of ethanol exposure in Chapters 1 and 2, the changes observed as a result of ethanol exposure do not appear to be functionally significant,
at least up to the blastocyst stage, however they must be considered when using ethanol in media for oocyte maturation.

*mRNA expression in GV and MII oocytes*

In the current study, we analyzed changes in mRNA expression between the GV and MII stages to offer perspective of the results obtained (Figure 12). Of the genes analyzed 3 were significantly downregulated in MII oocytes (CDC2, TRβ, and TUBA), and 4 genes (DAZL, p53, KIF5b, and AURKA) were upregulated in MII oocytes however expression levels in KIF5b were not significantly different. Results currently observed in GV and MII oocyte expression of KIF5b, CDC2, p53, HSP70, and AURKA are in line with previous reports (Manejwala et al., 1991; Juriscova et al., 1998; Swain et al., 2008; Uzbekova et al., 2008; Mamo et al., 2011; Niu et al., 2013; Macaulay et al., 2014). There was no observable correlation between GV/MII pattern and gene-specific BPA effect.

*Meiosis and spindle assembly*

Due to the ability of BPA to induce meiotic aberrations during oocyte maturation as was observed in Chapter 1, we analyzed the expression of several meiosis and spindle-related genes in MII oocytes. Significant differences were observed between treatment groups of three of the genes studied, (CDC2, AURKA, and DAZL) (Figure 13). CDC2, AURKA, and DAZL expression in MII oocytes was significantly increased in MII oocytes following exposure to 15 ng/mL BPA relative to that of the IVM group. Additionally, expression profiles for CDC2, AURKA, and KIF5b showed similar patterns of expression between the treatment groups, characterized by non-significant decreases in the 30 ng/mL BPA and E2 groups compared to the vehicle control, with no change or an increase in expression in the 15 ng/mL BPA groups. Further investigation is required to determine if the higher dose of BPA is interfering with processing of these genes that
may not be apparent at the mRNA level. Differences not observed through the current analysis, such as protein levels or timing of gene expression changes, will further help to elucidate the functional significance of the current findings and how BPA exposure is resulting in the meiotic defects observed under the same conditions in Chapter 1.

BPA exposure has previously resulted in upregulation of CDC2 expression in amalone embryos (Zhou et al., 2011), DAZL expression in mouse embryonic stem cells undergoing differentiation (Aoki & Takada, 2012), and AURKA expression in the human breast epithelial cell line MCF-10F (Fernandez et al., 2012). Conversely, DAZL and AURKA expression levels were decreased following BPA exposure in fetal germ cells (Zhang et al., 2012) and fetal mouse ovary, respectively (Lawson et al., 2012). These genes, as well as KIF5b which has not been studied following BPA exposure, are important to various aspects of the cell cycle and spindle assembly, and are thus critical regulators of meiosis (Andresson et al., 1998; Mendez et al., 2000; Yao et al., 2004; Uzbekova et al., 2008; Baumann et al., 2010; Chen et al., 2011). Alterations in mRNA expression of these genes during maturation has previously resulted in developmental arrest (CDC2) (reviewed by Uzbekova et al., 2008), disruptions to spindle assembly (DAZL) (Chen et al., 2011), and increased incidence of aneuploidy and chromosome alignment (AURKA) (Swain et al., 2008; Shuda et al., 2009; Lane et al., 2010).

Thus given BPA’s ability to disrupt meiosis and spindle formation (Can et al., 2005; Lenie et al., 2008; Machttinger et al., 2013; Ferris et al., 2015, Chapter 1), investigation of genes important to spindle assembly and function during meiosis is vital to analyze how these spindle effects may result. Further analysis observing polyadenylation, translation, and degradation of these genes is required to more fully analyze BPA’s implications regarding these genes during oocyte maturation. Furthermore, due to recent evidence that the cumulus cells can transport RNA molecules into the
oocyte during maturation (Macaulay et al., 2014), mRNA expression profiles throughout meiosis warrant investigation, as does investigation of oocyte-cumulus cell communications.

**Hormone receptors**

Exposure to 15 ng/mL BPA during oocyte maturation resulted in increased TRβ mRNA expression in MII oocytes compared to the IVM group (Figure 14B). There were no significant differences in ERβ mRNA expression as a result of BPA exposure in MII oocytes (Figure 14A). Due to the presence of TRs in granulosa cells, and GV, MI, and MII oocytes, it has been suggested that there may be a direct effect of THs on the maturing oocyte by interacting with thyroid receptors in the surrounding cumulus and granulosa cells (Wakim et al., 1993; Zhang et al., 1997; Zhang et al., 2007; Aghajanova et al., 2009; Costa et al., 2013). BPA has previously been found to decrease TRβ levels in *Xenopus* embryos (Iwamuro et al., 2003), however to the best of our knowledge, this is the first study analyzing the effects of BPA on TR levels following exposure during oocyte maturation.

Our lab has previously shown that embryo treatment with TH improves embryo quality, however when this treatment occurred at IVM no statistical differences were observed (Ashkar et al., 2010b). Similar results have since been reported by Costa et al. (2013) where cleavage and blastocyst rates were unaffected by IVM addition of THs, although increased blastocyst hatching rates were observed (Costa et al., 2013). Furthermore, TR levels in the mature oocyte were unaffected by TH supplementation (Ashkar et al., 2010b). In contrast we have observed alterations in TRβ following BPA exposure which may suggest interaction with the receptor, or interference with TRβ mRNA processing, resulting in altered expression in the MII oocyte. Therefore, BPA at the 15 ng/mL dose appears to be acting as an endocrine disruptor in the current model.
E2 is known to play an important role in oocyte and embryo development, and levels can improve developmental competence of *in vitro* matured oocytes (Kreiner et al., 1987; Zheng et al., 2003; Modina et al., 2007; Aardema et al., 201), however elevated levels of E2 have also been linked to poor developmental outcomes such as disruption to meiotic spindles (Beker-van Woudenberg et al., 2004). Currently we observed no significant alterations in ER mRNA expression in MII oocytes as a result of E2 or BPA exposure during oocyte maturation. Many non-genomic effects of BPA have been suggested such as interaction with membrane bound receptors (Alonso-Magdalena et al., 2012), and epigenetic effects (Susiarjo et al., 2007). In the current analysis BPA exposure at the doses tested does not influence ERβ mRNA expression in MII oocytes or resulting blastocysts, and further investigation of BPA’s estrogenic effects, possibly through non-genomic mechanisms, is required.

Although minor non-significant alterations in gene expression can be observed in the blastocyst, it is apparent that exposure to BPA during oocyte maturation alone does not alter gene expression of nuclear receptors (Figure 15). Thus new transcription arising from the embryo, at least for the genes studies currently, is unaffected. Additional investigation of gene and protein expression during early cleavage stages is important in further analysis of BPA’s influence on hormone receptors in the early embryo. Though the current study was intended to observe effects as a result of BPA exposure during oocyte maturation, it is established that gene expression in the blastocyst is more likely to be altered as a result of environmental perturbations during embryonic development. Therefore further analyses are required to assess exposure to BPA not only during oocyte maturation, but also during embryonic development.
Stress and metabolism genes

In the current analysis, exposure to 15 ng/mL BPA during oocyte maturation resulted in significantly higher p53 mRNA levels relative to the IVM and 30 ng/mL BPA groups in MII oocytes (Figure 16). There were no significant changes in gene expression in the HSP70 and GLUT1 mRNA expression levels. Due to the role that p53 plays in oxidative stress, DNA repair, cell cycle regulation, and centrosome function, alterations in p53 expression could lead to detrimental developmental effects in the embryo (Tchang & Méchali, 1999; Tritarelli et al., 2004; Pascreau et al., 2009). Oxidative stress can result in loss of meiotic spindle integrity and errors in chromosome segregation in oocytes, as well as increased apoptosis and decreased fertilization and development rates (Tarin et al., 1996; Tatemoto et al., 2000; Takahashi et al., 2003; Tatone et al., 2008; Vandeve et al., 2010). Additionally, p53 has been found to interact closely with AURKA in Xenopus oocytes and mammalian cells (Pascreau et al., 2009), and disruption to p53 results in similar phenotypes to that of AURKA such as centrosome amplification and aneuploidy (Pascreau et al., 2009). AURKA and the cell cycle regulator, microtubule-associated TPX2 regulate the phosphorylation of p53 (Pascreau et al., 2009). DAZL-directed translation during oocyte maturation has also been found to interact with AURKA and p53 during oocyte maturation through activation of various cell cycle regulators, such as TPX2 (Chen et al., 2011). Thus, the changes observed in p53 in the current study may be linked to alterations observed in AURKA and DAZL mRNA expression.

We found no significant alterations in mRNA levels of stress- and metabolism-related genes at the blastocyst stage between treatment groups (Figure 17). The lack of alterations in mRNA levels of these genes at the blastocyst stage indicates that embryos that survive to the blastocyst stage following in vitro oocyte exposure to BPA are not exhibiting a stress response at
that time. Expression of these genes at early cleavage stages and as a result of embryonic exposure is warranted to gain a fuller understanding of whether these genes are involved in BPA’s activities during early development.

It is important to recognize that an ethanol effect was observed in many of the groups, although this was only significant in the AURKA analysis. Thus although mRNA expression was significantly higher in the 15 ng/mL BPA group than that of the IVM group, the ethanol in which the BPA is dissolved is likely partly responsible for this increase. This increase in 15 ng/mL BPA was observed in several genes, and a seemingly opposite effect in the higher BPA group relative to the vehicle control, which although is not statistically significant, may indicate that BPA is causing subtle alterations to a variety of genes. Subtle alterations such as these may be responsible for larger effects such as changes in protein levels or epigenetic effects, and therefore these parameters require further investigation.

The molecular events of oocyte maturation are still largely unclear, and how changes in mRNA levels in the MII oocyte occur is not well understood. However it has been suggested that environmental stress imposed on the maturing oocyte can alter mRNA storage and stability, thereby affecting MII and embryonic gene expression (Gendelman & Roth, 2012). The alterations in mRNA expression in MII oocytes observed in the current study may therefore indicate that BPA can interfere with mechanisms responsible for gene processing, and may affect mRNA stability, translation, and degradation, possibly through altering polyadenylation of specific genes. Additional analyses are important to further investigate the molecular actions of BPA during maturation.
GENERAL DISCUSSION

Female fertility can be affected by a variety of factors, including physical, environmental, and lifestyle factors. The oocyte is at the center of a woman’s fertility and oocyte quality is critical for normal fertilization, embryo development, and production of a healthy offspring. The oocyte develops, grows, and matures within the ovarian follicle, and the follicular environment can be altered by maternal health, lifestyle, and exposures. For instance, toxic exposures such as alcohol, smoking, drugs, and various other chemicals may increase the risk of fertility issues (Mtango et al., 2008, Varghese, 2010). Alterations in the composition of follicular fluid can not only lead to poor oocyte quality, but can also result in a decreased embryo quality; thus, factors that can alter normal follicular fluid concentrations, or enter the follicular fluid itself, are of great concern.

The main goal of this study was to identify if BPA exposure of bovine oocytes during in vitro maturation leads to observable alterations in oocyte or embryo quality and developmental potential. All exposures in the current thesis occurred only during the window of oocyte maturation, thus all results observed are the consequence of alterations occurring during that period in development. BPA has been shown in vitro and in vivo to negatively affect various aspects of reproduction, and developmental exposure to the chemical has resulted in long-term, and even trans-generational, effects (Cantonwine et al., 2010; Wei et al., 2011; Wolstenholme et al., 2012). How BPA affects the mammalian, particularly the bovine, oocyte and early embryo is not as well understood. Considering the detection of BPA in follicular fluid (Ikezuki et al., 2002), the effects of BPA on the maturing oocyte are of great concern. Analysis of early parameters of developmental competency, such as oocyte maturation and embryonic viability, may provide insight into how developmental exposure prior to fertilization can disrupt early development and what implications this may have for further development.
The environment in which the oocyte matures is critical to the developmental fate of the oocyte, and can affect developmental capabilities of the subsequent embryo (Combelles & Albertini, 2002). Oocytes are a long-lived cell population, however, nuclear maturation, the period of time between the plasma LH surge and the oocyte’s rearrest at MII, is a critical window of development for the establishment of oocyte competence (Lonergan et al., 2003). The composition of follicular fluid is vital to and has the potential to affect oocyte maturation and quality as well as embryo quality and viability. Appropriate hormone levels in follicular fluid and IVM media are important in the acquisition of developmental competence (Kreiner et al., 1987; Zheng et al., 2003; Modina et al., 2007; Aardema et al., 2013). In addition to abnormal hormonal levels, environmental factors such as abnormal temperature, oxygen tension, in vitro culture conditions, and glucose levels in follicular fluid, can disrupt oocyte maturation, resulting in decreased embryonic developmental potential (Albertini & Carabatsos, 1998; Krisher & Bavister, 1999; Spindler et al., 2000;Trounson et al., 2001; Hodges et al., 2002; reviewed by Krisher 2013). Since oocyte competence is both critical to further developmental success and easily affected by environmental factors, suboptimal environmental conditions of the oocyte during maturation may have significant long-term effects on offspring arising from such oocytes.

Thus the current study investigated the effects of BPA exposure during in vitro oocyte maturation on the quality and developmental potential of both oocytes and embryos, as well as transcript levels of meiosis-, stress-, metabolism-, and hormone-related genes. The key findings of this thesis are as follows. BPA treatment at 30 ng/mL resulted in an average uptake of 2.48 ng/mL BPA per oocyte and led to decreased meiosis progression, increased MII spindle abnormalities, decreased embryonic first cleavage, decreased blastocyst formation rate, increased apoptosis in blastocysts and skewed sex ratio toward females. There were no significant gene expression
alterations in MII oocytes or blastocysts at this dosage level. The 15 ng/mL BPA treatment, which resulted in decreased blastocyst development compared with the no-treatment control, led to significantly higher p53 expression in MII oocytes than that resulting from 30 ng/mL BPA treatment. Finally, the E2 treatment did not have any effects in comparison to the controls.

The experiments presented in Chapter 1 were conducted in order to assess the effects of BPA exposure during oocyte maturation in vitro on meiosis progression and the MII spindle. The stage of meiosis at 24 hours post IVM culture, and MII spindle morphology and organization were assessed in order to determine BPA’s effects on oocyte quality and potential. Oocyte exposure to 30 ng/mL BPA resulted in an average uptake of 2.48 ng/mL per oocyte, and a decrease in oocyte quality and developmental potential due to disruption of meiosis and induction of MII spindle abnormalities. Due to the vulnerability of the oocyte to its microenvironment during maturation, a suboptimal environment can interfere with developmental potential and hinder embryonic development (van de Leemput et al., 1999; Sanfins et al., 2003; Ibáñez et al., 2005). Abnormalities occurring during oocyte maturation can decrease oocyte quality and developmental potential and can therefore compromise future embryonic development (Hyttel et al., 1989; Sirard et al., 2006).

The meiotic spindle, a key component of nuclear maturation, is a microtubular structure that acts primarily to assist in chromatid segregation and the associated second polar body extrusion occurring at the conclusion of meiosis (Coticchio et al., 2010). The meiotic spindle is exceptionally vulnerable to environmental factors such as oxidative stress (Hu et al., 2001; Eichenlaub-Ritter et al., 2002), which can induce meiotic abnormalities and chromosome instability, and lead to impaired embryo development and increased apoptosis (Liu et al., 2003) as was observed in the current study. The MII spindle can be adversely affected by factors such as changes in pH (Swain, 2010), high E2 levels (Beker et al., 2002; Beker-van Woudenberg et al.,
2004), and exposure to EDCs (Cecconi et al., 2007). Flattening of the spindle poles has been associated with increased chromosome misalignment (Coticchio et al., 2013; Ferris et al., 2015; Chapter 1). Chromosome misalignment may lead to improper chromosome segregation, which is relatively common in human oocytes (Hassold & Hunt, 2001). Though aneuploidy is common in humans, the proportion of aneuploidy cells within an embryo directly correlates with developmental potential (Baltaci et al., 2006; Mtango et al., 2008), and these errors have been linked to pregnancy failure, embryonic abnormalities, and chromosomal disorders (Hassold & Hunt, 2001; Mtango et al., 2008; Li & Albertini, 2013).

Meiosis progression, spindle morphology, and chromosome alignment were all negatively altered by exposure to 30 ng/mL BPA during maturation. This has been observed previously in mouse and human models (Hunt et al., 2003; Can et al., 2005; Susiarjo et al., 2007; Eichenlaub-Ritter et al., 2008), and BPA has been linked to embryonic aneuploidy in some (Hunt et al., 2003; Susiarjo et al., 2007), but not other experimental studies (Eichenlaub-Ritter et al., 2008). The disruption of meiosis progression and spindle organization as a result of BPA exposure during oocyte maturation indicates both unsuitable environmental conditions for the oocyte during this time, as well as an increased possibility of further developmental deficiencies. The current studies show that BPA at a sufficient concentration can be taken up by the oocyte and lead to disruption of meiosis and interference with normal MII spindle organization and chromosome alignment.

The mechanisms of spindle disruption are not clear, but oocyte-somatic cell communication is critical to spindle integrity. Disruptions to this communication may interfere with normal oocyte maturation (Albertini et al., 2001; Hodges et al., 2002). Furthermore, meiosis may be affected by BPA through disruption of the microtubules directly, and by interaction with proteins associated with the microtubules (Pfeiffer et al., 1997; Can et al., 2005). Meiosis
disruption may be due to selective interference with centrosome and microtubule organization (Can et al., 2005). Unlike other estrogenic agents that can disrupt meiosis through depolymerisation of microtubules, the actions of BPA appear to be a result of disorganization and fragmentation of centrosomes, resulting in spindle disorganization (Can et al., 2005). Motor proteins and regulatory mechanisms of transportation associated with the microtubules may be target sites for BPA during oocyte maturation. For instance, BPA may lead to errors in cell cycle progression, microtubule organization and centrosome assembly and function by interfering with the interaction between protein kinase C (PKC) and pericentrin, interfering with centrosomal proteins, disrupting protein transport, or by the disorganization of the microtubule organizing centers (MTOCs) (Takahashi et al., 2000; Can et al., 2005).

Because there appears to be a correlation between meiosis progression and spindle organization and later embryonic development, the present study assessed developmental parameters such as development rate, total cell number, apoptosis, sex ratio, and gene expression to evaluate oocyte and embryo quality and developmental potential. Oocyte and embryo quality are directly related, and poor quality oocytes exhibit decreased fertilization success, and embryo quality (Metwally et al., 2007). Although the embryonic environment has a great impact to the developmental fate of the embryo, oocyte quality can dictate the development and survival of an embryo, pregnancy establishment and maintenance, and fetal development (reviewed by Varghese et al., 2010). Taking into account that follicular fluid can alter the quality and developmental potential of oocytes, the results presented in Chapter 2 therefore indicate that oocyte exposure to BPA at certain concentrations can impact development of the preimplantation embryo, likely by decreasing quality of the mature oocyte. Cleavage and blastocyst rates were decreased, apoptosis
rate, as well as the proportion of cells with non-apoptotic DNA fragmentation, was increased, and there was a higher proportion of female embryos at blastocyst.

Oxidative stress, heat stress, abnormal hormone levels, in vitro culture conditions, abnormal oocyte-somatic cell signalling and exposure to toxic or xenobiotic chemicals can lead to decreased developmental competency of the embryo (Albertini & Carabatsos, 1998; Betts & King, 2001; Trounson et al., 2001; Hodges et al., 2002; Hamdoun & Epel, 2007). The effects of stress and/or abnormal hormone levels during preimplantation development have been linked to decreased development, increased apoptosis (Paula-Lopes & Hansen, 2002b), and skewed sex ratio (Krackow, 1997), but the evidence of embryonic effects as a result of oocyte impairments is not as well studied. Altered meiotic success and spindle abnormalities have been linked to a variety of developmental effects, however it is unclear if it is these meiotic changes themselves that influence further development, for instance by way of improper chromosome segregation leading to aneuploidies in embryos, or through other pathways that affect both maturation and further embryonic development such as poor oocyte-cumulus cell communication.

As mentioned previously, several studies indicating oocyte effects such as decreased meiosis success and spindle abnormalities also resulted in poor embryonic outcomes (van de Leemput et al., 1999; Ertzeid & Storeng, 2001; Van der Auwera & D’Hooghe, 2001; Sirard et al., 2006). Meiotic abnormalities have been linked to later developmental effects such as impaired fertilization, embryo development, and cell cycle progression as well as an increased incidence of apoptosis in blastomeres, embryonic abnormalities, and pregnancy loss (Volarcik et al., 1998; Sanfins et al., 2003; Cecconi et al., 2007; Bromfield et al., 2009). Furthermore, early cleaving embryos, which are considered to have greater developmental potential than later cleaving embryos, have been shown to have a greater spindle size and higher blastocyst formation rate.
whereas later cleaving embryos exhibited a smaller, compressed spindle and a lower blastocyst rate (Tomari et al., 2011). Meiotic spindle parameters are strong indicators of human embryo developmental potential, thus the decreased development rates currently observed following oocyte exposure to BPA could be directly related to the meiotic disturbances that were presented in Chapter 1. The mechanism by which this may occur is not clear; however, alterations in genes and protein expression, oocyte metabolism, and communication between the oocyte and surrounding cells may play a key roles in the observed effects (Van Blerkom et al., 1995; Tomari et al., 2011).

Embryos have been shown to respond to stress by undergoing apoptosis (Byrne et al, 1999). Apoptotic cell death in early embryos has been found to occur in a variety of mammalian species under certain stressful conditions once the genome of embryo has been activated. The degree of apoptotic response plays a role in the embryo’s ability to continue development. A limited apoptotic response allows the embryo to continue development by removing damaged cells without detrimentally affecting neighbouring cells. More extensive apoptosis may result in the removal of a relatively large proportion of cells thereby lowering the embryo’s viability or chance of survival (Betts & King, 2001; Paula-Lopes & Hansen, 2002b). Depending on the conditions and severity of the stress encountered, apoptosis may serve as a marker of an embryo at risk and/or as a mechanism to help the embryo survive suboptimal conditions. As was presented in Chapter 2, preimplantation embryos exhibited increased apoptosis under the same treatment conditions that resulted in increased meiotic abnormalities.

An altered sex ratio of blastocyst may also indicate stress experienced by the embryo (Krackow, 1997; Pratt & Lisk, 1989; Jiménez et al., 2003). The blastocyst sex-ratio was skewed towards females in both BPA groups, however this was only significantly different than the
expected 1:1 ratio in the 30 ng/mL BPA group (Chapter 2). This may indicate that the embryos experienced heightened stress, resulting in the disproportionate arrest of male embryos. Differences in how female and male embryos respond to environmental conditions are speculated to be a result of metabolic, genetic, and epigenetic differences. During the preimplantation period, male and female embryos differ only in the content of their sex chromosomes, and differences exhibited by embryos at this time may be a result of transcriptional dimorphism (Bermejo-Alvarez et al., 2011). Furthermore, epigenetic differences resulting from the presence of one versus two X-chromosomes may be a driving force in sex differences and how embryos respond to environmental conditions (reviewed by Gutiérrez-Adán et al., 2006).

Expression of genes encoded by the sex chromosomes, which may also affect autosomal gene expression, differs between male and female preimplantation embryos (reviewed by Bermejo-Alvarez et al., 2011). Y-linked genes are only expressed in male embryos, and X-linked genes are expressed doubly in females. X-chromosome inactivation (XCI) compensates for this disparity ensuring X-linked genes (in most cases) are transcribed equally in male and female adult tissues (reviewed by Bermejo-Alvarez et al 2011). However, if during early embryo development XCI is incomplete, or genes are reactivated, the female embryos exhibit higher expression of many X-linked genes (Kobayashi et al., 2006; Bermejo-Alvarez et al. 2010a). This phenomenon has been found in mouse (Kobayashi et al., 2006), bovine (Gutiérrez-Adán et al., 2000), and human embryos (Taylor et al., 2001).

These transcriptional alterations are responsible for variations between male and female embryos such as metabolic differences, and dimorphic susceptibilities to suboptimal in vivo and in vitro conditions (reviewed by Gutiérrez-Adán et al., 2006). Resulting alterations in molecular pathways, such as the pentose-phosphate pathway (PPP) that regulates glucose metabolism, may
result in varying susceptibilities of male and female embryos. For instance, male embryos have been reported to metabolize glucose at a higher rate than females (Tiffin et al., 1991) and male embryos appear to be more developmentally competent than females under hyperglycemic conditions \textit{in vitro}, however the opposite has also been found (Jimenez et al., 2003). Conversely, female embryos exhibit increased expression of X-linked genes such as those related to energetic metabolism, the regulation of oxygen radicals, and apoptosis inhibition (Gutiérrez-Adán et al., 2000; Jimenez et al., 2003). Since various stressors can lead to embryonic overproduction of reactive oxygen species (ROS), female embryos may be better equipped to survive such a stress due to enhanced ability to buffer the amount of cellular ROS (Perez-Crespo et al., 2005). Furthermore, male embryos have been shown to be more sensitive to oxidative damage induced by heat stress, which may be due to the increased expression of X-linked genes in females that result in an increased ability to buffer environmental stress (Perez-Crespo et al., 2005).

Therefore, whether the embryo possesses one or two X chromosomes may underlie the differences in the early embryo (Gutiérrez-Adán et al., 2006). This may explain observations that male embryos may be more vulnerable to stressors than female embryos under certain environmental conditions. Since male and female embryos evidently respond differently to stress, a skewed sex ratio may be indicative of environmental stress imposed on the embryo. Therefore, the skewed sex ratio observed in the present studies may indicate a stress response activated in the embryos resulting from oocytes exposed to 30 ng/mL BPA during maturation. Taken together, the results presented in Chapter 2 suggest that embryo quality and developmental potential were decreased. The decrease in quality as evidenced by increased apoptosis and a skew in sex ratio indicates that adverse environmental conditions may have elicited a stress response in the embryo.
Chapters 1 and 2 presented evidence that BPA exposure during oocyte maturation decreased oocyte and embryo quality and developmental potential. BPA has previously been shown to affect transcript levels of a number of genes. Hormone receptors (Rubin, 2011), cell cycle regulators (Peretz et al., 2012), apoptotic genes (Peretz et al., 2012), genes related to the stress response (Tabuchi et al., 2002), and those related to the onset of meiosis, chromatin modification, remodeling, and chromosome condensation (Lawson et al., 2011), among others have been altered as a result of BPA exposure. To determine if BPA was interfering with spindle-related gene processing, inducing embryonic stress, or altering hormone pathways, genes involved in meiosis and spindle assembly (CDC2, AURKA, DAZL, KIF5b), stress and metabolism (HSP70, p53, and GLUT1), and genes of nuclear hormone receptors (ERβ, and TRβ), were assessed at the oocyte and/or blastocyst stage. Alterations in mRNA levels in MII oocytes may indicate poor developmental competency and could result in poor developmental outcomes (Paczowski et al., 2011; Yuan et al., 2011). Blastocysts are a key developmental milestone in early embryonic development, and alterations in mRNA abundance at this stage may indicate an altered quality, or developmental competency of the blastocyst (reviewed by Duranthon et al., 2008). Thus, Chapter 3 consisted of mRNA quantification of genes involved in meiosis, embryonic stress, metabolism, and hormone-signalling in oocytes and blastocysts.

Oocyte maturation consists of a variety of tightly regulated sequential events mediated by transient production and phosphorylation of regulatory proteins (reviewed by Pelech et al., 2008; Brunet & Verlhac, 2011). Kinases and phosphatases act to initiate and maintain proper sequential activities throughout oocyte maturation (Yamasita et al., 2000). Meiotic progression and cell division are mediated by the selective and specific regulation of cellular components by translation and protein synthesis, cytoplasmic polyadenylation, phosphorylation, and protein degradation.
(Richter, 1991, 2007; reviewed by Uzbekova et al., 2008; Chen et al., 2011). Normal processing of genes involved in cell cycle progression and spindle-assembly is critical to the ability of the oocyte to acquire developmental competence (Tanaka et al., 1998; Takamiya et al., 2004; Uzbekova et al., 2008; Lane et al., 2010; Chen et al., 2011). Considering the disruption to meiosis as well as the spindle abnormalities observed in Chapter 1, several genes important to meiotic cell cycle progression and spindle assembly and integrity were analyzed in MII oocytes following maturation under various treatment conditions.

Treatment with 15 ng/mL BPA resulted in increased expression of CDC2, AURKA, DAZL in MII oocytes. There appeared to be a slight vehicle effect, with the vehicle group (0.1% ethanol) resulting in significantly increased expression of AURKA mRNA and non-significant increases in several other genes analyzed. Similar gene expression patterns were observed in MII oocytes of CDC2, AURKA, and KIF5b between treatment groups, characterized by non-significant decreases in gene expression in the E2 and the 30 ng/mL BPA groups, and no change or increased expression in the 15 ng/mL BPA group relative to the vehicle control (0.1% ethanol). Thus although some non-significant decreases were observed in the E2 and 30 ng/mL BPA groups compared to the vehicle control, considering the increase in gene expression and lack of developmental effects observed as a result of ethanol exposure, the functional significance of these observations cannot be ascertained.

BPA has been shown to interact with the ERs and TRs (Matthews et al., 2001; Kang et al., 2002; Moriyama et al., 2002; Iwamuro et al., 2003; Takao et al., 2003; Levy et al., 2004; Lahnsteiner et al., 2005; Zoeller et al., 2005; Vandenberg et al., 2009; Meeker & Ferguson, 2011). BPA has caused alterations in mRNA levels of these genes in reproductive and non-reproductive tissues (Tabuchi et al., 2002; Ramos et al., 2003; Markey et al., 2005; Berger et al., 2010;
Aghajanová & Giudice, 2011). In Chapter 3 the lower dose of BPA (15 ng/mL; 1.69 ng/mL BPA/oocyte), resulted in an increased TRβ mRNA expression in MII oocytes compared to the IVM group, and no effects were observed in ERβ mRNA expression in MII oocytes. There were no significant alterations in either ERβ or TRβ in blastocysts. Alterations in TRβ following BPA exposure may suggest interaction with the receptor, or interference with TRβ mRNA processing, resulting in altered expression in the MII oocyte. Therefore, BPA at the 15 ng/mL dose appears to be acting as an endocrine disruptor in the current model, however these results must be interpreted with caution due to the vehicle effect observed. Further analysis of BPA’s non-genomic effects, as well as analysis of exposure during embryonic development, may further help to elucidate hormone-disrupting properties of BPA in the current model.

Due to the embryonic effects observed in Chapter 2, it was decided to evaluate stress-related genes to see if transcripts of stress-related genes were altered in MII oocytes, and if a stress-response was induced in the blastocyst. Exposure to 15 ng/mL BPA during oocyte maturation resulted in significantly higher p53 mRNA levels relative to the IVM and 30 ng/mL BPA groups in MII oocytes. There were no differences in HSP70 and GLUT1 mRNA expression levels in MII oocytes. The significant difference observed between the two BPA groups is interesting and supports the idea that the effects induced by BPA differ according to the exposure doses administered. Given that p53 and AURKA are known to interact during oocyte maturation (Pascreau et al., 2009), the results presented indicate a disruption to this molecular pathway. No significant alterations were found in any of the groups for the three genes (HSP70, p53, and GLUT1) in the blastocyst stage, suggesting that new transcription following the MET may not be affected by BPA exposure at the oocyte stage. However earlier stages both before and during the MET are necessary to determine whether BPA can alter mRNA expression of these genes.
Alterations observed in p53 mRNA in MII oocytes by BPA treatment may indicate that BPA is inducing oxidative stress. Oxidative stress is thought to be a common mechanism by which endocrine disruptors exert their effects (Wang et al., 2012), and is also a well characterized example of how oocyte stress can affect embryonic development (reviewed by Guérin et al., 2001). Stress experienced during oocyte maturation can lead to the production of ROS in embryo development and lead to alterations in embryonic gene expression (Maître et al., 1993; Schultz, 1993; Guérin et al., 2001). The mRNA transcripts of various antioxidant enzymes have been found in human, bovine, and murine GV and MII oocytes (El Mouatassim et al., 1999), indicating their importance for further development (Harvey et al., 1995; reviewed by Guérin et al., 2001). Due to the correlation between mRNA, proteins and enzyme activity levels of antioxidant enzymes, it has been suggested that these enzymes are primarily regulated at the pre-translational level (Guérin et al., 2001). Therefore, since maternal transcripts decrease until the maternal to embryonic transition (MET), variations in the synthesis or accumulation of mRNAs of antioxidant enzymes during oocyte maturation may impact embryonic development (Guérin et al., 2001). Thus, the developmental effects observed in Chapter 2, taken together with the current findings may indicate that BPA is affecting oocyte quality and developmental potential resulting in abnormal early embryonic development. Further analysis of early cleavage stages is required to expand on this observation.

All of the effects discussed in this thesis must be brought back to the oocyte. Since exposure only occurred over a short period of time during in vitro oocyte maturation, key events occurring during that window set the stage for all of the results observed in the current study. How BPA exerts its effects at the oocyte may provide key insight into the mechanism by which the embryonic effects are elicited. Clearly BPA affects oocyte quality, which can lead to developmental effects
in the preimplantation embryo. But exactly how these oocyte and embryonic effects are elicited is difficult to determine. The developmental effects observed in the oocyte and embryo following BPA exposure during oocyte maturation may be due to a number of mechanisms. Interfering with microtubule assembly, interruption of proper metabolism, epigenetic alterations, improper degradation of maternal mRNAs and proteins, and interference with communication between cumulus cells and the oocyte, can all lead to interruptions to normal oocyte and embryonic development (Pfeiffer et al., 1997; Hassold & Hunt, 2001; Can et al., 2005; Wetherill et al., 2007; Lenie et al., 2008).

The communication between the oocyte and cumulus cells may be a key factor involved in BPA’s effects. This bidirectional signaling is critical to oocyte development, maturation, and the acquisition of developmental competence (reviewed by Hassold & Hunt, 2008). The oocyte drives development by secreting factors that influence the growth, proliferation, and differentiation of its surrounding cells, to which it depends on for growth and competence (Eppig, 2001; Sugiura et al., 2005; Diaz et al., 2006, 2007; Mtango et al., 2008). Furthermore, the cumulus cells regulate meiosis (Chesnel et al., 1994), and modulate oocyte genomic transcriptional activity (De La Fuente & Eppig, 2001). Disruptions to these processes can result in reduced ovulation, fertilization and developmental potential (Yan et al., 2001; Di Pasquale et al., 2004). BPA exposure may interfere with these communications, altering the quality and developmental potential of both oocytes and resulting embryos.

Oocyte maturation is an intricate event and alterations in this process can lead to poor developmental outcomes down the line. Factors such as the xenoestrogen BPA can contaminate follicular fluid and disrupt oocyte maturation and are thus of great concern. Although many reproductive effects of BPA have been reported, the mechanism of action of BPA on oocyte
maturation is still being elucidated. Although E2 has been shown to impair meiotic progression and spindle morphology in some cases during *in vitro* maturation (Beker-van Woudenberg et al., 2004), in the current study E2 did not show any detrimental effects of oocyte maturation and embryo development. BPA may not be acting as an estrogen mimic in the current analysis, but it appears that BPA is acting, at least in part, as an endocrine disruptor, possibly by interfering with endocrine pathways in ways such as activation of hormone receptors or alterations in hormone metabolism. Disruptions to normal oocyte maturation may influence short- and long-term viability. The mechanisms by which environmental factors influence oocyte maturation are important in order to determine the risk of exposure and how chronic exposure could impact long-term fertility.
SUMMARY AND CONCLUSIONS

Disruption to the environment in which an oocyte matures can result in short- and long-term developmental effects. Exposure to 30 ng/mL BPA during oocyte maturation resulted in an average uptake levels of 2.48 ng/mL per oocyte, and an associated disruption to oocyte maturation, compromised oocyte and embryo quality and reduced developmental potential.

Exposure to a BPA concentration of 30 ng/mL during oocyte maturation decreased meiosis success and increased the incidence of MII spindle abnormalities including an abnormally shaped spindle (compressed with loss of pole focus) as well as chromosome misalignment.

Oocyte exposure to 30 ng/mL BPA decreased embryo cleavage and development rates and increased the proportion of apoptotic blastomeres and nuclei containing non-apoptotic DNA damage in the blastocyst, and caused a sex skew towards femaleness. The 15 ng/mL BPA group had decreased blastocyst formation rate in comparison to the no-treatment control. Total cell number did not differ between any of the groups.

Treatment with 15 ng/mL BPA resulted in increased expression of CDC2, AURKA, DAZL, TRβ and p53 in MII oocytes relative to that of the IVM group. This increased p53 expression resulting from the 15 ng/mL BPA group was also significantly greater than the expression levels resulting from 30 ng/mL BPA. There appeared to be a slight vehicle effect, with the vehicle group (0.1% ethanol) resulting in significantly increased expression of AURKA mRNA and non-significant increases in several other genes analyzed. There were no significant differences observed in blastocyst mRNA expression levels of the genes analyzed.

The results obtained support the working hypothesis of the study that exposure to BPA during oocyte maturation can, in a dose-dependent way, decrease oocyte and embryo quality and
developmental potential. Exposure of bovine oocytes *in vitro* to 30 ng/mL BPA during maturation induced meiotic perturbations as well as poor embryonic outcomes including decreased development rates, increased apoptosis and DNA fragmentation, and a skewed sex ratio, however did not result in significant changes in mRNA expression relative to controls. Although gene expression was not affected at this dose, it is clear that exposure to BPA only during *in vitro* maturation can induce effects that are observable in the blastocyst.
**FUTURE DIRECTIONS**

Since the current study was the first, to our knowledge, to assess BPA exposure during oocyte maturation in a bovine model, much of the thesis was dedicated to whether or not there was an observable effect in the oocyte as well as in the subsequent embryo. Further analyses of gene expression as a result of embryonic exposure to BPA would clarify if the blastocyst is more vulnerable to embryonic exposures rather than those at the oocyte stage.

Oocyte maturation can be further assessed by analyzing temporal gene transcript and protein levels for genes essential to developmental competence, those involved in oocyte-cumulus cell communication, as well as genes that have been altered as a result of oocyte stress such as those of antioxidant enzymes. Analysis of mRNA stability and degradation during oocyte maturation will help to determine if BPA is altering mRNA expression by disrupting transcript degradation. Because of the importance of communication between cumulus cells and the oocyte during maturation, evaluation of whether BPA interrupts or alters signalling during maturation may provide key understanding of the disruptive capabilities of BPA at this time. Additionally, looking at transcriptional and translational alterations in the cumulus cells during maturation is likely critical in the evaluation of communication within the COC.

Embryo developmental parameters measured can be expanded by observing earlier developmental effects such as fertilization success, the stage and sex of arrested embryos, apoptosis levels during the MET, embryonic aneuploidy, and further analysis of stress and hormonal pathways. Co-supplementation of IVM media with E2 or THs as well as antagonists may provide information on whether BPA is acting through E2 or TH pathways. Furthermore, incubation with antioxidants or HSP antibodies may provide further information as to whether maturational exposure to BPA is resulting in stress-related effects. Alterations in both oocyte and
embryonic metabolism may also be important in gaining a greater understanding of the effects of BPA on early developmental stages.

Further analysis of gene and protein expression at the blastocyst as well as earlier in development will continue to provide insight into the developmental effects of BPA. Analysis at all stages of development would also help to provide information on whether BPA affects maternal transcripts, embryonic, or both and when the greatest disruption is. Protein expression during embryo development would also provide key information on how these alterations lead to functional differences in the early embryo. Furthermore, epigenetic analysis, such as methylation levels of genes, will be key to understanding how early developmental effects can be linked to the later developmental effects observed in offspring, adults, and the subsequent generation. Further analysis of the delicate interactions during oocyte maturation is required to further elucidate the mechanism by which BPA is affecting meiosis, and how this may be linked to the embryonic effects observed.
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