Mechanisms of seasonal reproduction in cattle

By

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Declaration

I hereby declare that all the studies in this thesis is my own work, except were acknowledgement is made by reference. The work described here has not been submitted anywhere for any other degree of qualification. All assistance given to me during the preparation of this thesis is also acknowledged

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Abstract

This study was conducted to evaluate the effect of season on reproductive parameters in cattle in the temperate climate of the UK.

In the first study, reproductive fertility data were collected from a local dairy herd. The results revealed that cows born in autumn were inseminated at an earlier age ($P<0.05$) and calved earlier ($P<0.001$) than spring and summer born animals. In addition, the conception rate within 90 days after calving was higher ($P<0.05$) in autumn calving animals. Conception rate was higher ($P<0.05$) when insemination was performed at a temperature range from 7 to 15°C compared with <7 and >15°C and a temperature humidity index (THI) range from 40 to 59 compared with >60 units (10 days before and 21 days after insemination).

In the second study, ovarian tissues were collected from a local abattoir to investigate the effect of season on follicular populations, corpus luteum (CL) development and incidence of multiple ovulations. There were no effects of season on antral follicle count. However, individual and total CL weight was heavier in the autumn. Additionally, season influenced multiple ovulations with a higher incidence ($P<0.05$) in summer and autumn compared to winter and spring. The number of follicles >7mm in cows with multiple CLs was higher ($P<0.05$) than cows with a single CL. The individual CL weight was heavier in single ovulation cows. However, the total luteal tissue weight and total progesterone (P_4) content of luteal tissue was higher in cows with multiple ovulations.

The third study investigated the effects of culturing granulosa cells (GCs) under low (5%; physiological oxygen (O_2)) conditions rather than traditional culture (20%; atmospheric O_2) systems. Granulosa cells from antral follicles were cultured in fibronectin coated plates in M199 for up to 144 hour (h) under physiological (5%) and atmospheric (20%) O_2 tension. Melatonin was added at one of four concentrations (0, 20, 200, 2000 pg/ml). The number of viable GCs was greater ($P<0.05$) under 5% O_2 than 20% O_2. Reactive oxygen species (ROS) generation was similar under both physiological and atmospheric O_2, but was reduced ($P<0.05$) by treatment with melatonin. Oestrogen concentration ($P<0.001$) and aromatase activity ($P<0.014$) were also influenced by O_2 tension in a time dependent manner. Both oestradiol (E_2) production and aromatase enzyme activity

were maintained for up to 144 h of culture under 5% O_2 conditions. Progesterone production was increased under 20% O_2 compared with 5% O_2 ($P<0.05$). Additionally, the expression of *hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 (HSD3B1)* mRNA increased ($P<0.05$) with time under 20% O_2, but remained unchanged at 5% O_2. Haemoglobin subunit alpha 1 (HBA) transcript was increased ($P<0.05$) under 5% O_2 levels.

The final study quantified the effect of temperature and melatonin on GC function. Cells were cultured for up to 144 h under 5% O_2 tension. Treatments commenced after 48 h of culture and consisted of two incubation temperatures (37.5 *vs* 40.0°C) and four melatonin treatments. Melatonin increased cell number at high temperature (40.0°C). However, *BCL2-associated X protein (BAX)* mRNA expression was greater ($P<0.05$) in GCs cultured at 40.0°C than at 37.5°C by 144 h. Culture temperature did not affect ROS, but melatonin reduced ($P<0.001$) generation of ROS. Oestradiol production increased with time ($P<0.001$) and was not affected by temperature. In contrast, high temperature reduced P_4 production ($P<0.001$) at 144 h of culture. Similarly, the effect of melatonin treatment depended on temperature; melatonin increased P_4 production at 37.5°C, while reducing P_4 at 40.0°C. Temperature increased *acetylserotonin O-methyltransferase* (*ASMT)* mRNA expression ($P<0.05$) though there was no significant effect of temperature and melatonin on *tumour protein p53* (*P53)*, *HSD3B1, superoxide dismutase (SOD1 and SOD2), HBA* and *heat shock protein family A* (Hsp70) member 1A (*HSPA1A)* gene expression.

The results of this thesis contribute to our understanding of the effects of season on ovarian function and seasonal variation in cattle fertility particular in temperate climate regions where season influenced puberty, conception rate, incidence of multiple ovulations and CL development. In *in vitro* studies, low O_2 (5%) enhance cell proliferation, reduced luteinisation and altered steroidogenesis as well as increasing the expression of *HBA* mRNA. Culture at higher temperature reduced P_4 production and increased apoptotic mRNA while addition of melatonin reduced ROS generation and influenced P_4 production. This new approach to culture could offer a valuable system for future investigation of the physiological function of cells *in vitro*.

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List of abbreviations

Symbol	Name
°C	Celsius
AANAT	Aryl alkyl amine N-acetyltransferase
AI	Artificial insemination
ASMT	Acetyl serotonin O-methyltransferase
B2M	Beta-2-microglobulin
BAX	BCL2-associated X protein (BAX)
BCA	Bicinchoninic acid assay
BCL2	B-cell CLL/lymphoma 2
BSA	Bovine serum albumin
CASP3	Caspase 3, apoptosis-related cysteine peptidase
CC	Cumulus cells
cDNA	Complementary deoxyribonucleic acid
CL	Corpus luteum
CO_2	Carbon dioxide
COC	Cumulus oocyte complex
CR	Conception rate
Ct	Threshold cycles
CV	Coefficient variation
CYP17A1	Cytochrome P450, family 17 alpha-hydroxylase
CYP19A1	Cytochrome P450, family 19, subfamily A, polypeptide 1
D	Day
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
E	Efficiency
E_2	Oestradiol
E_2:P_4	Oestradiol to progesterone ratio
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
Exp	Experiment
FAM	Fluorophore 60 carboxyfluorescin
FSH	Follicle stimulating hormone
FSHR	Follicle stimulating hormone receptor
G	Gram
GC	Granulosa cell
GnRH	Gonadotropin releasing hormone
GPCR	G protein couple receptor
H_2O_2	Hydrogen peroxide
HB	Haemoglobin
HBA	Haemoglobin, alpha
HBB	Haemoglobin, beta
H	Hour
HSD3B1	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1
HSPA1A	Heat shock 70kDa protein 1A

IF	Immunofluorescence
IHC	Immunohistochemistry
IGF	Insulin growth factor
IMS	Industrial methylated spirit
KCl	Potassium chloride
LH	luteinizing hormone
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimolar
mm	Millimetre
mRNA	messenger ribonucleic acid
MTNR1A	Melatonin receptor 1A
MTNR1B	Melatonin receptor 1B
n	Number
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
ng	Nanogram
nM	Nanomolar
NTB	Nitro blue tetrazolium blue chloride
O_2	Oxygen
P/S	Penicillin/streptomycin
P_4	Progesterone
P53	Tumour protein p53
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
pg	Picogram
PRL	Prolactin
QC	Quality control
qPCR	Quantitative reverse transcriptase polymerase chain reaction
RH	Relative humidity
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPL19	Ribosomal protein L19 (RPL19)
RPLP0	Ribosomal protein, large, P0 (RPLP0)
rpm	Revolutions per minute
RT	Reverse transcription
SAM	S-adenosylmethionine
sec	Second
SED	Standard error difference
SOD1	Superoxide dismutase 1
SOD2	Superoxide dismutase 2
STAR	Steroidogenic acute regulatory protein
TAMRA	60-carboxy-N,N,N0,N0 tetra methyl rhodamine
TBP	TATA-box-binding protein
TC	Theca cells

TES	N-TRIS (Hydroxymethyl) methyl-2 aminoethan sulfuric acid
THI	Temperature humidity index
TGF	Transforming growth factor
µg	microgram
µl	microliter
µm	micro molar

Chapter 1: Literature Review

1.1 General introduction

Cattle are not classified as seasonal breeders. However, there is some evidence that some species of cattle show seasonal breeding patterns (Borisenkov et al., 2004, Koos and Feiertag, 1989). Season is known to influence various aspects of cattle fertility and reproductive performance (Hansen, 1985, Ismael et al., 2016) and has a substantial effect on overall profitability in dairy cattle.

The different geographical location of specific breeds and genetic and /or physiological alterations to increase milk production could have increased the negative environmental outcomes on cattle fertility. The financial loss caused by heat stress has been estimated at about £700 million in dairy and £300 million/year in beef cattle in the USA (St-Pierre et al., 2003). High temperature, photoperiod and changes in melatonin have commanded considerable research attention to identify the effect of season on reproductive activity including animal growth, oestrous cycles, embryonic development, pregnancy rate, milk production and the postpartum period (Miglior et al., 1998, García-Ispierto et al., 2007, Dahl et al., 2012, Wakayo et al., 2015, Allen et al., 2015).

Animals maintain normal physiological body temperature within the thermo-neutral zone. However, ambient temperature higher than 20 to 25°C can lead to increases in internal body temperature and induce heat stress (Yousef, 1985). It has been reported that ovarian temperature is about 1°C cooler than rectal temperatures and cooler temperatures were recorded in pre ovulatory follicles than ovarian stroma (Grøndahl et al., 1996). This suggests that ovarian tissue could be more sensitive to temperature level than other parts of the body. Therefore, the effect of season on reproductive function in cattle may not be restricted to the tropical regions of the world. Furthermore, several studies have reported that reproductive performance is affected by photoperiod (Youngquist and Threlfall, 2006, Andreu-Vazquez et al., 2012).

Some research has been done on genetically improving genes which are resistance to thermal stress. Heat tolerance is heritable (Ravagnolo and

Misztal, 2002) and it has been reported that cross breed animals (Holstein x Brown Swiss) had higher adaptability to heat stress than pure Holstein (El-Tarabany and El-Bayoumi, 2015). Furthermore, improving the environment around the animals has been attempted to increase dairy cattle production (Collier et al., 2006). However, seasonal alterations in dark light cycles have been overlooked as a main factor that could compromise reproductive activity.

Melatonin is the main hormone for regulating responses to photoperiod and signalling environmental information to the brain (Walton et al., 2011). Melatonin treatment can induce ovarian cyclicity in anoestrous buffalo during summer anoestrous (Ghuman et al., 2010). Melatonin not only functions as a circadian and circannual rhythm regulator but also has a major role in the prevention of oxidative stress (Johnsson, 2008). Furthermore, the presence of melatonin in follicular fluid may indicate that melatonin plays an important role during ovarian follicular development, ovulation and luteinisation. Thus seasonal effects may also be important in temperate climates where high temperatures are not seen as a problem. It appears from the literature that there are few studies about seasonal variation and its effect on cattle fertility in the temperate climate region particularly in UK.

1.2 Mechanism of folliculogenesis

1.2.1 Oestrous cycle

The first study of the oestrous cycle was reported in 1927(McNutt, 1927) and the theory of follicular waves in cattle throughout the oestrous cycle proposed in 1960 (Rajakoski, 1960). Cattle have two or three follicular waves during the oestrous cycle (Adams, 1998). Normally, the length of oestrous cycle is 20 days in heifers and 21 days in cows. However, this ranges from 18 to 22 days and 18 to 24 days in heifers and cows respectively (Noakes et al., 2001). The first and second waves emerge around days 0 and 10 in two wave cows and days 0, 9 and 16 in three wave cows (Ginther et al., 1989). The duration of oestrus is about 15 h but ranges from 2 to 30 h (Noakes et al., 2001). The morphological changes

and many physiological mechanisms occurring during normal oestrous cycles are reviewed by Adams et al. (2008).

1.2.2 Follicular development and multiple ovulations

Cyclic patterns of pituitary gonadotropic hormones control ovarian functions. These are stimulated by gonadotropin releasing hormone (GnRH) from the hypothalamus and has been extensively reviewed by, (Echternkamp, 2000, Wiltbank et al., 2000, Çobanoğlu, 2011). A high level of circulating follicle-stimulating hormone (FSH) is required for the onset of follicular wave development. Additionally, selection of the dominant follicle may depend upon a fall in FSH level (Ginther, 2000, Çobanoğlu, 2011). At the time of deviation, the concentrations of FSH are not adequate for developing subordinate follicles through the deviation point (Wiltbank et al., 2000). Decreasing or nadir FSH concentrations during the oestrous cycle lead to atresia of subordinate follicles and enhance the first dominant follicle selection (Mihm et al., 1997).

The subordinate follicles regress after follicular selection due to the low levels of FSH (Day of Oestrous; Figure 1.1A). However, in some cases more than one follicle within the same follicular wave, in one or both ovaries, are selected to become dominant follicles (Day of Oestrous; Figure 1.1B). Conceivably, the mechanisms to select one dominate follicle and regression of subordinate follicles is altered, resulting in multiple ovulation and the phenomenon of co-dominance (Wiltbank et al., 2000, Kulick et al., 2001, Çobanoğlu, 2011). The continuing growth of selected dominant follicles is dependent on the transition from FSH to LH and ovulation is controlled by increasing LH concentration (Son et al., 2011).

Twinning can be categorized into two types: during ovulation each ovulatory follicle contain a single oocyte which expelled into oviduct then fertilize and converted to a zygote. So, twins that arise from fertilization of one oocyte, that subsequently cleaves and forms two embryos during development called monozygous twinning (identical twins) which are phenotypically and genetically identical and they are always same sex. However, twins that arise from fertilization of multiple oocytes during ovulation of multiple follicles in the same oestrous cycle termed dizygous

twinning (fraternal twins) (Wiltbank et al., 2000, Fricke, 2001). Most cattle twins are dizygotic resulting from multiple follicle ovulations (Silva del Río et al., 2006).

Several mechanisms impact follicular development to enhance multiple ovulation and subsequent multiple births. Small doses of exogenous FSH can increase the incidence of multiple ovulations by about 25% (Glick et al., 2013). Wiltbank et al. (2000) proposed that slightly increased FSH near the time of deviation could lead to increased ovulation rate. The factors that regulate FSH levels do not reduce sufficiently to prevent further growing of subordinate follicles. The key factors that diminish FSH levels are steroid hormone, while other factors such as insulin-like growth factors (IGF) and transforming growth factors (TGF) affect FSH action. Increased E_2 production by developing follicles around the time of selection may lead to the final depression of FSH and subsequent regression of subordinate follicles. It has been suggested that increased dry matter intake in high yielding cows may result in increased blood flow to the digestive tract and subsequently increased steroid metabolism by liver. The increased E_2 clearance by the liver and associated elevated FSH around follicular deviation may be sufficient to allow multiple follicles to undergo physiological changes and proceed to ovulation (Wiltbank et al., 2000, Çobanoğlu, 2011). However, dominant follicles could be selected at earlier stage of follicle development as the future dominant follicle can be identified at a follicular diameter about 1 mm and it emerges earlier than the first subordinate follicle (Jaiswal et al., 2004).

Multiple ovulations occur at the same time from different ovulatory follicles of the same follicular wave rather than two consecutive waves (Wiltbank et al., 2000, Echternkamp, 2000). The evidence of multiple corpora lutea at the same stage of development on the ovarian surface indicates multiple ovulations. Ovulation rate for the right ovary has been reported to be significantly greater than for the left side (Cushman et al., 2005). Perhaps, the variation of ovulation rate in right and left ovaries may due to the functional activity of the right ovary. Reported incidences of multiple ovulations and twinning are presented in (Table 1.1).

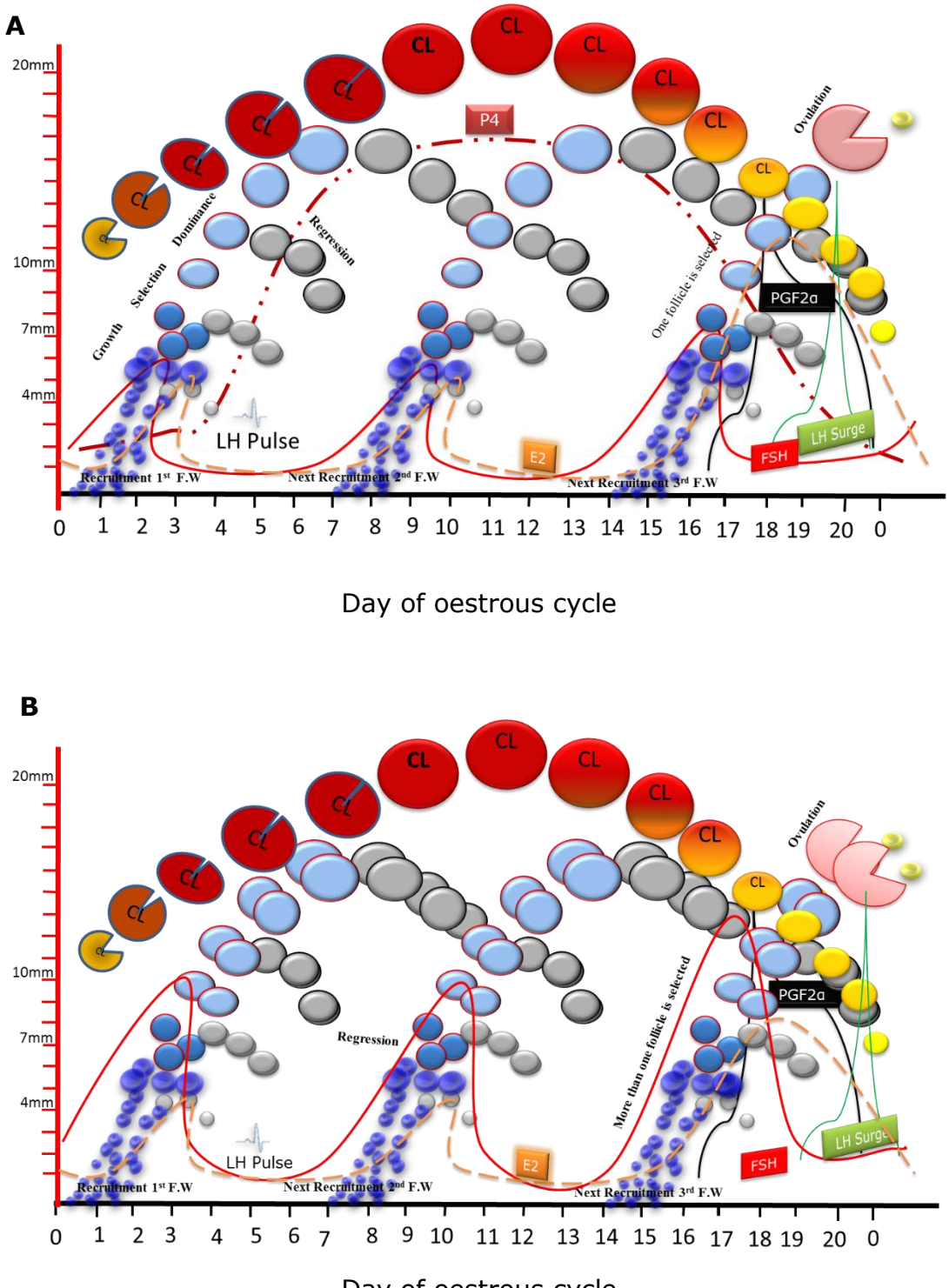


Figure 1.1 *Diagrammatic representation of the oestrous cycle describing the endocrine system controlling folliculogenesis, follicular wave, selection, ovulation, CL development and regression in animals having single ovulations (**A**) and multiple ovulations (**B**). Circle= follicle, F.W= follicular wave, FSH= follicle stimulating hormone, LH= luteinizing hormone, E_2= oestradiol, P_4= progesterone, $PGF_2\alpha$= Prostaglandin F2 alpha and CL= Corpus luteum.*

1.3 Underlying mechanisms involved in multiple ovulations

1.3.1 Follicular development

Several studies have shown that the ovulatory follicle size plays a significant role in reproductive fertility, affecting oocyte quality, embryonic development and pregnancy rates (Vasconcelos et al., 2001, Stevenson et al., 2007, Perry et al., 2007, Atkins, 2009). The ovulation of larger follicles resulted in increased pregnancy rates compared to the ovulation of smaller follicles (Atkins, 2009). A diameter of ovulatory follicles less than 10 mm was associated with fewer conceptuses than follicles greater than 10 mm (Echternkamp et al., 2009). In multiple ovulation cows, the diameter of the individual ovulatory follicles decreased with higher in ovulation rates (Echternkamp et al., 1990a, Echternkamp, 2000). The frequency of multiple ovulations was increased in cows that had more follicles greater than 5mm. However, the diameter of the largest follicle decreased when the CL number increased and the regression of CL was reduced in animals that had multiple CL compared with those had one CL (Stevenson et al., 2007). Mann et al. (2007) recorded that there was no difference in size of ovulatory follicle between single and multiple ovulations in non-lactating dairy cattle. The size of the individual CL resulting from these ovulatory follicles reduced with increased ovulation rate, whereas the total volume of CL per cow increased with each extra ovulation (Echternkamp et al., 2009).

Table 1.1 *The percentage of multiple ovulations and multiple births in cattle throughout the season of the year demonstrated in the literature from the different countries.*

Region	Type	Winter			Spring			Summer			Autumn			No. of calving and animals	Total Rate%	References
		12	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*			
Netherlands	Twinning					↑↑	↑↑	↑↑	↑↑					11,951	3.2	(Nielen et al., 1989)
Saudi Arabia	Twinning						↑↑	↑↑						28,843	1.0-8.0	(Ryan and Boland, 1991)
Norway	Twinning				↑↑			↑↑	↑↑					0.5 million	0.6-4.0	(Karlsen et al., 2000)
USA and Canada	Twinning					↑↑	↑↑	↑↑						1.3 million	5.0	(Johanson et al., 2001)
USA	Multiple ovulation							↑↑	↑↑	↑↑				245	22.27	(Sartori et al ., 2002)
Northeast Spain	Multiple ovulation	↑↑	↑↑	↑↑	↑↑	↑↑								1,792	15.5	(López-Gatius et al., 2005)
USA	Multiple ovulation								↑↑	↑↑	↑↑			249	14.9	(Stevenson et al., 2007)
Egypt	Twinning										↑↑	↑↑	↑↑	1243	4.7	(Shamiah et al., 2007)
Italy	Multiple ovulation						↑↑	↑↑	↑↑	↑↑	↑↑			1,200	15.5	(De Rensis et al., 2008).
Ireland	Twinning									↑↑	↑↑	↑↑	↑↑	23,658,351	1.7	(Fitzgerald et al., 2014)
	Multiple ovulation	↑↑										↑↑	↑↑	75,966	6.8	

↑↑*means increased*

1.3.2 Hormonal variation in single and multiple ovulations

Hormonal secretion during the oestrous cycle controls follicular development and may play a role in follicular selection and dominance of one or more follicles. It has been suggested that lower circulating inhibin and P_4 concentrations are responsible for increasing FSH and LH respectively in cow that had codominant follicles (Lopez et al., 2005b). Therefore, hormonal changes around/during follicle selection may encourage a dominant phenotype to develop and deviation of other subordinate follicles and continue to develop as second dominant follicles. Thus, the largest subordinate follicle could also continue to grow despite FSH depression after selection

The IGF-1 level in both peripheral blood and follicular fluid is closely associated with the incidence of twinning rate in cattle (Echternkamp et al., 1990a). IGF-1 affects proliferation and differentiation and reduces apoptosis of follicular cells, thus increased IGF-1 in genetically selected twin females may contributed to enhanced follicular development (Echternkamp et al., 2004a). In the two largest follicles, greater IGF-1 concentrations were recorded in twin producing compared with control cows, whereas the IGF-I concentrations in small follicles (<4 mm) in twin cow were similar to control cows (Echternkamp et al., 1990a).

The mean level of plasma P_4 increases with increased ovulation rate from 1 to 3 in the days following insemination (Echternkamp et al., 2009). The serum concentration of P_4 and the size of the subsequent CL formed from the ovulatory follicle are strongly related (Vasconcelos et al., 2001). Steinman (2006) proposed that the CL originating from a smaller ovulatory follicle may have fewer luteal cells. It has been proposed that these smaller follicles may produce lower E_2 concentration prior to ovulation followed by lower P_4 production by the resulting CL (Vasconcelos et al., 2001). Low P_4 concentration before and after insemination in dairy cow could be responsible for low fertility (Sartori et al., 2002). It has been reported previously that large luteal cells are responsible for the secretion of the majority of P_4. Therefore, an increase in the large luteal cell number,

resulting from an increased number of GCs, may lead subsequently to increased P_4 secretion (Niswender et al., 1985).

Perhaps the mechanism of luteinizing GCs is a key factor to producing a mature and active CL. It has been shown that melatonin protects GCs undergoing luteinisation and resulting in increased P_4 secretion (Taketani et al., 2011). Melatonin secretion may play a crucial role in GCs luteinization and subsequent enhanced P_4 secretion either through protection of mitochondrial activity, or as an antioxidant and as a free radical scavenger.

1.3.3 Factors affecting multiple ovulations

Generally, cattle are a monotocous species, which mean cattle produce one calf per pregnancy. However, cattle reproductive processes can result in multiple births. Multiple births in cattle have been considered as an undesirable trait that reduces general cow reproductive efficiency, productivity and thus the profitability of enterprises. It has been estimated that the negative effect of twinning in dairy cows cost about £75/cow in Great Britain (Eddy et al., 1991) whereas profitability in beef cattle may increase (De Rose and Wilton, 1991, Echternkamp and Gregory, 2002). People with experience in beef and dairy programs identify many problems that can arise with twin births (Nielen et al., 1989, Wiltbank et al., 2000, Çobanoğlu et al., 2010). Twinning in cattle leads to increases the incidence of health problems, lower conception rate (CR), perinatal mortality, abortion, retained placenta, dystocia, and metritis (Gregory et al., 1990, Kinsel et al., 1998, Çobanoğlu et al., 2010, Cockcroft and Sorrell, 2015). Additionally, it causes metabolic disorders, freemartinism and increased culling rates (Fricke, 2001). The incidence of multiple births in dairy cow are unavoidable because of the factors that contribute to follicular development and the mechanisms of follicular selection are not fully understood (Çobanoğlu, 2011). The factors such as parity, genetics, milk yield, nutrition, season of the year and some other factors greatly influence multiple ovulation rates.

1.3.3.1 Parity and age of animals

In general, twinning rate and multiple ovulations are low in heifers but continue to increase during subsequent parities (Wood, 1984). It has also been reported that the incidence of multiple ovulations is increased from younger non lactating cows to older ones (Mann et al., 2007). In another study, the twinning rate from first parity to parity six was increased and then slightly decreased in parity seven (Karlsen et al., 2000). Conceivably, the increase in the proportion of multiple ovulations and births with increased parity may be associated with increased milk production (Walsh et al., 2007).

1.3.3.2 Milk production

In high producing dairy cows, there is a positive correlation between multiple ovulations and milk yield (Fricke and Wiltbank, 1999, Lopez et al., 2005a). High milk production may affect hormonal changes that are responsible for follicular selection and dominance prior to oestrus (Lopez et al., 2005b). Increased milk production might be a factor contributing to increased incidence of multiple ovulations in dairy cows via altered metabolism of steroid hormone in liver. However, a high incidence of multiple ovulations in non-lactating dairy cows was recorded by Mann et al. (2007). The author recommended that high milk yield may increase multiple ovulations, but it is not a prerequisite for higher occurrence of multiple ovulations, and it is unlikely to be a key factor.

Several studies through America and Europe confirmed that the milk production is affected by photoperiod that in turn is related with endocrine changes. In winter and autumn, animals exposed to extra light produce more milk without affecting milk composition and had higher plasma circulating IGF1 than natural daylight (Dahl et al., 1997). It has also been reported that cows bearing singles had lower milk production than cows bearing twins (Wood, 1984, Nielen et al., 1989, Guerra-Martinez et al., 1990). These factors could be the reasons for the inherited multiple ovulations in cows. Again, the possibility of photoperiod increasing milk production and the interaction of multiple ovulations with milk production

could be the reason for increasing the incidence of multiple ovulations during long day photoperiod (Figure 1.2).

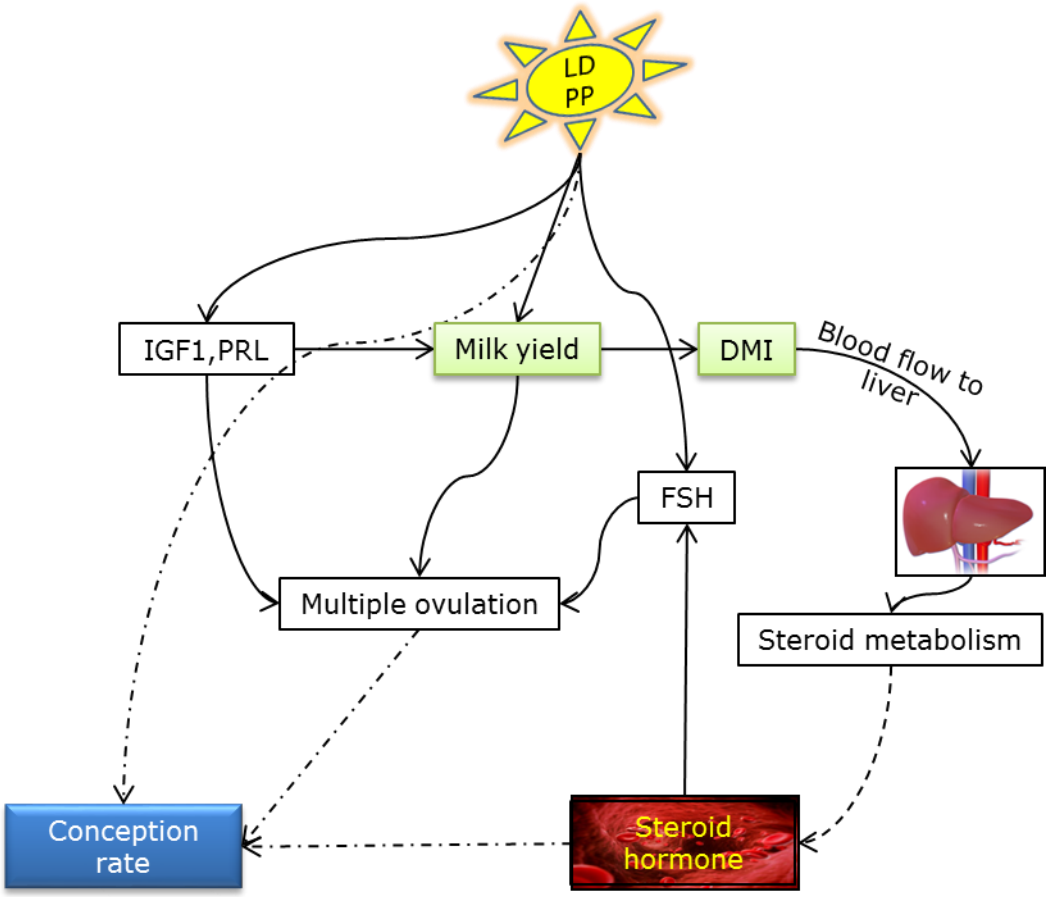


Figure 1.2 *The diagram show the mechanism causing multiple ovulations in dairy cows (⇢) inhibit and (→) increased. LDPP=Long day photoperiod, PRL=Prolactin, DMI=Dry matter intake and FSH=Follicle stimulating hormone and IGF1=Insulin like growth factor 1.*

1.3.3.3 Season

The occurrence of multiple ovulations and multiple births could be associated with the month and season of the year and the frequency of multiple ovulations and births have been reported to vary among studies in different regions (Table 1.1). However, some studies reported that the frequency of twinning was not associated with season, but with other factors contributing to increased twinning rates such as parity and milk production (Kinsel et al., 1998, Stevenson et al., 2007, Mann et al., 2007). The incidence of multiple ovulations in lactating cows increased unexpectedly in the summer and was considerably greater than in heifers.

However, the ovulation rates in lactating cows were similar to those in dry cows during winter (Sartori et al., 2002).

It should be considered that ovulation failure increased during the warm season (López-Gatius et al., 2005). Pregnancy rate in cows was decreased during the summer and further reductions were observed as ovulation rate increased (Echternkamp et al., 2009). However, the initial pregnancy rate following twin ovulations was greater than single ovulations, though premature death was higher in multiple pregnancies. Perhaps the capacity of the uterus to support more than one foetus could be insufficient for embryonic development (Echternkamp et al., 1990b). These could be reasons that twinning rate increased in dairy cows inseminated during cold and short daylight period (Andreu-Vazquez et al., 2012). The incidences of multiple ovulations are not a true reflection of the incidence of multiple births (Kidder et al., 1952).

It has been reported that the frequency of multiple ovulation increased in cattle with more than two follicular waves per oestrous cycle (Bleach et al., 2004) and more follicular waves have been reported in heifers and cows exposed to high temperature (33°C) (Wilson et al., 1998a, Wilson et al., 1998b). The above results could support the idea that season could influence multiple ovulation rate and that the hot seasonal effect on follicular development could continue to the subsequent season. However, it has been suggested that reduced temperature in autumn and high levels of feeding could result in an increased twinning rate in May, June, and July (Johansson, 1932, Cady and Van Vleck, 1978).

An in vivo study of embryo production and embryo survival after transfer to recipient cows found that the efficiency of embryo production following multiple ovulation decreased during the hot season and the recipient cows were less susceptible than donor cows (Vieira et al., 2014). Effect of hot season on steroid hormones, insemination, implantation and early embryonic death may lead to increase the frequency of early pregnancy losses during summer hot season in animals had multiple ovulation.

In temperate climates, Fitzgerald et al. (2014) reported that the high occurrence of multiple ovulations and multiple births were associated with the month of the year. It could be that the photoperiod had an effect on

follicular development and the temperature interacts to increase this outcome. Melatonin secretion could interact with the follicular development and may play a role in the mechanisms of ovulation and maintenance of pregnancy through various actions.

1.4 Effect of season on female reproductive physiology

In the Northern hemisphere, the winter and summer solstice are associated with shortest and longest daylight periods. The spring and autumn equinoxes are associated with equal daylight and dark. Winter season usually start on 21-22 December (winter solstice) followed by spring equinox on 20-21 March, summer solstice on 20-21 June and autumn equinox on 22-23 September. However, in the Southern hemisphere these patterns are reversed (Figure 1.3).

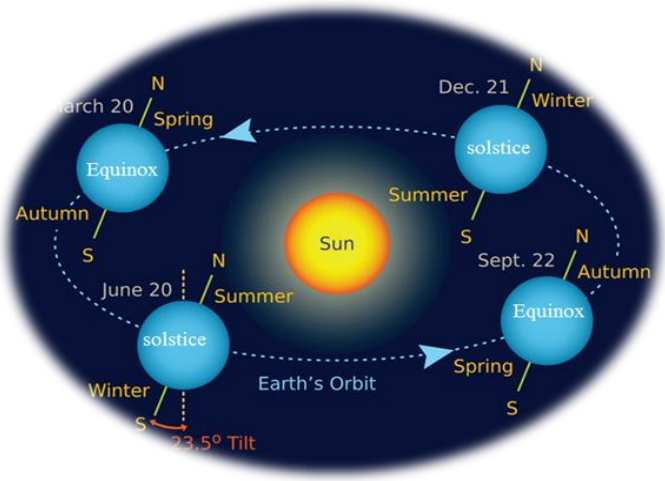


Figure 1.3 *Classified of season of the years according to the summer-winter solstice and the spring–autumn equinox. N= Northern hemisphere, S=Southern hemisphere. Figure was taken from the website (http://astronomy.starrynight.com/content/free-astronomyteaching-resources).*

1.4.1 Effect of season on oestrous cycle

Several physiological changes occur in animals during seasonal changes. A number of studies have investigated the effect of seasonal exposure to heat stress (Dunlap and Vincent, 1971, Trout et al., 1998, Sankar and

Archunan, 2012, Cooke et al., 2012, Lacerda and Loureiro, 2014) in different regions though results have not been consistent. Heat stress can increase the incidence of silent ovulation and anoestrous, as well as reducing oestrus intensity and duration (Kadokawa et al., 2012). Additionally, the numbers of inseminations per animal were increased during the hot season. Recently, oestrus duration was shown to be reduced during the hot season (Sakatani et al., 2016). However, in another study, the length of the oestrous cycle was not changed between summer and winter (Wolfenson et al., 2002) and between heat stressed and non-stressed cows (Trout et al., 1998). Also, increased oestrous activity during summer has been reported by Homer (2013). Oestrus in *Bos taurus* was decreased significantly during heat stress, whereas any effect of stress on oestrus in *Bos indicus* was rare (Youngquist and Threlfall, 2006).

1.4.2 Effect of season on hypothalamus pituitary axis

There is evidence that GnRH and pituitary gonadotrophins are affected through the different seasons and are sensitive to thermal stress (Gilad et al., 1993). Furthermore, dairy cattle are more susceptible to heat stress than beef cattle (O'Brien et al., 2008). Animals with high internal heat production have been produced through genetic selection to increase milk yield (Youngquist and Threlfall, 2006).

1.4.2.1 Follicle stimulating hormone

The main role of FSH is the regulation of follicular development through action on specific receptors in the GCs (Ulloa-Aguirre et al., 1995). It is generally thought that the secretion of FSH is increased during summer, perhaps due to the reduction of inhibin from small follicles (Khodaei-Motlagh et al., 2011, Rensis and Scaramuzzi, 2003). The amount of FSH receptors in GCs from follicles was lower in heat stressed than control rats (Shimizu et al., 2005). They concluded that lowering FSH receptors in GCs reduced E_2 concentration which may cause ovarian dysfunction during the hot season. Roth et al. (2000) reported that the plasma concentration of FSH in synchronized animals increased in heat stressed cows compared with cooled cows. Furthermore, heat stress had a delayed effect on FSH, stating that the FSH concentrations were increased, which could lead to

the low fertility of cow during the summer and subsequent autumn (Roth et al., 2000). Therefore, during follicular phase, the proportions of large follicles are increased. Also, cattle exposed to heat reduced dominant follicle size (Badinga et al., 1993, Badinga et al., 1994, Roth et al., 2000, De Rensis and Scaramuzzi, 2003).

1.4.2.2 Luteinizing hormone

The effect of season on LH is a controversial matter. The concentration of LH in plasma has been reported to increase (Roman-Ponce et al., 1981), decrease (Shimizu et al., 2005) or remain unchanged (Gwazdauskas et al., 1981, Gauthier et al., 1986) during the hot season. It is important to note that the reasons for different findings were unclear. Variation in LH concentration is essential for the full development of the dominant follicle, the final development and differentiation of dominant follicle is affected negatively by lower LH (Guzeloglu et al., 2001).

It has been suggested by Khodaei-Motlagh et al. (2011) that the reduction of fertility may be due to the poor oestrous behaviour because the dominant follicle developed in a low LH environment resulting in reduced E_2 production. Gilad et al. (1993) have indicated that GnRH induced LH release and tonic LH secretion were unchanged during chronic heat stress in cows with high E_2 concentrations in plasma of cyclic animals. However, reduction of LH was noted in cases of low E_2. Chronic summer heat did not affect the plasma concentration of LH, LH baseline or pulse frequency. However, LH pulse amplitude was greater in cooled cows compared with non-cooled cows (Gilad et al., 1993).

1.4.2.3 Prolactin

Prolactin (PRL) is known to play a role in mammary gland function and reproduction in general (Bachelot and Binart, 2007). Prolactin is synthesized not only in the anterior pituitary but also in other organs including the ovary (Shibaya et al., 2006). High temperature exposure increased circulating PRL (Collier et al., 2008). It is also well documented that circulating PRL displays a circannual rhythm with increased levels in summer and decreased levels in winter (Borromeo et al., 1998). Additionally, the maximal concentration in the day occurred at midday

(Ginther et al., 2013). Among its countless functions, PRL is a one of the important factors causing postpartum anoestrous as a suckling and presence of offspring induced its production (Ahmed et al., 2015). Prolactin, in rodents, is considered as the most important constituent of the luteotropic complex (Murphy and Rajkumar, 1985) and stimulated P_4 production in porcine luteal cells (Ciereszko et al., 1998).

1.4.3 Effect of season on steroid hormones

1.4.3.1 Oestrogen

Oestrogen concentrations during hot and cold seasons are shown in (Table 1.2). Both aromatase activity and E_2 concentration in follicular fluid were decreased in September, suggesting that animal exposure to heat stress could affect GCs and TCs, in this manner causing long term negative effects on ovarian follicular quality (Badinga et al., 1993). Wilson et al. (1998b) claimed that the reduction in follicular size under heat stress may lead to a decrease in the concentration of E_2 because this mechanism will lead to a decrease of steroidogenesis by both GCs and TCs. Wolfenson et al. (1997) found that the E_2 concentration in follicular fluid was lower in summer and autumn compared to winter. Additionally, cows under heat stress during the winter season had lower E_2 than winter control.

In vitro studies have found no significant effect of temperature on E_2 production by cultured GCs at 37.5°C and 40.5°C collected during winter, winter heat stressed cows and during summer (Wolfenson et al., 1997). Recently, *in vitro* cultured bovine GCs exposed to heat produced lower E_2 concentrations; this study was supported by the lower expression of steroidogenic factor 1 (SF-1) and *CYP19A1* mRNA in GCs (Li *e*t al., 2016). Also, the reduction of E_2 production by cumulus cells during *in vitro* maturation was demonstrated by (Paes et al., 2016).

The plasma concentration of E_2 in cows was not significantly different between winter and summer ventilation groups. However, in the summer group the level of E_2 underwent a dramatic increase at the onset of the oestrous cycle (Rosenberg et al., 1982). However, during the luteal phase of the treated oestrous cycle, the plasma concentration of E_2 was

unchanged between cooled and heat stressed groups (Roth et al., 2000). But, during the follicular phase, the concentration of E_2 tended to be lower in cows exposed to heat stress. Impaired E_2 secretion during follicular development disrupts oestrus signs, the gonadotropin surge, and ovulation, which could cause infertility during hot season.

1.4.3.2 Progesterone

The environmental effects on the blood concentration of P_4 are still a controversial matter. It has been reported that heat stress had no direct effect on plasma P_4 production but delay luteolysis processes (Wilson et al., 1998b). However, elevated, unchanged or decreased P_4 have all been reported (Table 1.2). These different data among scientists could be due to the difficulties in controlling factors that affect P_4 production. For instance, the circulating P_4 concentration will be affected directly or indirectly by the temperature and dry matter intake (Rensis and Scaramuzzi, 2003). The plasma P_4 does not only depend on its production but also its metabolism, blood volume, nutrition, lactation, age and other factors could contribute to effect the P_4 concentration (Roth, 2015).

The effect of season has on P_4 production could depend on the stage of the oestrous cycle. Trout et al. (1998) recorded that the P_4 concentration was elevated from day 11 to 17, whereas from day 19 to 21 it was lower in heat-exposed cows. Also, Wolfenson et al. (2002) stated that the P_4 concentration was similar during the first 7 days of the oestrous cycle, whereas P_4 concentration at the mid-luteal phase was higher in winter than summer. They found no detectable effects of parity and milk production on P_4. The reduction of P_4 production in summer could result from impaired follicle and/or CL function (Wolfenson et al., 2002).

Gir cows exposed to heat stress had no immediate effect on fertility and P_4 production. However, the reduction of P_4 in heat stressed cow was observed after removing the stress (Torres-Junior et al., 2008). Zeitoun et al. (1996) reported that the lower fertility in autumn than spring is supported by lower plasma P_4 in autumn.

In vitro, elevated temperature did not affect luteal function suggesting the lower fertility during the summer season was not due to the direct effect

of temperature on luteal cells (Iwazawa and Acosta, 2014). Recently, an *in vitro* study revealed no effect of temperature on P_4 secretion by GCs, despite the expression of steroidogenic acute regulatory protein (*STAR*) and cytochrome P450 (*CYP11A1*) mRNA were decreased by heat treatment (Li et al., 2016). Lower P_4 production could lead to abnormal oocyte development, early embryonic death (Ahmad et al., 1995) and implantation failure (Mann et al., 1999). Briefly, molecular mechanisms of P_4 synthesis are described in (Figure 1. 4).

It has been reported that cows subjected to chronic and acute heat stress decreased and increased P_4 concentrations respectively (Howell et al., 1994). Badinga et al. (1994) reported that the variation in the peripheral concentration of gonadal steroids during month of the year was not dependent on the cow's energy status. Khodaei-Motlagh et al. (2011) concluded that the effect of heat stress on plasma P_4, which leads to infertility during the summer, is still unclear. Furthermore, there are several other reasons that could lead to infertility during this season (Rensis and Scaramuzzi, 2003). However, exogenous P_4 supplementation 5 days after insemination improved conception rates during summer season (Friedman et al., 2012). Ovulation induced by hCG using timed artificial insemination protocol during hot season, improves the fertility of dairy cattle through increasing plasma P_4 concentration (De Rensis et al., 2008). Recently, it has been demonstrated that the lower plasma P_4 concentration in summer resulted from lower luteal activity during this period (Satheshkumar et al., 2015).

Table 1.2 *Changes in the concentration of E_2 and P_4 collected during the summer season and/or after heat exposure in cattle reported in the previous literature.*

Reference	Steroid hormone		Body fluid	Protein/Gene
	E_2	P_4		
Roman-Ponce et al. (1981)	↔	↔	Plasma	
Gwazdauskas et al. (1981)	↓	↔	Plasma	
Rosenberg et al. (1982)	↑↑	↓	Plasma	
Badinga et al. (1993)	↓	↔	Plasma	
Badinga et al. (1994)	↑↑	↑↑	Plasma	
Wolfenson et al. (1995)	↓	↔	Plasma	
Wolfenson et al. (1997)	↓	↔	FF	
Jonsson et al. (1997)		↓	Plasma	
Trout et al. (1998)		↔	Plasma	
Wilson et al. (1998a)	↓	↔	Plasma	
Roth et al.(2000)	↔	↔	Plasma	
Guzeloglu et al. (2001)	↔	↔	Plasma, FF	
Ronchi et al. (2001)		↓	Plasma	
Wolfenson et al. (2002)		↓	Plasma, GCs, TCs	
Bridges et al. (2005)	↓	↑↑	Follicle wall	
Rispoli et al. (2013)		↑↑	COC	↓MMP9
Iwazawa and Acosta (2014)		↑↑	Luteal cells	↑*HSP70*, ↔HSP70,↑$PGF_2\alpha$,PGE2
Ghanem and Nishibori. (2015)		↑↑	Plasma	
Ghanem and Nishibori. (2015)		↔	Plasma	
Paes et al. (2016)	↓	↑↑	Follicles	↑BAX,HSP70
Li et al. (2016)	↓	↔	GCs	↑caspase-3, ↑*BAX*/*BCL2* ratio

E_2=oestradiol, P_4= progesterone, FF= follicular fluid, GCs= granulosa cells, TCs= theca cells, COC=cumulus oocyte cells, MMP9=latent matrix metallopeptidase 9, HSP70=heat shock protein 70, $PGF_2\alpha$=Prostaglandin F2 alpha, PGE2=Prostaglandin E2, BAX, caspase-3, BCL2= apoptotic gene, ↑↑=increased, ↓= decreased and ↔=no effect

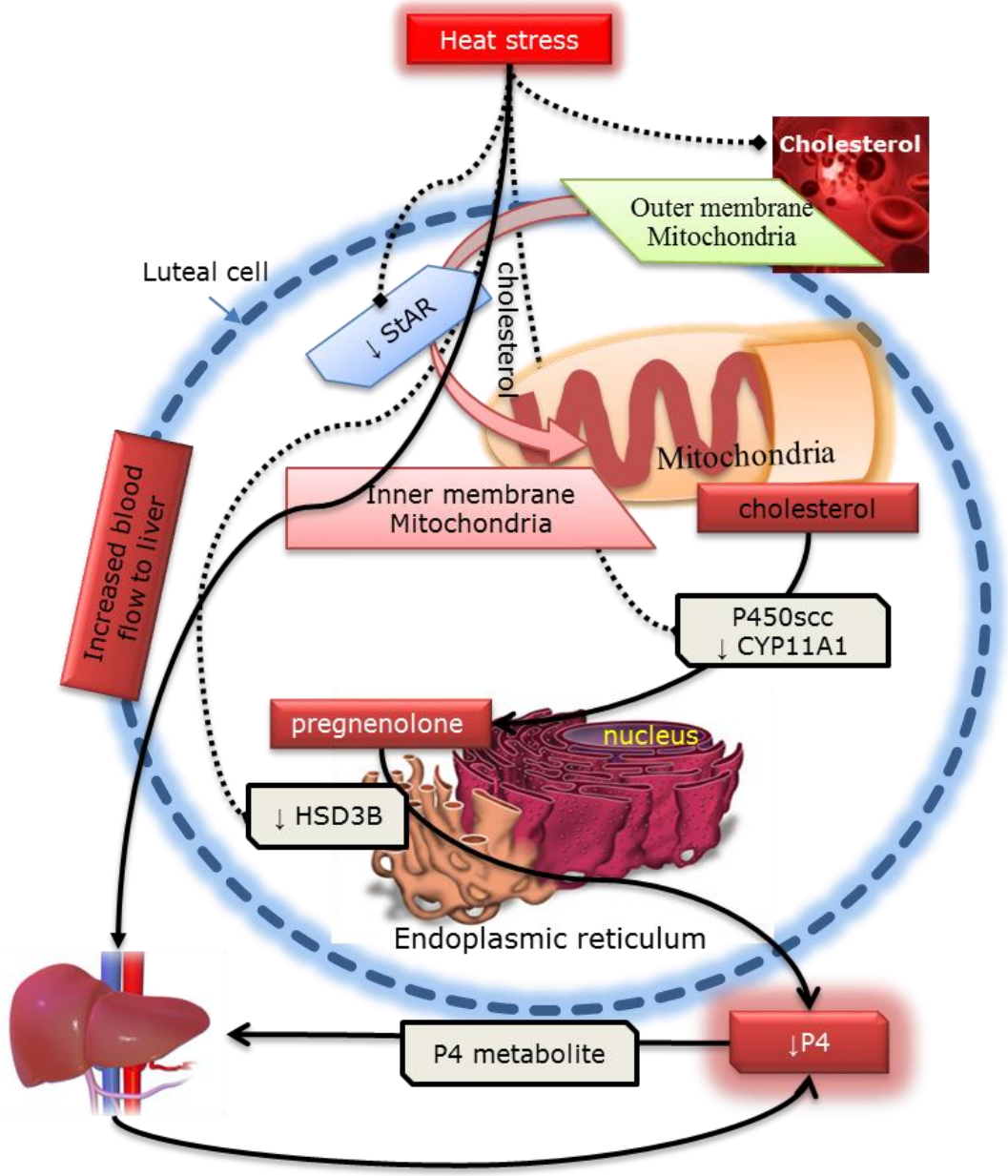


Figure 1. 4 *Physiological model of how heat stress affects the mechanisms of P_4 production pathway. Cholesterol from outside of membrane is transported into inner membrane by Steroidogenic acute regulatory protein (STAR). The activity of cytochrome P450 family 11 subfamily A member 1 (CYP11A1), HSD3B converted cholesterol to pregnenolone and finally hydroxy-delta-5-steroid dehydrogenase, 3 beta (HSD3B) participate to release progesterone (P_4) into blood circulation and tissue. The mechanisms of P_4 biosynthesis was reviewed from (Wiltbank et al., 2014) with slight modification*

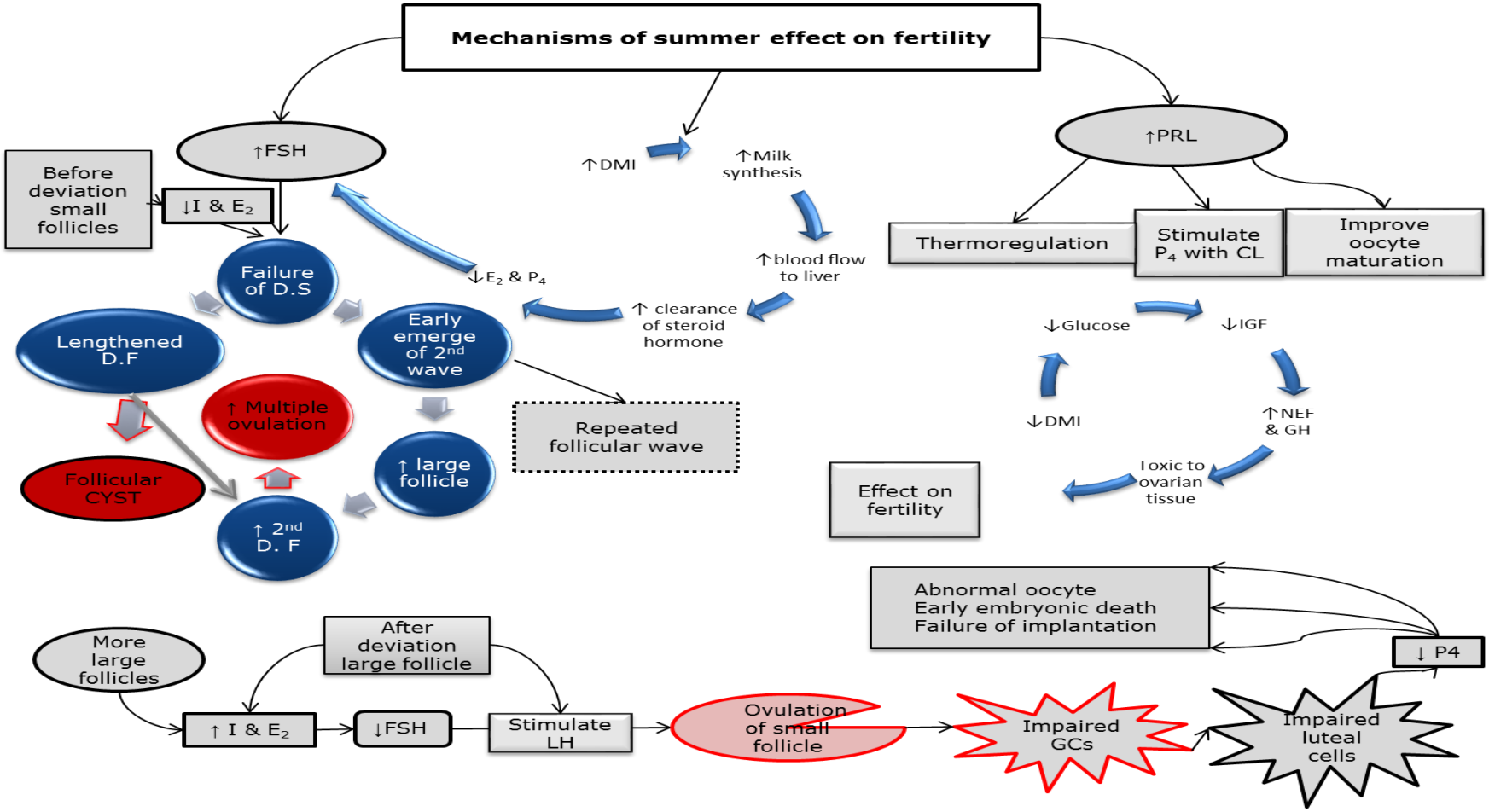


Figure 1.5 *Schematic representation of the general effect of season on reproductive system described in literature that could support the hypothesis of this study in term of ovulation rate, follicular population and CL development.* ↑=increased, ↓=decreased, *FSH=Follicle stimulating hormone,* LH=*luteinizing hormone, PRL=prolactin,* I=inhibin, E_2=oestradiol, P_4=progesterone, D.S=dominant follicle selection, D.F=dominant follicle, IGF=insulin growth factor, GCs=granulosa cells, CL=corpus luteum, NEF=non-esterified fatty acid, GH=growth hormone and *DMI=dry matter intake.*

1.4.4 Effect of season on ovarian function

1.4.4.1 Follicular development

Season of the year can lead to altered follicular populations and follicular diameter. The dominant follicle in the first follicular wave grew faster and were greater in size in April than June, August and November. Furthermore, these follicles could be related with regression of the largest subordinate follicle (Badinga et al., 1994). Shehab El Deen (2011) found that the average diameter of the dominant follicle in dairy cows during the early postpartum period was higher in winter than in summer. In heifers, the development of preovulatory dominant follicle was inhibited by heat stress (Wilson et al., 1998a). However, the diameters of follicles did not change during the seasons of the year (Wolfenson et al., 1997). The dominant follicular size during the follicular phase under heat stress was less or equal to that of the control group (Guzeloglu et al., 2001). Roth et al. (2000) suggested that neither the size nor growth and reduction of the first and second wave dominant follicles were altered after heat exposure.

With respect to the follicular waves, Wilson et al. (1998b) reported that 18% of cows under heat stress had two follicular wave cycles compared with 91% in the normal group. The remaining non-stressed cows had an oestrous cycle with three follicular waves, whereas stressed cows had 45%, 27% and 9% with three, four and five follicular waves respectively. These may by supported by the (fact that the) finding of a reduction of follicular dominance and prolonged dominance of ovulatory follicle under heat stress as well as increased number of follicles greater than 10 mm (Hansen, 2009). Dairy cattle exposed to heat during the entire oestrous cycle showed a 50% increase in the number of large follicles (>10mm) during the first follicular wave (Wolfenson et al., 1997).

The pattern of follicular development was not affected directly by heat stress. However, after removal of heat stress, the number of the large follicles and the diameter of the first and second largest follicles increased in the heat stress group. Additionally, the number of follicles >9 mm was increased by heat stress known as follicular codominance. After cessation

of heat stress, plasma P_4 concentration was decreased and this reduction was associated with increased follicular diameter (Torres-Junior et al., 2008). However, the number of follicle ranging from 3 to 4 mm was higher during the cold period than the warm period (Satheshkumar et al., 2015). These finding could support the idea that the incidence of multiple ovulation is affected by environmental factors.

1.4.4.2 Granulosa / theca cell function

Granulosa cells within the follicle play a vital role during follicular development because of their endocrinological capabilities to produce hormones as well as cell proliferation and differentiation (Petro et al., 2012). Granulosa cells within the follicle are set in three different zones; cells attached to the basal membrane named mural cells, cells surrounding the antrum of the follicle named antral cell and cumulus cells situated internally surrounding the oocyte (Rouillier et al., 1998, Galas, 2012). The viability of GCs was not changed at 40.0°C compared to 37.5°C (Wolfenson et al., 1997). However, the apoptotic rate of GCs, activity of caspase 3, pro-caspase 3 expression and the ratio between anti-apoptotic gene (*Bcl2*) and pro-apoptotic gene (*BAX*) was higher under heat stress (Li et al., 2016). Heat stress inhibits murine GC proliferation and induced apoptosis (Luo et al., 2016). The mechanisms through which heat stress leads to apoptosis is thought to be through the mitochondrial pathway. Also the reduction of E_2, P_4 and steroidogenic enzyme mRNA expression (*STAR*, *CYP19A1*, and *CYP11A1*) could cause murine GC dysfunction and apoptosis (Luo et al., 2016). In addition, under heat stress, the colony formation of sheep GCs was reduced and GC apoptosis were increased at 43.0°C (Fu et al., 2014).

It has been shown that high temperatures *in vitro* enhance follicular cells luteinisation (Bridges et al., 2005). The study revealed that production of androstenedione and E_2 were reduced at 41°C, whereas P_4 production was increased. In this study, pieces of follicular wall, isolated from dominant follicles, were incubated for 96 h. They suggested that reduction of cattle fertility under heat stress may be due to the premature differentiation of follicular cells (Bridges et al., 2005).

1.4.4.3 Corpus luteum

The CL is a temporary endocrine gland developed from ovarian follicle after ovulation. CL plays a vital role in P_4 production and maintains pregnancy development. It has been reported that there is a reduction of P_4 secretion indicating lower luteal activity during the hot season (Satheshkumar et al., 2015). Wilson et al. (1998a) demonstrated that the size of CL was similar until day 16 of the cycle, however, the regression of the CL was slower in heat stressed animals than thermo-neutral ones. The same authors, in another study, revealed that about 20% of heat stressed cows had a regressed CL, whereas non stressed cows had 80% regressed CL by day 21 (Wilson et al., 1998b).

The number and the viability of luteal cells after dispersion were higher in winter than summer. However, no effect of temperature on both cell number and viability were seen after incubation at 38.0 and 40.0°C (Wolfenson et al., 1993b). *In vitro culture*, of luteal cells revealed no significant effect of temperature on luteal cell viability. Whereas P_4, $PGF_2\alpha$ and PGE2 production increased at high temperature and the expression of *HSP70* and *HSP90* mRNA were higher at 41.0°C than the 37.5°C (Iwazawa and Acosta, 2014).

1.4.5 Effect of season on establishment of pregnancy

1.4.5.1 Conception rate

In the last few decades, CR in high producing dairy cattle has reduced worldwide and further reductions have been predicted in the future (Lucy, 2001). Several research studies have investigated the emerging crisis of infertility in dairy cows. It should be noted that there has been an industry trend toward housing more cows under one roof (Winsten et al., 2010) which could increase the risk of adverse environmental conditions (Wagner-Storch and Palmer, 2002). Seasonal variation has a dramatic effect on pregnancy rate especially in the hot months throughout the different region of the world. Furthermore, effects of heat stress on CR in the moderate climate regions has been demonstrated as well (Schüller et al., 2014).

The reduction of CR during summer or hot periods in multiparous and primiparous cows has been demonstrated (Rosenberg et al., 1982, Azzam et al., 1989, Zeron et al., 2001, Sönmez et al., 2005). The pregnancy rate was increased in cows inseminated during cold periods (October to April) (López-Gatius et al., 2005). The pregnancy rate of dairy cattle underwent a significant fall in the summer at day 90 and at day 135 postpartum compared with winter (Rensis et al., 2002).

It has been suggested that dairy cattle in moderate climate regions are more susceptible to heat stress than those in tropical and subtropical climates. Temperature humidity index (THI) greater than 73 for 1h reduced CR while heat stress before and after insemination had a negative effect on CR. However, 21 days before insemination is believed to be the most sensitive period to stress (Schüller et al., 2014). In subtropical environments, the conception and pregnancy rates of Holstein cows was negatively correlated with THI. Furthermore, embryonic loss was increased at high THI (El-Tarabany and El-Bayoumi, 2015). The pregnancy rate was reduced in heat stressed cows than non-stressed (Khan et al., 2013).

The critical period around the day of breeding was consistently related with the reduction of CR (Morton et al., 2007, Mellado et al., 2013, Schüller et al., 2014). One cause of pregnancy loss is a negative effect of season on oocyte development. The three mechanisms which could compromise the oocytes ability during the hot season are follicular development, follicular steroidogenesis and direct oocyte sensitivity to the high temperature (Youngquist and Threlfall, 2006).

1.4.5.2 Embryonic development

The percentage of normal oocytes was higher in the cool season than in hot season. Also, in the cold season, embryo development and the proportion of embryos reaching the morula stages was greater than in the warm season for *Bos taurus* cows (Rocha et al., 1998). Fertilized oocytes collected in the summer were less developed by the blastocyst stage than those collected in winter (Youngquist and Threlfall, 2006). In a UK study, Silva et al. (2006) recorded that the development of the embryo was greater in autumn (September and November) than in spring (March and

May). The proportion of fertilized oocytes cleaving to the two to four cell stage and further developing to blastocysts was lower in the hot season. While the mRNA levels of *POU5F1 (OCT4),* which is essential for maintain pluripotency of embryonic stem cells and primordial germ cells, was higher at oocyte, 2, 4, 8 cell stage and blastocysts in the cold season (Gendelman and Roth, 2012).

High temperature compromised the oocyte capacity to developed into a blastocyst (Hansen, 2013), increased the proportion of apoptotic blastomeres (Silva et al., 2013), reduced cleavage rate and embryonic development (Sakatani et al., 2015). Higher hydrogen peroxidase was generated by elevated temperature (41.0°C) during fertilization (Sakatani et al., 2015). Thus, one approach to reducing the effect of heat stress on fertilization is to limit the effect of oxidative stress. The effect of heat stress on oocyte function can commence early during folliculogenesis, therefore, the effects of heat stress on oocyte competence can continue after the end of the stress (Hansen, 2013). In a study by Torres-Junior et al. (2008) they demonstrated that after removal of heat stress, total blastocyst rates were reduced. In addition, the follicular pool and oocyte quality restoration took two or three oestrous cycles to improve from the previous effect of heat stress (Torres-Junior et al., 2008).

1.4.5.3 Uterine function

Generally, the uterus, as other visceral tissues, will respond to high environmental temperature with a reduction of blood flow and its redistribution to the peripheral tissue. While the nutrient, hormones and O_2 are carried by blood, elevated temperature may compromise uterine function.

In an *in vitro* study, Putney et al. (1988b) found that high temperature from 36°C to 42°C for 6 h led to an increase in the secretion of prostaglandins from endometrial tissue and reduced conceptus protein production. Jordan (2003) commented on this evidence that if the same effect happened *in vivo* it could lead to premature CL regression. Furthermore, in response to oxytocin, uterine production of prostaglandin is increased (Wolfenson et al., 1993a). In the UK, longer postpartum

anoestrous period was found in cows calving in spring than in autumn (Lamming et al., 1981). During late pregnancy, the effect of thermal stress causes a decline in the function of placenta, placental size and weight of calf. This could be due to the reduction of blood flow into the genital tract. Also immunoglobulin transfer to the neonate was reduced and calf mortality rates were increased during high environmental temperature in summer (Youngquist and Threlfall, 2006). In an ovine model, intrauterine infusion of melatonin increased umbilical blood flow suggesting a role of melatonin on the placenta (Lemley et al., 2013). This factor could be related to the reduction of CR and increased early embryonic death in summer when melatonin levels are low.

1.4.6 Genetics and thermo-tolerance

Genetic selection has been used on livestock to decrease the effect of climate on animals. Perhaps, there may be specific genes which are sensitive or tolerant to the environmental temperature and photoperiod. The multifactorial problems in dairy cattle fertility caused by summer heat are various and depend on the breed of cattle. For example, *Bos indicus* has been reported as a more tolerance breed to heat stress than *Bos taurus* (Paula-Lopes et al., 2013).

The ability of animals to maintain body temperature could influence pregnancy especially in highly producing dairy cattle. The pregnancy rate was similar between high yield cows and low yield cows when the rectal temperature of high producing milk was maintained below 39.0°C (Vasconcelos et al., 2011). Rocha et al. (1998) have drawn attention to the effect of season differences on folliculogenesis in *Bos taurus* cows, while the Brahman breed showed no effects. Apparently, the embryos of Holstein or Angus breeds developed at high temperatures were more sensitive to heat than Brahman embryos (Youngquist and Threlfall, 2006). The dramatic differences in thermoregulation lead to the idea of variation in thermotolerance between *Bos taurus* and *B. indicus.* Probably, these results indicated that specific gene could be identified and transferred to non-tolerant breeds to improve the fertility and thus select cows resistant to heat stress (Youngquist and Threlfall, 2006).

In subtropical condition crossbred animals (Holstein x Brown Swiss) had higher fertility and adaptability to the environmental stress than pure Holstein (El-Tarabany and El-Bayoumi, 2015). Another approach looking to increase thermal tolerance of cattle is to identify the characteristics of hair pigment and hair length as well as controlling cellular resistant to heat stress. It has been suggested that Black Holstein cows are more sensitive to thermal stress than white ones (Becerril et al., 1993, Hansen, 1990). Moreover, the gene that is responsible for controlling hair length in the Senepol breed may increase ability to regulate body temperature during thermal stress (Olson et al., 2003). A published study in lactating Holstein cows identified slick hair haplotype as having greater ability not only to regulate body temperature but also to sustain milk production during the summer season in a hot environment (Dikmen et al., 2014).

The effect of heat stress on lower fertility could be influenced by single nucleotide polymorphisms (SNPs) (Lacerda and Loureiro, 2014). In response to heat stress, heat shock protein (HSP) is secreted in the cytosol and binds to denatured protein (Chrenek et al., 2003). Kamwanja et al. (1994) claimed that during heat shock, the lymphocyte survival was less in the Angus breed than in both Brahman and Senepol cattle. Basiricò et al. (2011) suggested that the presence of polymorphism (C/- and G/T) in the 5-UTR region of inducible HSP70.1 ameliorates responses of bovine peripheral blood mononuclear cell to heat stress. The polymorphism within the intron 3 in gene *HSP90AB1* (Charoensook et al., 2012), two SNPs in the *HSF1* gene (Li et al., 2011), SNPs in *ATP1A1* gene which is coded for Na, K-ATPase (Liu et al., 2011b) showed better thermal tolerance in Holstein cows. In the genome wide association study of rectal temperature, in Holstein cows done by Dikmen et al. (2013) results were reported that the gene involved in metabolic rate regulation *SLCO1C1* plays an important role in the processes controlling body temperature during stress. Possibly SNPs will be valuable as molecular markers to promote genetic selection for heat tolerance.

The discovery of thermotolerance biomarkers without any impact on milk yield or reproductive performance could consequently give superior profit.

1.4.7 Effect of photoperiod on female reproductive physiology

Daylight is the one of the most important factors effecting reproductive performance in mammals. The duration of daylight varies in different seasons in UK. Long daylight is about 17 h of light with 7 h of darkness (Dahl et al., 2000). Cattle appear to discriminate light as low as 5 lux (Phillips and Weiguo, 1991). There are several studies that suggest the reproductive activities in cows are affected by the length of the photoperiod (Rius et al., 2005, Andreu-Vazquez et al., 2012, Dahl et al., 2012). The photoperiod is known to affect puberty, postpartum and pregnancy rate, (Youngquist and Threlfall, 2006) milk production, growth rate (Dahl, 2008) and immune response (Nelson, 2004, Collier et al., 2006).

Autumn born animals reached puberty earlier than those born in spring (Youngquist and Threlfall, 2006). Although, ear implanting with melatonin for a maximum of 100 days at age 5 weeks to simulate short days accelerated puberty (Youngquist and Threlfall, 2006). Under long photoperiod animals achieved puberty earlier than short daylight via increasing LH secretion in response to E_2, which reduced age at first ovulation and oestrus (Hansen et al., 1983). Furthermore, calves growing under long photoperiod achieved greater body weight, lean tissue and hastened puberty (Rius et al., 2005). Also the concentration of blood IGF1 increased in heifers exposed to long photoperiods (Spicer et al., 2007). The physiological processes of early puberty could be due the effect of photoperiod on hormonal production such as prolactin, melatonin, and other growth factors. Furthermore, nutritional factors also need to be considered during animal growth. It seems that melatonin or short day photoperiod are important during earlier life of animals and long photoperiod hastened the onset of puberty and maturation.

To understand more about the mechanisms of photoperiod and how animals keep time physiologically, melatonin, is the main signalling hormone that responds to photoperiodic time in mammals, (Walton et al., 2011). For example in buffalo melatonin treatment, stopped summer anoestrus and induced ovarian cyclicity and ovulation of anoestrous buffalo heifers (Ghuman et al., 2010).

1.5 Effect of melatonin on female reproductive

After the discovery of melatonin in 1958 by Lerner et al. (1958) in the bovine pineal gland, a new field of research in reproductive physiology was heralded. Since its discovery, several studies have investigated the role of this indole amine in fertility.

1.5.1 Melatonin signalling

Melatonin (N-acetyl-5methoxytryptamine) is produced from the pineal gland during dark. The initial precursor of this indole amine is tryptophan; the classic pathway of melatonin synthesis is illustrated in Figure 1.6. Melatonin biosynthesis has been reported not only in the pineal gland but also in other tissues including reproductive tissue. Reiter et al. (2014) proposed that mitochondria as a site for melatonin synthesis, therefore, all cells that possess mitochondria could produce melatonin for their function.

The rate limiting enzymes, aralkylamine-N-acetyltransferase (AANAT) and acetyl serotonin-O-methyltransferase (ASMT) for biosynthesis of melatonin has been discovered in extra pineal gland tissues such as; ram testis (Gonzalez-Arto et al., 2015), human ovary (Itoh et al., 1999), human placenta (Soliman et al., 2015), rat ovary (Itoh et al., 1997) and bovine ovary (El-Raey et al., 2011). These findings support the synthesis of melatonin in tissues other than the pineal gland. The two enzyme AANAT and ASMT control the amount of melatonin production (Tan et al., 2015). The production of melatonin in pineal gland was positively correlated with increased ASMT activity (Ceinos et al., 2004). Once melatonin is synthesized by the gland, it is quickly released into the blood (Tamura et al., 2013). Melatonin as a signalling molecule has many functions in controlling circadian rhythms and reproduction (e.g. the start of the breeding season) it also plays a role as an antioxidant molecule (Reiter, 1991, Reiter, 1993, Tamura et al., 2013, Tan et al., 2015).

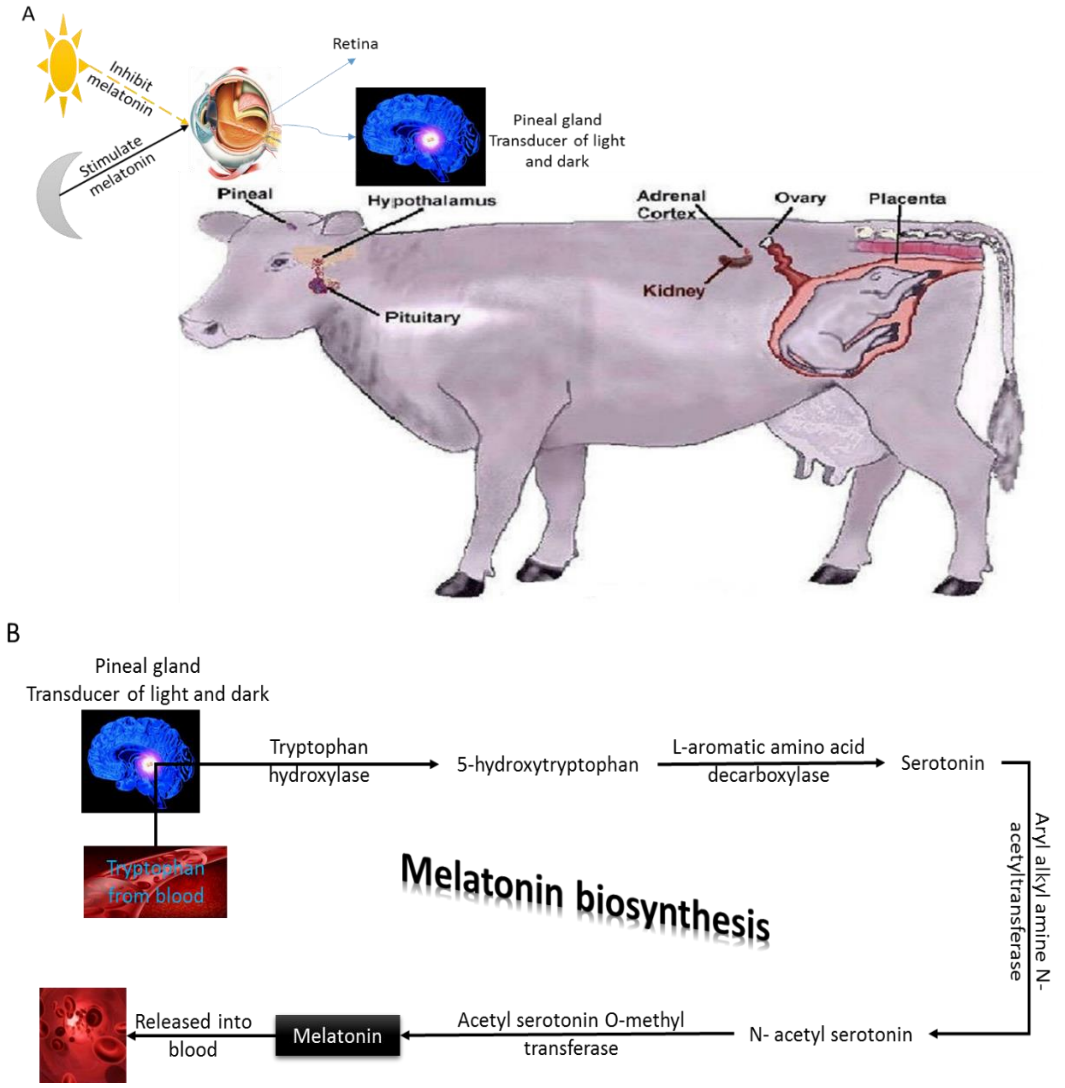


Figure 1.6 *The mechanisms of melatonin biosynthesis. Region of melatonin biosynthesis (**A**) and melatonin synthesis pathway (**B**) started by taken up the tryptophan from blood and hydrolysed by tryptophan hydroxylase to 5-hydroxytryptophan and converted to melatonin via pineal gland by the action of the four melatonin biosynthesis enzymes.*

1.5.2 Melatonin pathway

The physiological action of melatonin involves its interaction with a transmembrane G protein coupled melatonin receptors. There are two types of melatonin receptors: MTNR1A and MTNR1B. The expression levels and distribution of MTNRs vary in different tissues (Pandi-Perumal *et al.*, 2008). In humans, MTNR1A structurally has fewer amino acids and 60% sequence homology with MTNR1B (Reppert et al., 1995). Another class of MTNR is the nucleus receptor for tyrosine kinase such as orphan (ROR) and retinoid Z receptor (RZR) (Rafii-El-Idrissi et al., 1998).

The activation of melatonin receptors by melatonin appears to influence second messenger cascades by inhibiting adenylyl cyclase (AC)/ cyclic adenosine monophosphate (cAMP)/ protein kinase A (PKA)/ cAMP response element-binding protein (CREB) cascade (Fustin et al., 2009). Further credence to the role of melatonin, is its lipophilic and hydrophilic character. Therefore, it can move across all cell membranes and easily passes into subcellular compartments and reaches mitochondria and nuclei, thereby increasing mitochondrial respiration and ATP synthesis (Leon et al., 2005). The free passage of melatonin into cells suggests interaction with intracellular enzymes, proteins and transporters and generate independent signal or with dependent receptor pathways (Figure 1.7).

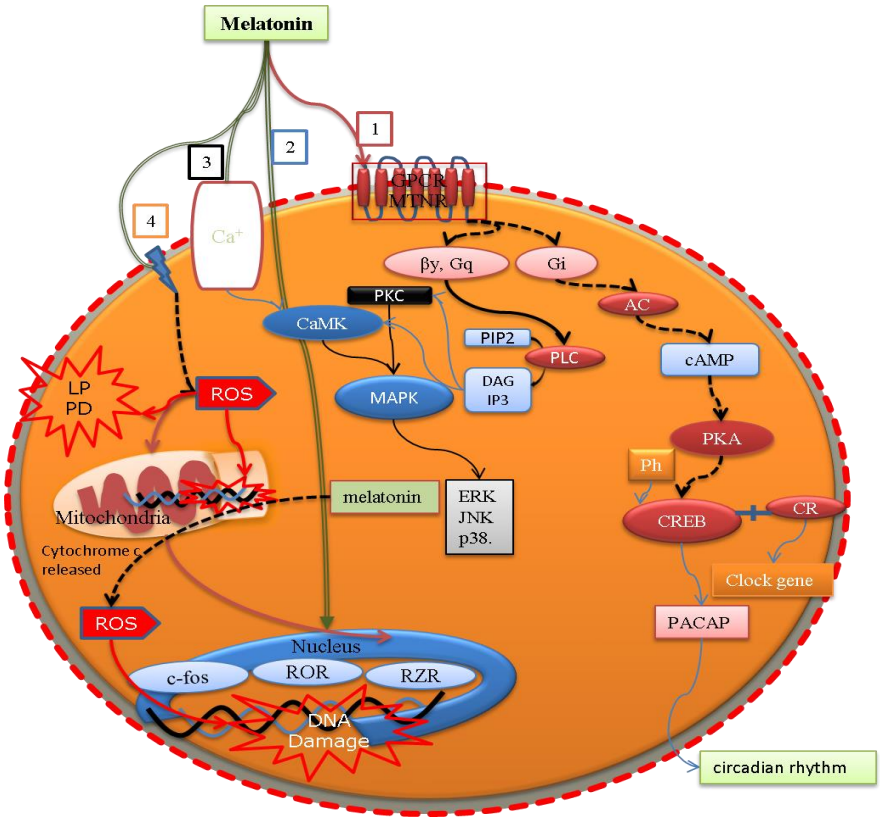


Figure 1.7 *Proposed melatonin pathway. The physiological action of melatonin involves its action through GPCR receptor (1) nucleus receptor (2), Ca channel (3) and can across cell membrane (4) to affect cell functions. Adenylyl cyclase (AC), cyclic adenosine monophosphate (cAMP), Protein kinase A (PKA), cAMP response element-binding protein (CREB), pituitary adenylyl cyclase activating peptide (PACAP), phospholipase C (PLC), phosphatidylinositol (PIP2), diacylglycerol (DAG), inositol 1,4,5-triphosphatase (IP3), calmodulin kinases (CaMK), mitogen-activated protein kinase (MAPK) reactive oxygen species (ROS), long photoperiod day (LPPD) reviewed by (Chan and Wong, 2013). Solid line mean increase and dashed line mean reduce.*

1.5.3 Effect of melatonin on the oestrous cycle

Several studies have investigated the effects of melatonin on the oestrous cycle of different animals; ewe (Waller et al., 1988), cat (Schäfer-Somi, 2015), mouse (Bondi et al., 2014), buffalo (Ramadan et al., 2016). A previous study reported that melatonin sustained the activity of cyclic breeding in anoestrous ewes (Waller et al., 1988). Exposure of cats to 24 h light induced ovarian follicular waves (Leyva et al., 1988) while in cats the suppression of cyclicity by melatonin administration is widely accepted (Schäfer-Somi, 2015). Also, in cows reports indicate that most of cows have a higher incidence of oestrus at night (Pinheiro et al., 1998). Furthermore, melatonin treatment at 220 days of gestation reduced the number of days "open" in the subsequent postpartum period in dairy cows (Garcia-Ispierto et al., 2013).

The suppression of ovarian function or irregular menstrual cycles can be achieved by melatonin treatment (Miyauchi et al., 1992). It has been suggested that night shift workers have more irregular cycles than day time workers. They demonstrated similar concentrations of FSH and LH between night and day shift but melatonin levels varied (Miyauchi et al., 1992). The concentration of melatonin in ovaries was higher during proestrous at mid-night than other days of the oestrous cycle. Also it was higher in preovulatory follicles than small follicles suggesting that melatonin may have an important role in the ovulation processes (Tamura et al., 2013).

1.5.4 Effect of melatonin on hypothalamus pituitary axis

There is no doubt that melatonin serves more than one purpose inside the body, particularly in the reproductive system. This is possibly due to melatonin's ability to interfere with the hypothalamic pituitary axis. The action of melatonin on the reproductive system is regulated by GnRH and perhaps depends on cAMP and calcium signalling in the hypothalamus (Vanecek, 1995). Melatonin significantly reduced the mRNA expression level of *GnRH receptor* in human GCs (Woo et al., 2001). Melatonin stimulated GnRH secretion in human (Cagnacci et al., 1995a) though other

studies have reported no effect of melatonin on pituitary/gonadal hormone (Rajaratnam et al., 2003).

Exogenous melatonin administration during the menstrual cycle induced FSH and LH secretion during the follicular phase in responses to GnRH, whereas no effect of melatonin was seen during the luteal phase (Cagnacci et al., 1995a). *In vitro*, melatonin treatment upregulate the expression level of *LH* receptor in sheep GCs (Fu et al., 2014). In a previous study, increased photoperiod increased circulating LH concentrations in pubertal heifers (Critser et al., 1987). However, in buffalos, melatonin implantation reduced plasma LH production (Ramadan et al., 2014).

It has been reported that prolactin levels are increased during summer but reduced during winter, in contrast to melatonin secretion which showed the opposite trend (Tenorio et al., 2015). In heifers, prolactin concentrations were increased during long photoperiod (Critser et al., 1987). Also implantation of melatonin during pregnancy decreased plasma prolactin concentrations (Garcia-Ispierto et al., 2013).

1.5.5 Effect of melatonin on steroid hormone

Melatonin could enhance steroid hormone production by acting as a multifunctional molecule with a role as an antioxidant as well as its role in signalling pathway. Melatonin effect on steroidogenesis are shown in (Table 1.3)

1.5.5.1 Oestrogen

The effect of melatonin on the E_2 level could be an essential factor in regulating follicular development and ovulation. In culture, melatonin did not affect E_2 production by rat GCs (Nakamura et al., 2014), hamster GCs (Tamura et al., 1998) or ovine GCs (Baratta and Tamanini, 1992). Additionally, in cancer cells melatonin is considered as an antioestrogen, due to a reduction of aromatase activity, aromatase mRNA expression and cAMP (González et al., 2007, Martinez-Campa et al., 2009). However, melatonin increased E_2 production in human GCs (Bodis et al., 2001). The expression *CREM* and cytochrome *P45017A1* genes in rat ovarian tissue was downregulated by melatonin treatment (Lima et al., 2015).

1.5.5.2 Progesterone

An outcome of ROS imbalance during ovulation is oxidative stress and this could lead to reduce P_4 production by newly formed luteal cells (Sugino et al., 1999, Fernando and Rombauts, 2014). Progesterone production by cultured human and bovine GCs was increased in the presence of melatonin (Webley and Luck, 1986).

Progesterone secretion by cultured GC was not affected directly by melatonin. However, melatonin reversed the inhibitory effect of hydrogen peroxide (H_2O_2) on human GCs (Taniguchi et al., 2009, Taketani et al., 2011) and the inhibitory effect of BMP-6 on P_4 production in the rat (Nakamura et al., 2014). Also, human GCs treated with melatonin and hCG increased P_4 accumulation (Woo et al., 2001). Melatonin reduced P_4 production by hamster TCs, whereas there was no effect of melatonin on GCs (Tamura et al., 1998). Maganhin et al. (2013) demonstrated that the density of P_4 receptors in ovaries during proestrus was lower in pinealectomized rats than both control and pinealectomized rats treated with melatonin during the night for two months.

1.5.6 Effect of melatonin on ovarian function

Melatonin concentration in the follicular fluid and its role in ovarian tissue have been described most extensively in human. Significantly higher concentrations of melatonin are found in human follicular fluid than in the plasma (Brzezinski et al., 1987, Nakamura et al., 2003). This suggests that melatonin presumably plays an important role in regulation of ovarian function. The higher melatonin concentration in follicular fluid apparently results from transfer from the blood into the follicle and/or by ovarian synthesis. Melatonin concentration in ovarian follicular fluid of human (Tamura et al., 2008b) and cat (Wurtman et al., 1964) increased with melatonin administration. On other hand, *in vitro* study showed that melatonin can be produced by ovarian cells (Kim et al., 2013).

1.5.6.1 Follicular development

The concentration of melatonin in immature smaller follicles is lower than larger pre-ovulatory follicles. Furthermore, anti-oxidant capacity in large

mature follicles was also higher than small follicles (Nakamura et al., 2003). The mechanisms of follicular selection are dependent on the transfer of dependency from FSH to LH, which is probably linked to the expression of mRNA encoding *LH* receptors and *HSD3B* in the GCs (Webb et al., 2003). Interestingly, melatonin has been reported to increase the expression of *LH* receptors in GCs (Woo et al., 2001). Maganhin et al. (2013) indicated that melatonin had a role to maintain proper function of follicles in rats. Another study reported that melatonin interacts with FSH to promote folliculogenesis in goats and maintain the viability and ultrastructural integrity of cultured pre-antral follicles (Rocha et al., 2013).

In buffalo heifers during seasonal anoestrus, melatonin increased the diameter of large follicles and reduced plasma P_4 and LH production (Ramadan et al., 2014). Melatonin function could be dependent on the age of follicle, as it has been shown *in vitro* that melatonin reduced steroid hormone production in pre ovulatory hamster follicles (Tamura et al., 1998) and increased P_4 production after the ovulation processes had been stimulated in mice (Adriaens et al., 2006).

With respect to the ovulation process, prostaglandin E2 is an important factor for successful ovulation (Clark and Myatt, 2008). Melatonin treatment has been reported to increased prostaglandin E2 in rat gastric mucosa (Cabeza et al., 2003) and rat oesophageal tissue (Konturek et al., 2007). The biochemical and physiological events occurring during ovulation are an inflammatory processes (Espey, 1980). Therefore, high production of potent antioxidants is needed to protect the ovum until fertilization and luteinizing cells during CL formation. The increased melatonin concentration at the time of ovulation in preovulatory follicles and the synthesis of melatonin by the ovarian cells may protect the ovum and luteinisation processes of follicular cells from the oxidative stress during the ovulation processes.

1.5.6.2 Granulosa / theca cell function

Both membrane and nuclear melatonin receptors have been identified in ovary (Wang et al., 2012, Tamura et al., 2013, Reiter et al., 2013). Some actions of melatonin in the ovary probably involve the conventional

membrane receptors. These receptors have been identified in both the GCs and TCs, which are found in mature follicles and CL, indicating that melatonin acts directly on the ovary (Yonei et al., 2010). Melatonin receptors (MTNR1A and MTNR1B) were localized in bovine granulosa cytoplasm and nuclear membrane (Wang et al., 2012).

Melatonin synthesis by ovarian GCs, including cumulus oophorous cells and oocytes has been reported (Kim et al., 2013, Reiter et al., 2014). Melatonin has been showed to protect ovine GCs integrity; by reducing mitochondrial dysfunction, lipid peroxidation, DNA damage and apoptosis through the reduction of oxidative stress (Tanabe et al., 2015). It has been demonstrated that melatonin has protective effect on ovine GCs under heat stress. It reduced the proportion of the apoptotic cells and down regulated the expression of apoptotic gene *P53* (Fu et al., 2014).

Melatonin altered steroidogeneses through altering cAMP level. Rats exposed to melatonin treatment showed downregulation of *CREB*, which acts through *cAMP* and *CYP17A* gene expression in rats (Lima et al., 2015). The increase in *cAMP* by FSH was not affected by melatonin treatment, whereas melatonin inhibited or reversed the reduction of *cAMP*, *STAR*, *P450scc* and *HSD3B* by BMP-6 in GCs cultured for 48 h in serum free conditions (Nakamura et al., 2014). Melatonin has been shown to increase MAPK activity in human GCs and increase Elk-1 phosphorylation (Woo et al., 2001). However, at high melatonin concentrations, MAPK activity was inhibited. It has been shown that sustained MAPK activity increased cell differentiation and transient MAPK activation leads to cell proliferation (Heasley and Johnson, 1992). Furthermore, melatonin has been reported to increase tissue oxygenations (Hlutkin and Zinchuk, 2008) and increased O_2 concentration stimulate stem cells differentiation (Ivanovic, 2009). Whilst considerable efforts have been directed towards melatonin as a potent antioxidant, enhancing the differentiation cascade and tissue oxygenation could be crucial during the ovulation processes and CL development after ovulation.

1.5.6.3 Corpus luteum

Several reports have indicated the expression of melatonin receptors in luteal tissue in the human, rat (Soares et al., 2003) and equine (Pedreros et al., 2011). In humans, melatonin treatment protects luteinized GCs from the oxidative stress and melatonin improved P_4 production in woman with defective luteal phases (Taketani et al., 2011). It has been reported that during out of season breeding, melatonin had the ability to maintain CL after insemination in buffalo heifers (Ramadan et al., 2014).

Presumably, melatonin's role on ovarian cells depends on several factors such as incubation period and culture condition (Table 1.3). According to its signalling pathway melatonin reduced cAMP, and oxidative stress. Therefore, melatonin could reduce steroidogenic hormone as it reduced cAMP and increased P_4 through reduced oxidative stress and enhanced differentiation. Prostaglandins and ROS interact synergistically in the luteolytic process (Cruz et al., 2014). As prostaglandin F2α increased ROS generation and induced luteal cell apoptosis (Tanaka et al., 2000). Consequently, the application of melatonin for CL development may be important to reduce the negative effect of ROS and support luteal development.

1.5.7 Effect of melatonin on establishment of pregnancy

1.5.7.1 Conception rate

Reduction of oxidative stress by melatonin during fertilization increased pregnancy rates. Tamura et al. (2008b) suggested that melatonin improved oocyte maturation and enhanced fertility rates. In anoestrous buffalo, melatonin implantation increased the percentage of CR about threefold higher than the control group (Ramadan et al., 2014). Also in beef cattle melatonin treatment (4mg/100 kg) during the postpartum period reduced days open and increased CR during the first ovulation (Wettemann et al., 1994).

1.5.7.2 Embryonic development

Embryo production *in vitro* plays an indispensable role in bovine production system and also in human reproductive technology. It has been reported throughout the world that the transferrable bovine embryos *in vitro* were augmented in the last decade (Stroud and Callesen, 2012). However, embryo development *in vitro* cannot produce embryos of a quality similar to those *in vivo* (Lonergan et al., 2006). Several researchers have drawn attention to the role of melatonin in embryonic development. Supplementation of melatonin in embryo culture media improved embryonic growth rate at various stages especially, cleavage rate and blastocyst rate in mice (Ishizuka et al., 2000, Bahadori et al., 2013), pigs (Rodriguez-Osorio et al., 2007) and cattle (Papis et al., 2007, Wang et al., 2014). However, no effects of melatonin on bovine embryo development *in vitro* have been reported (Takada et al., 2010, Tsantarliotou et al., 2010).

The gene expression of melatonin receptors in bovine embryos produced *in vitro* appeared to enhance bovine embryonic development *in vitro* and *in vivo* (Wang et al., 2014). The ROS outcome was reduced by melatonin (Tamura et al., 2013) and the production of ROS during ovulation may be reduced maturation of the oocyte, development of the embryo and luteinisation of GCs. *In vitro*, melatonin has been reported to improved bovine oocyte maturation through upregulation of oocyte maturation related genes (Tian et al., 2014). However, the role of melatonin on bovine embryo development *in vitro* differs depending on the O_2 concentration. The beneficial effect of melatonin at atmospheric O_2 level was demonstrated in bovine embryo development, whereas it reduced embryos reaching the blastocyst stage at 7% O_2 (Papis et al., 2007).

Table 1.3 *Effects of melatonin on follicular cells.*

References	Sample	Species	Culture time	Melatonin dose	Effect
Webley and Luck (1986)	Granulosa cells	Human & Bovine	1-4 d	100-400 pg/ml	↑P_4
Baratta and Tamanini (1992)	Granulosa cells	Ovine	9 d	20pg-20 ng/ml	↑P_4, ~E_2
Yie et al. (1995)	Granulosa cells	Human	1-7 d	10 -1000 pg/ml	↑P_4
Woo et al. (2001)	Granulosa cells	Human	1-5 d	2.3 pg– 23 ng/ml	↑P_4 ↑LHR,MAPK
Bodis et al. (2001)	Granulosa cells	Human		2.3 ng-230 µg/ml	↑E_2, ~↓ P_4, ↓FSH & ↓LH
Tanavde and Maitra (2003)	Follicles	Pig	30 h	10-100 ng/ml	↑P_4&A ~E_2,↓CYP11A,17, ~19
Adriaens et al. (2006)	Follicles	Rat	12 d	230 pg- 460 µg/ml	↓ IVM ↑P_4&A ~E_2
Taketani et al. (2011)	Granulosa cells	Human	2 d	1- 100 µg/ml	↑P_4
El-Raey et al. (2011)	Cumulus cells	Bovine	12 d	10-100 ng/ml	↑nuclear maturation CC ↑Expansion
Wang et al. (2012)	Granulosa cells	Bovine	1-3 d	400-2000 pg/ml	↑P_4, ~E_2,↓apoptotic rates
Takada et al. (2012)	Cumulus cells	Bovine	8 d	230 pg/ml	↓ DNA damage
Wang et al. (2014)	Cumulus cells	Bovine	8 d	2.3 pg-230 µg/ml	↑IVM, Gpx4, SOD1, Bcl-2.↓P53, BAX, and Caspase3 genes
He et al. (2016)	Granulosa cells	Pig	2 d	0.001-10 ng/mL	↑E_2, ↓ P_4

D=days, h=hour, ↑= increase, ↓=decrease, ~ =no effect. E_2=oestradiol, P_4=progesterone, A= androstenedione IVM=in vitro maturation, Bcl2=anti apoptotic gene, P53, BAX, and Caspase-3, =apoptotic gene, Gpx4= Glutathione peroxidase 4, SOD1= Superoxide dismutase 1, LH=luteinizing hormone, LHR= LH receptor, FSH= follicle stimulating hormone, CYP11A,17, 19 =Cytochrome P450 family 17,19.

1.5.7.3 Uterine function

The concentration of melatonin in pregnant woman appeared to be increased (Carlomagno et al., 2011). Melatonin concentration in pregnant woman was higher up to 35 weeks and suddenly reduced at week 40 until delivery and remains low during the postpartum period compared to non-pregnant woman (Yadav et al., 2008). The authors suggested that the relationship between melatonin and steroid hormone might be essential for maintaining pregnancy and signalling parturition (Yadav et *al*., 2008). In an ovine model intrauterine infusion of melatonin resulted in an increase in the placenta fetal weight ratio and increased umbilical blood flow (Lemley et al., 2013). In non-photoperiodic animals, Dair et al. (2008) reported that melatonin plays a role in the endometrium and improves implantation of rat embryos. In mice thicker endometrium, higher uterine gland density and upregulation of *HB-EGF* (an important gene for implantation) were increased by melatonin treatment (He et al., 2015). The melatonin generating system and melatonin receptors in human placenta has been established throughout the pregnancy period (Soliman et al., 2015). Also in human, melatonin supplementation increased the implantation rate (Kim et al., 2013). Placenta is often a site of ROS production and melatonin as a direct free radical scavenger and indirect antioxidant effect and has an essential function in fetal development (Reiter et al., 2014).

1.5.8 Antioxidant role of melatonin

Oxidative stress results from a faster rate of production of reactive forms of O_2 than can be safely neutralized by antioxidants leading to health disorders (Sies, 1997). Reactive oxygen species are generated as a result of cellular metabolism (electron transport), several oxidase enzymes (NADPH and cytochrome P450 oxidase) and the reaction of metal catalase. Low concentration of ROS may be beneficial for regulating cellular function (intracellular messaging, immunity, cell proliferation and apoptosis), whereas high amount of ROS causes cellular damage (expression of gene, enzyme, protein and DNA oxidation). The compound which is the first line of defence against ROS production is superoxide dismutase (Cu/Zn SOD

(SOD1) and MnSOD (SOD2)). SODs can dismutase superoxide anions to H_2O_2 then catalysed to H_2O by catalase (Matés, 1999, Luchetti et al., 2010, Fear and Hansen, 2011, Emamgholipour et al., 2016). It has been reported that oxidative stress and the activity of SOD in dairy cattle were increased during the hot summer season (Bernabucci et al., 2002). Additionally, oestrogen induced oxidative damage to DNA in liver and kidney was attenuated by melatonin treatment (Karbownik et al., 2001).

Recently, melatonin has received a great deal of attention in the prevention of oxidative stress. Melatonin, besides its functions in circadian rhythms, has an effect on oxidative stress pathways. As a potent antioxidant and free radical scavenger, melatonin cascade reaction distinguished from other antioxidants. Melatonin can neutralize numerous O_2 toxins and has more efficient capacity to scavenge ROS than the classic antioxidants (Tan et al., 2003). The reasons why melatonin is considered as a potent antioxidant is the secondary (cyclic 3-hydroxymelatonin (Cyclic3OHM)) and tertiary (N1-acetyl-N2-formyl-5-methoxykynuramine (FAMK)) products of melatonin metabolism, which have the capability to scavenge and donate electrons to neutralize free radicals (Figure 1.8). The above cascade marked melatonin as a unique antioxidant and cascade action (Tan et al., 2003, Tan et al., 2015). Melatonin was shown to detoxify hydroxyl radical (·OH), hydrogen peroxide (H_2O_2), superoxide anion radical ($O2^{\cdot -}$), nitric oxide (NO·), peroxynitrite anions ($ONOO^-$) and singlet O2 ($1O2$) (Reiter et al., 2007).

It seems that perturbation of the normal level of melatonin or disturbance of the dark-light environment could compromise reproduction. The widespread use of manufactured light interrupts natural period of darkness, especially in dairy cattle and, as reported previously, increased light enhances milk production. Melatonin's role in circadian and circannual rhythms as well as an antioxidant (Lincoln, 2006, Johnsson, 2008) could be the reason for fertility reduction in dairy cattle particularly during long photoperiods.

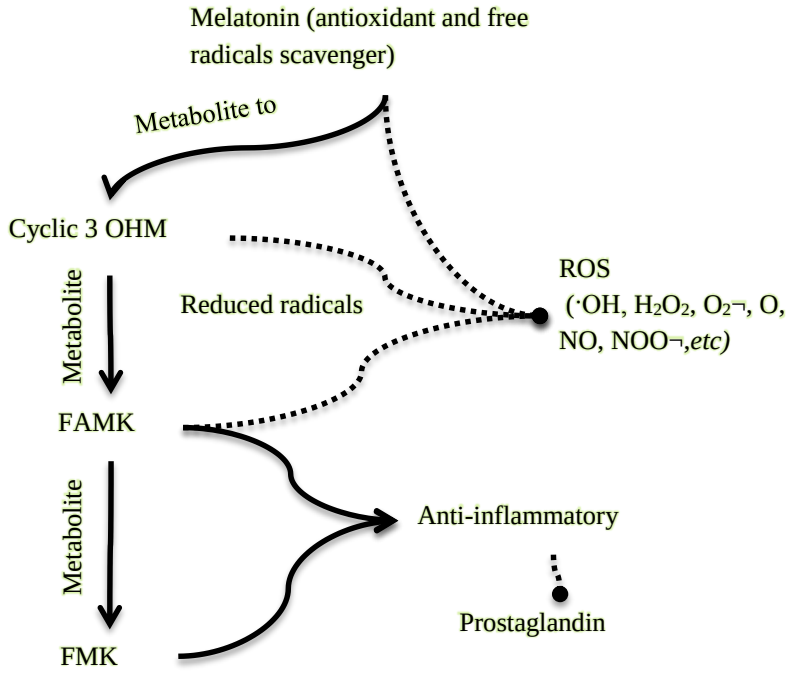


Figure 1.8 *Melatonin and it metabolite cascade reaction interact with ROS. Dot line mean reduce, solid line mean stimulate. Cyclic 3-hydroxymelatonin (Cyclic3OHM), N1-acetyl-N2-formyl-5-methoxykynuramine (FAMK), hydroxyl radical (·OH), hydrogen peroxide (H_2O_2), superoxide anion radical ($O_2^{\cdot -}$), nitric oxide ($NO^{\cdot}$), peroxynitrite anions ($ONOO^-$) and singlet O2 ($1O_2$)*

Table 1.4 *Melatonin concentrations within the body fluid reported in the previous literature.*

Reference	Species	Melatonin Concentration pg/ml	Sample	Time
Brzezinski et al. (1987)	Human	36.5	Follicular fluid	
		10	Serum	
KivelÄ et al. (1989)	Human	38	Serum	Night
		7		Day
		23	amniotic fluid	Night
		14		Day
Yie et al. (1995)	Human	213	Follicular fluid	Dark Season
		138		Light season
Kim et al. (2013)	Human	11.7 to 77.7	GCs Culture	
Martin et al. (1983)	Bovine	6 to 58	Plasma	Day
		44 to 328		Night
Wettemann et al. (1994)	Bovine	65 to 106	Plasma	

1.6 Role of oxygen tension in culture

Oxygen plays an important role in the processes of ovarian follicular development. It is essential for cellular nutrition, growth, metabolism and maintenance. Analyses of O_2 concentration in follicular fluid and the COC throughout follicle development have been explored by several researchers (Van Blerkom et al., 1997, Redding, 2007, de Castro e Paula et al., 2008, Clark and Stokes, 2011, Li et al., 2013a). They documented that normoxia or O_2 concentration inside tissue is much lower than the atmospheric O_2 (20-21% O_2). In actual fact, the O_2 concentration inside the body is varies from 1 to 12% and is dependent on the distance of the tissue from arterial blood vessels (Bates, 2012).

For many decades, somatic cells have been cultured in atmospheric O_2 conditions but new applications are required to allow *in vitro* systems to mimic those *in vivo*. It has been demonstrated that culturing cells in low O_2 could provide beneficial results *in vitro* (Bates, 2012). Group of researchers revealed that culturing immune cells under atmospheric O_2 resulted in signalling against uncontrolled inflammation, whereas cells at 5% O_2 operated in a healthy state (Atkuri et al., 2007). The O_2 concentration in the follicle may be affected by the size of follicle, degree of vascularization, blood supply and haemoglobin (HB) level.

1.6.1 Haemoglobin role in oxygen transportation

Haemoglobin is the main functional gas binding molecule in erythrocytes (Rahbar, 1983) carrying oxygen from lung to target tissue via the circulation. Haemoglobin is found in cattle with alleles HB alpha (HBA) and beta (HBB) (Bangham and Blumberg, 1958, Naik et al., 1969, Pieragostini et al., 2010). Haemoglobin is not only produced by erythrocytes but also in other tissues (Saha et al., 2014). In microarray study of mouse COCs both alpha and beta HB were expressed *in vivo* but not *in vitro* (Kind et al., 2013). Also, both HB alleles are expressed in human GCs and COCS (Brown et al., 2015). Furthermore, hormonal regulation of HB transcripts and upregulation around the time of ovulation were reported. Importantly, the discovery of *HB* genes in follicular cells could make these cells more

tolerant to low O_2 (Thompson et al., 2015) as the ovarian blood supply reduces towards ovulation (Wise et al., 1982).

Another factor that could interfere with HB and oxygenation is melatonin. It is clear that ovarian follicles are at a low O_2 level (Basini et al., 2004) with a high melatonin concentration (Nakamura et al., 2003). It has been reported that melatonin interacts with HB to increased tissue oxygenation through haemoglobin-oxygen affinity in blood cells (Hlutkin and Zinchuk, 2008). It seems there are strong correlations between melatonin and O_2 concentration in the follicular fluid. There are still gaps in the published literature about the role of O_2 tension on long term bovine GCs culture systems particularly in term of oxidative stress and it would be useful to understand *HB* expression. In a study that assessed oxidative stress in cultured swine GCs under O_2 tension it was demonstrated that in GCs cultured at 5% and 1% O_2 reduced ROS (both O_2^- and H_2O_2) generation and increased scavenger enzyme activity (superoxide dismutase and peroxidase) catalase (Basini *et al*., 2004).

1.6.2 Oxygen level in ovarian follicles

The concentration of O_2 supplied to GCs and oocytes depends on the antral follicle size. The concentration of O_2 in the follicle decreases as the follicle develops (Basini et al., 2004, De Rensis et al., 2008). This is not unexpected, because the vasculature is limited to the theca cells (Fraser, 2006). Increased blood flow during follicular maturation is inadequate to bridge the growing distance between the blood vasculature and the follicle centre (Fischer et al., 1992). The mean O_2 concentration within body is much lower than atmospheric O_2 level (Table 1.5). This could mean that cells within the follicle grow in a low O_2 environment. It has been reported that the physioxia is lower (1.5 to 11%) than atmospheric O_2 (20%) (Carreau et al., 2011).

Table 1.5 *Oxygen (O_2) concentrations in the atmosphere and within the body.*

Tissue	O_2 Level %	References
Atmosphere	20-21	
Lung Liver, Hearts and circulation	4.0-14	Reviewed by (Ivanovic, 2009)
Oviduct, uterus	2-8	(Fischer and Bavister, 1993)
Follicle	4-9 (1) 2-4 (2)	1. (de Castro e Paula et al., 2008). 2. (Van Blerkom et al., 1997)
Eyes	1-5	(Yu and Cringle, 2005)
Brain	0.5-7	(Hemphill et al., 2005)
Bone marrow	0.0-4	(Chow et al., 2001)

1.6.3 Effect of oxygen tension on steroid hormone production

1.6.3.1 Oestrogen

Oxygen concentration has a significant effect on cytochrome P450 enzymes involved in steroidogenesis (Koos and Feiertag, 1989). The key enzyme for oestradiol biosynthesis is aromatase (CYP19A), which is responsible for the conversion of androgen to E_2. It has an essential role in maintaining the homeostatic balance between hormones (Simpson et al., 1994). The first study culturing bovine GCs in 5% O_2 was done in 1994. In that study, E_2 production at 5% O_2 increased after 2 to 4 days of culture compared to atmospheric O_2 (Roberts and Echternkamp, 1994). Another study reported that E_2 production increased by culturing sheep ovarian follicles under low O_2 tension (5% O_2) (Cecconi et al., 1999). The upregulation of Vascular endothelial growth factor (*VEGF)* was seen under low O_2 levels (Jiang et al., 2011) and rapid stimulation by E_2 through Hypoxia-inducible factor (HIF)-1 and oestrogen receptors (Koos, 2011) indicated that E_2 secretion under low O_2 conditions could be related to angiogenesis. The O_2 environment for follicular cells in culture is an integral part closely associated with steroid production and gene regulation.

1.6.3.2 Progesterone

During CL development O_2 level is influenced in steroidogenesis and angiogenesis (Nishimura and Okuda, 2010). In a study designated to investigate the role of O_2 on P_4 production and relative gene expression, it was found that P_4 concentration was reduced in cells cultured at 1% O_2. In addition the expression of *STAR*, *CYP11A1* and *HSD3B* were downregulated

by hypoxic conditions, suggested that the inhibition of steroidogenesis under low O_2 could be controlled via the inhibition of protein kinase A (PKA) activity (Jiang et al., 2011). The P_4 secretion by luteal cells cultured under 3% O_2 was reduced in LH and non LH treated groups (Nishimura et al., 2006). In addition, the expression of cytochrome *P450* mRNA was inhibited by low O_2. Progesterone production by non-luteinized GCs was not affected by O_2 tension, whereas P_4 production by luteinized GCs (stimulated by insulin and forskolin or combination) increased at low O_2 concentration. Furthermore, 10% O_2 increased *STAR* mRNA as well as STAR and HSD3B protein in luteinized GCs (Fadhillah et al., 2014).

Cumulatively, there is strong evidence of an important role of O_2 concentration in steroidogenesis. Therefore, it is important to address the effect of melatonin and temperature on the GCs function under physiological O_2 concentration to find the mechanisms of seasonal effect on steroid production.

1.6.4 Effect of oxygen on ovarian function

Follicles and GCs, around the time of ovulation, are in a hypoxic environment. Furthermore, recently formed CL, expresses high levels of hypoxic inducible factor -1 α (HIF) (Nishimura and Okuda, 2010) transcript response to low O_2 level.

1.6.5 Follicular development

Oxygen concentration *in vitro* that closely mimic that *in vivo* seemed to be a vital parameter in establishing an optimal culture system. Recently, many efforts have been focused on the role of O_2 tension in follicular growth (Gook et al., 2014, Connolly et al., 2015, Thompson et al., 2015). Atmospheric O_2 (20% O_2) concentration reduced follicular growth (Van Blerkom et al., 1997), antrum formation (Silva et al., 2010) and healthy COCs (Cecconi et al., 1999). The viability of growing follicles in serum free culture increased significantly under 5% O_2 compared to atmospheric O_2 (Gook et al., 2014). In contrast, atmospheric O_2 increased the follicular growth rate compared to those cultured at 5% O_2 (Connolly et al., 2015). Inconsistency of results among researchers may possibly be due to

variations in culture systems or due to the absence of factors in the culture system. Preantral follicles of sheep cultured in the presence of 1 µg/ml FSH, increased E_2 concentration significantly at 5% O_2 which reached high levels on day 4 of culture. However, when culturing in air the culture time was unable to increase E_2 levels (Cecconi et al., 1999). *In vivo,* comparison of the O_2 concentration in follicular fluid from preovulatory follicle of dairy cows exposed to the heat stress (de Castro e Paula et al., 2008) concluded that there was no effect of heat stress on follicular O_2 level and steroid production.

1.6.5.1 Granulosa /theca cells function

Hypoxia-inducible factor (HIF) activity is expressed in GCs and reached a peak level around the time of ovulation *in vivo* and in *vitro* (Tam et al., 2010). The proliferation of luteinized GCs could be induced by low O_2, as proliferating cell nuclear antigen (PCNA) was upregulated in low O_2 (Jiang et al., 2011). The above factors indicated that the GCs may grow and proliferate more when O_2 tension is reduced.

1.6.5.2 Corpus luteum

In view of the finding that hypoxia stimulates angiogenesis by increasing the expression of angiogenic factors in cultured GCs. It has been resulted that hypoxic conditions activate HIF1A which is a factor that promotes VEGFA to induce angiogenesis during luteal establishment. After raptured ovarian follicle, low O_2 could contributed to develop CL vascularization in cattle (Nishimura and Okuda, 2010). The origination of luteal cells and subsequent CL formation and luteolysis could be regulated by O_2 concentration (Jiang et al., 2011). There is also increasing support for the suggestion that low O_2 contributes to vascularization and may be critical in follicular development, ovulation and subsequent CL formation. Exposure to low O_2 not only regulates HIF but also other transcription factors and cellular pathways such as translation (Kenneth and Rocha, 2008) .

1.6.6 Embryo development

Oxygen tension during *in vitro* production of embryo has been an important area of research. Recently, the *in vitro* production of bovine embryo has been increased dramatically. However, the overall outcome or embryo development are still not ideal (Perry, 2014). Oxygen levels in oviduct range from 2 to 5% O_2 and within the uterus O_2 concentration is about 5% (Table 1.5). Thus embryos develop under low O_2 *in vivo* compared to atmospheric O_2 especially during preimplantation (Fischer and Bavister, 1993). Recently, reduction of histone markers (*H3K4me2* and *H3K9me2*) were demonstrated under low O_2 tension on embryo culture (Gaspar et al., 2015).

1.7 Hypothesis and objectives

The overall hypothesis tested by this research is that season could affect bovine reproductive function in a temperate climate zone.

The objective of Chapter 2 was to understand the effect of season on reproductive performance in a dairy herd maintained within a temperate climate and to investigate the effects of temperature and THI at important physiological time points around mating on conception rate.

The objective of Chapter 3 was to investigate the influence of season on patterns of follicle development, CL development and function and the incidence of multiple ovulations.

There are limited data on the effects of either melatonin or O_2 tension on GC function and so the objective of Chapter 4 was to examine the effects and interactions of melatonin and O_2 tension on the endocrinological capabilities of GCs.

It has been reported that melatonin could improve reproductive performance in heat stressed cows (De Rensis et al., 2015) by reducing oxidative stress. Therefore, the objective of Chapter 5 was to determine the influence of melatonin and O_2 tension on the negative effects of culturing GCs at elevated temperature.

Chapter 2: Is there an effect of season on the fertility of UK dairy cows?

2.1 Introduction

While cattle are not generally regarded as seasonal breeders, several researchers have documented that reproductive performance varies during seasons of the year (Badinga et al., 1993, Wolfenson et al., 1997, Wolfenson et al., 2002, Borisenkov et al., 2004, El-Tarabany and El-Tarabany, 2015). This is a particular problem in hot regions where high temperature and relative humidity reduce fertility (Al-Katanani et al., 1999, Bell, 2008). The high rate of metabolism in high producing dairy cattle makes them especially sensitive to heat stress with only 10 to 20% of cows inseminated when under heat stress were diagnosed as pregnant (Hansen, 2013). In UK, the milk production per cow has doubled during the last 4 decades. This increase causes a higher energy burden to meet lactational need and results in cows dissipating more heat making them more susceptible to heat stress (West et al., 2003, Oltenacu and Broom, 2010). Whether heat stress is a problem in the temperate climate of the UK is less clear. However, it has been reported that heat stress in UK reduced milk yield (Dunn et al., 2014), also summer can increase mastitis risk, reduced feed intake, and fertility (Ohnstad, 2008). Seasonal increased somatic cell count in Irish, England and Welsh herds have been reported by (Archer et al., 2013).

Seasonal influences on cattle fertility are associated with changes in air temperature (AT), relative humidity (RH) and/or temperature humidity index (THI) as well as photoperiod. These factors may impact on fertility through effects on impaired hormonal secretion (Khodaei-Motlagh et al., 2011), follicular development (Guzeloglu et al., 2001, Torres-Junior et al., 2008) and embryonic development (Silva et al., 2006). In contrast, some studies have claimed that the cold season could lead to fertility problems (Refsdal, 2007).

In many cases, probably the problem has been made worse by technological advances in transportation and management, which have changed the geographical location of specific breed of cattle. Hence, altering the genetic and physiological adaptation to new environments has become increasingly important (Hansen, 2014)

While few studies have investigated seasonal effects on reproduction in the UK some effects have been observed. For example, in suckling cows a longer anoestrous period was reported in spring compared with autumn calving cows (Lamming et al., 1981) while Silva et al. (2006) reported that a higher proportion of oocytes developed to blastocysts *in vitro* in the autumn compared to the spring. In recent studies, Homer (2013) reported increased oestrous activity during the summer while Hudson (2015) reported lower pregnancy rates. Recently, it has been suggested that THI in the UK could increase in the near future through global warming (Dunn et al., 2014) highlighting the need to gain increased insight into seasonal effects in the UK.

Hypothesis

The hypothesis in the current chapter is that season and climate could affect reproductive performance of dairy cattle in the temperate UK climate. The purpose of the current research was to evaluate the impact of elevated temperature and season on reproductive performance of dairy cows at the University of Nottingham Dairy Centre.

Specific aims were to determine:

1. The effect of season of birth on time to first mating, conception and calving.

2. The influence of season on aspects of reproductive performance (conception rate, days open, calving interval).

3. The relationship between conception rate and temperature and THI at physiologically important time points.

2.2 Materials and Methods

2.2.1 Herd location, management and data collection

2.2.1.1 Experimental animals

Reproductive management data were collected during a 5-year period from January 2008 until December 2012 from the University of Nottingham Dairy Centre. All cows were housed throughout the year with access to individual cubicles with rubber mats. All cows were milked by robots on an average 2-3 times per day and managed according to normal farm practice with no researcher intervention. Cows were fed *ad lib* on a total mixed ration based on maize and grass silage (with additional ingredients such as rolled wheat, sugar beet pulp nuts, molasses, and minerals). Additional concentrates were fed during robotic milking according to yield. Pregnancy was diagnosed by ultrasound and/or rectal palpation at routine vet visits according to normal herd practice (usually from 30 – 60 days post AI).

2.2.1.2 Metrological data

Sutton Bonington is located at latitude 52.82° North and longitude 1.25° West (01.10W) at 65 metres above sea level. Data were collected from the weather station located adjacent to the dairy centre, including mean daily ambient temperature and relative humidity. This data is collected automatically as part of ongoing meteorological recording and was downloaded from the Metadata system at the University of Nottingham. Details of environmental condition and the average, minimum and maximum metrological data are been collated in Table 2.1.

2.2.1.3 Reproductive data

The record system included information on fertility and health in all cows with herdsmen and veterinarians recording; date of birth, inseminated dates, result of pregnancy diagnoses, gestation lengths, calving dates, milk production, dry off dates, gestational disorders, calving difficulty and veterinary treatments. Cows that had at least one artificial insemination were included from which an interval from calving to first service could be calculated. After calving, calf was left with cow for about 24 h then

automatic feeding machine in group pens. Cow also returned to main herd within 48 h of calve and mated at observed oestrus and/or basis of changes in activity monitors after voluntary waiting period of 45 days by genus AI Technician

The data collected consisted of records from 244 Holstein Friesian cows, covering a 5 year period (2008 to 2012) with 410 calvings and 1550 inseminations. In cows inseminated for the first time in 2008, earlier data on date of birth was also collected.

A range of reproductive parameters were determined (Figure 2.1) and analysed in relation to season and climate data. The metrological data in this study were limited to AT, RH and THI.

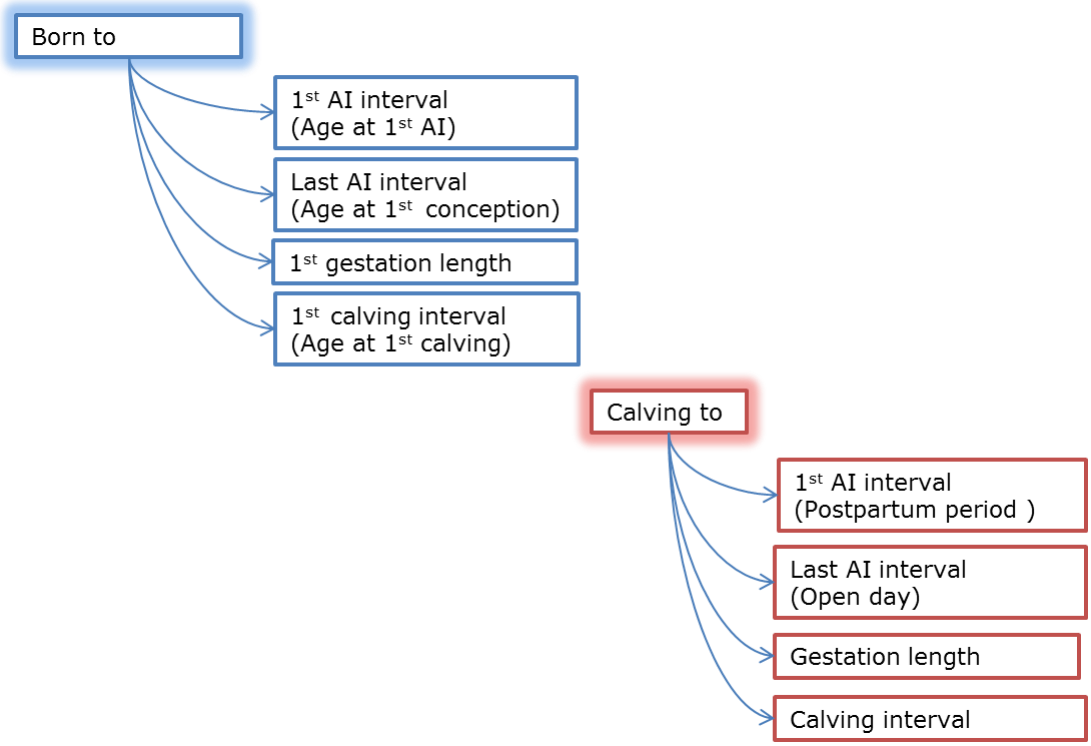


Figure 2.1 *Reproductive data recorded in the present study for analysis in relation to season. AI mean artificial insemination.*

Table 2.1 *Monthly mean, minimum and maximum meteorological data = air temperature (AT) (°C), relative humidity (RH %) and temperature humidity index (THI) values during a 5-year period from 2008 to 2012 recorded at the weather station at Sutton Bonington adjacent to the University of Nottingham Dairy Centre.*

Month	AT °C			RH %			THI		
	Min	**Mean**	Max	Min	**Mean**	Max	Min	**Mean**	Max
January	-3.0	4.4	12.3	71.5	89.4	101.6	31.5	40.9	54.1
February	-3.0	4.7	12.9	68.2	87.4	101.4	32.1	41.6	55.2
March	-1.1	6.7	12.8	62.5	81.6	101.8	35.9	45.5	55.1
April	2.0	9.2	15.7	57.6	82.5	101.7	40.8	49.4	60.2
May	5.0	12.0	20.1	59.9	80.6	101.8	44.7	54.1	68.2
June	8.4	14.6	22.1	61.2	80.9	101.7	49.5	58.2	72.0
July	12.4	16.2	23.4	61.3	80.8	101.7	55.1	60.9	74.2
August	11.3	16.3	21.3	63.1	81.5	101.2	53.5	61.0	70.5
September	8.3	14.0	20.6	60.8	82.7	101.7	49.3	57.3	69.2
October	2.1	10.9	20.3	63.4	86.3	101.7	40.3	52.1	68.6
November	-5.7	7.6	15.8	67.0	90.6	101.7	28.3	46.3	60.4
December	-8.1	3.2	11.8	73.4	90.8	101.7	23.4	38.8	53.2

Min= minimum, Max= maximum degrees during each month.

2.2.2 Classification of season

The seasons were defined as winter (December, January and February), spring (March, April and May), summer (June, July and August) and autumn (September, October and November).

To further evaluate the relationship between weather parameters and outcome of insemination the effects of temperature and THI at specific times relative to insemination (10 days before insemination, at insemination and 21 days after insemination) were investigated.

- Temperature during the 10-day period preceding insemination was selected to determine the impact on final development of the ovulatory follicle.
- Temperature at day of insemination was selected to determine the direct effect on conception.
- Temperature during the 21-day period after insemination was analysed to determine the effects on early embryonic development, luteal development and the preimplantation period.

For the purposes of analysis temperature was categorised as below (<7°C), within (7-15°C) and above (>15°C) the accepted thermos-neutral range in high producing dairy cattle.

The temperature humidity index (THI) values were calculated using the formula:

$$THI = (1.8 * AT + 32) - \big((0.55 - 0.0055 * RH) * (1.8 * AT - 26)\big).$$

The THI were grouped into four categories; <40, 40 to 49, 50 to 59 and ≥60.

2.2.3 Statistical analysis

Statistical analyses were conducted to investigate association between seasonal factors and reproductive traits. In the first analysis, reproductive traits (born to first and last insemination, age at first calving, calving to first and last insemination, service interval, calving interval and gestation length) were categorized according to season. The effect of season was then analysed by linear mixed models using GenStat (GenStat 17th edition, 2014) software. Reproductive data were included as variates, season was fixed and season within year (season/year) as a random effect. To compare the mean value

and to verify the difference among the means of seasons. Least significant differences were used to estimate significant differences between means.

In the second analysis, the initial data on conception rate was converted into percentage. A logistic regression model was then used to determine the effect of season on conception rate and the relationships between artificial insemination and conception rate during each season and month, as well as effects of temperature and THI.

Data are presented as mean with standard error of differences (SED) and least significant differences (LSD) used to estimate significant differences between means and different letters used to indicate significant differences between/among groups.

2.3 Results

2.3.1 Metrological data

Metrological data collected from 2008 to 2012 at Sutton Bonington are shown in (Figure 2.2). The minimum temperature per day was -8.1°C recorded in December 2010, whereas in July 2009 it reached the highest level of 23.4°C. Generally, the temperature and temperature humidity index (THI) were higher from May to October.

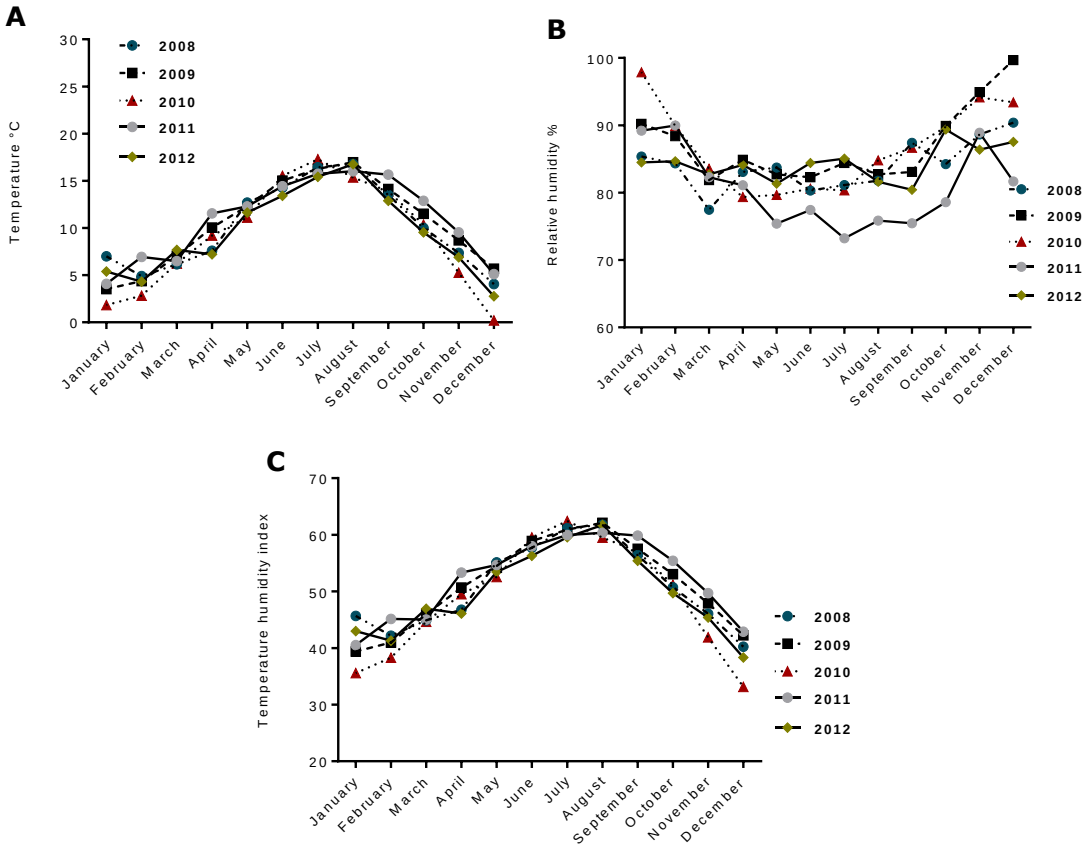


Figure 2.2 *Monthly mean metrological data. Ambient temperature (**A**), relative humidity% (**B**) and temperature humidity index (**C**) during a 5 year period from 2008 to 2012 at the weather station at Sutton Bonington, Nottingham.*

2.3.2 Effect of season of birth on parameters associated with first breeding

2.3.2.1 Age at first AI and first conception

The results show season had a significant effect on age at first insemination. Cows born in autumn and winter were inseminated 39 and 28 days earlier ($P<0.05$) than those born in summer respectively (Figure 2.3). Season also

influenced age at first conception. Cows born in autumn conceived earlier (P<0.05) than spring and summer born animals (Figure 2.4).

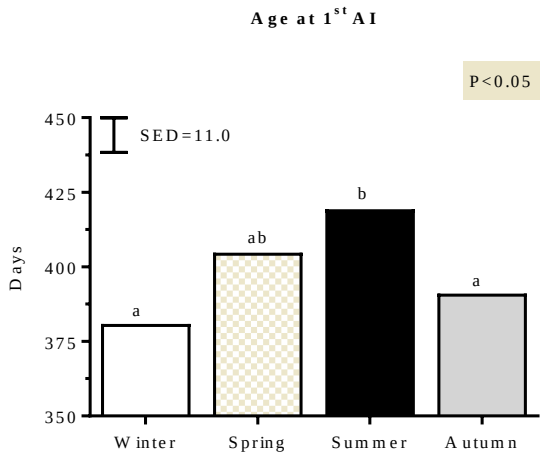


Figure 2.3 *Seasonal variation in age at first artificial insemination (AI). The data are presented as mean of winter (n=45), spring (n=57), summer (n=68) and autumn (n=36) with SED with LSD used to estimate significant differences between means (ab=P<0.05).*

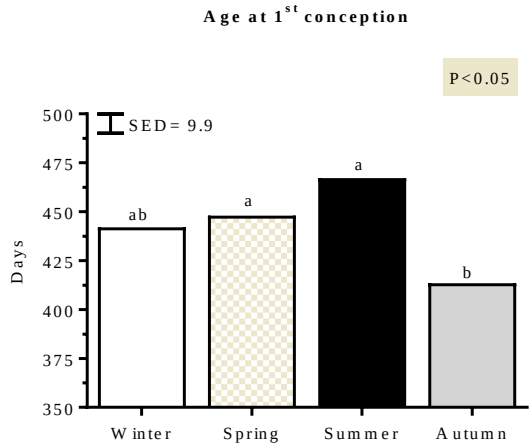


Figure 2.4 *Seasonal variation in age at first conception (Date of the last artificial insemination at which pregnancy was established). The data are presented as mean of winter (n=45), spring (n=57), summer (n=68) and autumn (n=36) with SED and LSD used to estimate significant differences between means (ab=P<0.05).*

2.3.2.2 First gestation length and age at first calving

The first gestation length for dairy heifer was 277.7; SED=2.5 days and was not affected by season of birth (P=0.30). However, the age at first calving for cows born in autumn was shorter (P<0.001) than those born in spring and summer (Figure 2.5). With regard to the effect of season on heifer fertility, the first postpartum period (calving to first AI), open days (calving to last AI) and service interval (the period from first insemination to last insemination) were not significantly affected by season (P>0.10).

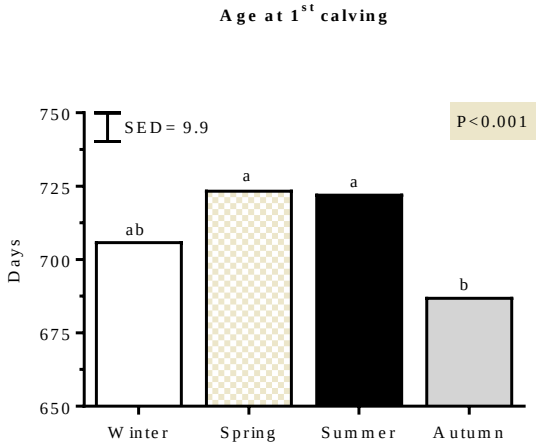


Figure 2.5 *Seasonal variation in age at first calving as affected by season of birth. The data are presented as mean of winter (n=49), spring (n=45), summer (n=39) and autumn (n=31) with SED with LSD used to estimate significant differences between means (ab=P<0.001).*

2.3.3 The relationship between calving date and fertility

2.3.3.1 Calving to 1st and last AI

The mean value (days) for the effect of season on calving to first AI (postpartum period) and calving to last AI (day open) were not affected by season (Figure 2.6).

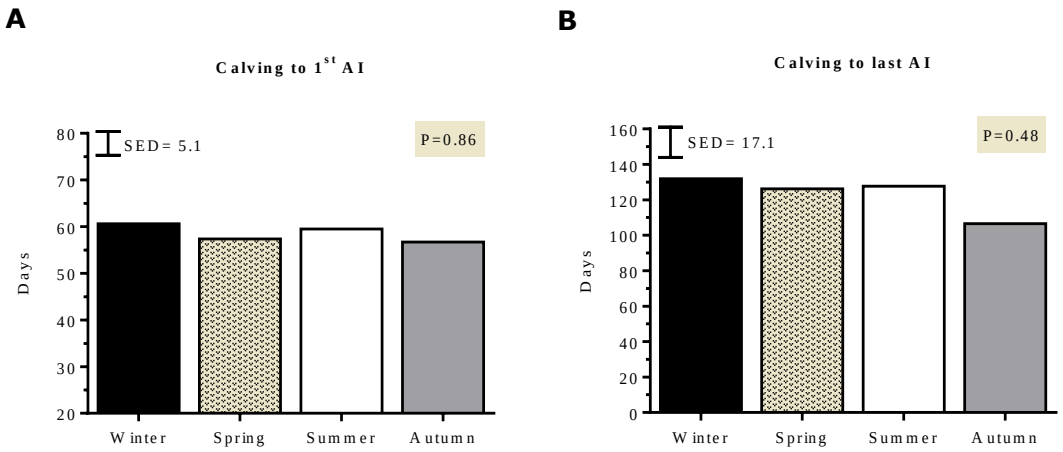


Figure 2.6 *Time of calving to first artificial insemination (AI) and the last insemination at which pregnancy was established during different seasons. Calving to 1st AI (**A**) and last AI (**B**). The data are presented as mean of winter (n=70), spring (n=87), summer (n=110) and autumn (n=85) with SED with LSD used to estimate significant differences between means.*

2.3.3.2 Gestation length and calving interval

The period from last insemination to calving was not affected by the season (P=0.97). Also, the number of days between two successive calvings (calving interval) was not affected by season (Figure 2.7). Additionally, service interval was not affect by season.

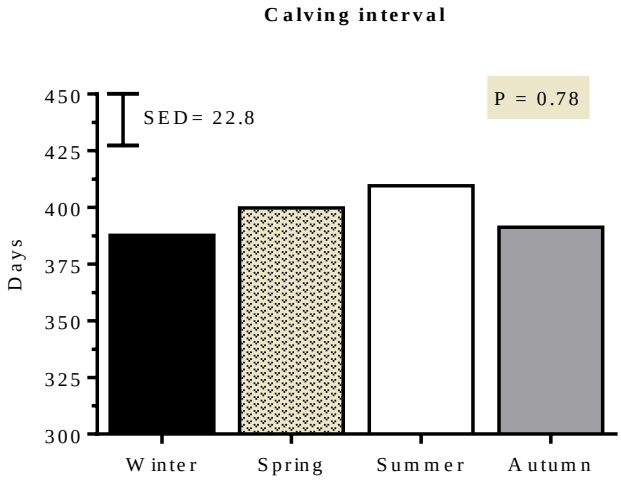


Figure 2.7 *Calving interval in cows calving during different seasons. The data are presented as mean of winter (n=46), spring (n=46), summer (n=58) and autumn (n=51) with SED and LSD used to estimate significant differences between means.*

2.3.4 Impact of season on conception rate

While the conception rate showed some variation this was not significant (P=0.32) overall. However, comparing the frequency of conception rate against August revealed that March, April and November had the higher conception rate (P<0.05; Table 2.2). Furthermore, the conception rat was higher (P<0.05) in spring than those observed in summer and winter (*Table 2.3*). The proportion of cows that conceived was separated into two groups the first group included animals that conceived less than 90 days after calving. The second group included all cows that conceived after 90 days. The conception rate within 90 day post calving increased in autumn compared to winter, spring and summer. Whereas the proportion of cows that conceived after 90 days of calving decreased in autumn compared with winter, spring and summer (*Table 2.4* A). Additionally, according to the insemination season, the conception rate within 90 days after calving increased in winter compared to spring and autumn (*Table 2.4* B).

Table 2.2 *Effect of month of AI on conception rate (CR) through the year. P values compare the frequency of conception rate in the month against that found in the August.*

Month	Total AI	Failed AI	Successful AI	CR%	P value	Odd ratio	Confidence interval 95%	
							Lower	Upper
January	115	79	36	***31.3***	0.50	1.21	0.7	2.1
February	89	54	35	***39.3***	0.06	1.72	1.0	3.1
March	96	56	40	***41.7***	**0.03**	1.90	1.1	3.3
April	102	59	43	***42.2***	**0.02**	1.94	1.1	3.4
May	113	70	43	***38.1***	0.08	1.63	0.9	2.8
June	139	87	52	***37.4***	0.08	1.59	0.9	2.7
July	127	86	41	***32.3***	0.39	1.27	0.7	2.2
August	128	93	35	***27.3***	Reference			
September	149	96	53	***35.6***	0.14	1.47	0.9	2.5
October	176	119	57	***32.4***	0.35	1.27	0.8	2.1
November	173	107	66	***38.2***	**0.05**	1.64	1.0	2.7
December	143	99	44	***30.8***	0.54	1.18	0.7	2.0

Table 2.3 *Effect of season of AI on conception rate. P values compare the frequency of conception rate in the spring, summer and autumn against that found in the winter season (reference; ab=P<0.05).*

Season	Total AI	Failed AI	Successful AI	CR%	P value	Odd ratio	Confidence interval 95%	
							Lower	Upper
winter	347	232	115	***33.1***^{a}	Reference			
Spring	311	185	126	***40.5***^{b}	**0.05**	1.37	1.0	1.9
Summer	394	266	128	***32.5***^{a}	0.85	0.97	0.7	1.3
Autumn	498	322	176	***35.3***^{ab}	0.51	1.10	0.8	1.5

Table 2.4 *Effect of season on conception rate within 90 days post calving and after 90 days post calving during the four different seasons. P values compare the frequency of conception rate in the spring, summer and autumn against that found in the winter season (reference; ab=P<0.05).*

A. Conception rate according to the calving season

Season of AI	Total Conception (n)	Conception (n)		Conception rate %		P value	Odd ratio	Confidence interval 95%	
		≤90d	>90d	≤90d	>90d			Lower	Upper
Winter	70	25	45	**35.7a**	64.3	Reference			
Spring	86	33	53	**38.4a**	61.6	0.73	1.12	0.46	1.72
Summer	109	41	68	**37.6a**	62.4	0.80	1.09	0.49	1.72
Autumn	85	46	39	**54.1b**	45.9	0.02	2.12	0.25	0.90

B. Conception rate according to the insemination season

Season of AI	Total Conception (n)	Conception (n)		Conception rate %		P value	Odd ratio	Confidence interval 95%	
		≤90d	>90d	≤90d	>90d			Lower	Upper
Winter	86	46	40	***53.5^{a}***	46.5	Reference			
Spring	72	27	45	***37.5^{b}***	62.5	0.05	1.0	1.0	3.6
Summer	83	32	51	***38.6^{ab}***	61.4	0.05	1.0	1.0	3.4
Autumn	109	40	69	***36.7^{b}***	63.3	0.02	1.1	1.1	3.5

2.3.5 Effect of season on conception rate at first, second, third, and subsequent insemination

Assessment of the effect of season on conception rate at first, second, third and subsequent inseminations revealed no statistically significant effect of season on conception rate performed at specific (first, second, third and subsequent) inseminations (P>0.10;Table 2.5).

Table 2.5 *Effect of season on conception rate at first, second, third and subsequent insemination during the four different seasons. P values compare the frequency of conception rate in the spring, summer and autumn against that found in the winter season (reference).*

Season	Total 1st AI	Successful 1st AI	CR% at 1stAI	Odd ratio	P value	Total 2nd AI	Successful 2nd AI	CR% at 2ndAI	Odd ratio	P value
Winter	118	29	24.6	Reference		82	28	34.1	Reference	
Spring	130	46	35.4	1.7	0.1	84	33	39.3	1.2	0.5
Summer	158	46	29.1	1.3	0.4	102	34	33.3	1.0	0.9
Autumn	180	48	26.7	1.1	0.7	133	56	42.1	1.4	0.2

Season	Total 3rd AI	Successful 3rd AI	CR% at 3rdAI	Odd ratio	P value	Total >3rd AI	Successful >3rd AI	CR% at >3rdAI	Odd ratio	P value
Winter	64.0	29.0	45.3	Reference		87.0	30.0	34.5	Reference	
Spring	44.0	22.0	50.0	1.2	0.6	53.0	25.0	47.2	1.7	0.1
Summer	62.0	23.0	37.1	0.7	0.4	72.0	25.0	34.7	1.0	1.0
Autumn	70.0	31.0	44.3	1.0	0.9	111.0	40.0	36.0	1.1	0.8

2.3.6 Impact of temperature at physiologically important time points relative to insemination

In this analysis, comparison was made between air temperature and conception rate at physiological time points (preovulatory follicle development, insemination and early embryo development), ignoring the specific season. In general, reduced reproductive performance was observed at both high and low temperatures while conception rate was highest when insemination was performed when temperature ranged from 7 to 15°C. The effects of temperature differed at the three physiological time points investigated.

2.3.6.1 Effect of temperature 10 days before insemination

The conception rate was significantly different (P=0.006) among the three temperature groups during the 10 days before insemination. The lowest conception rate was observed when ambient temperature was higher than 15°C during the 10 day period before AI was performed (Table 2.6A). The maximum air temperature also showed a significant (P<0.027) effect on conception rate performed 10 days later. Conception rate was lower when the maximum temperature was lower than 7°C compared to temperature ranging from 7 to 15°C (Table 2.6 B).

***Table 2**.6 Effect of the daily temperature over the preceding 10 days period on the outcome of AI. Temperatures were grouped according to 3 temperature ranges; <7°C, 7 to 15°C and >15°C (reference; ab=P<0.05).*

A. Average temperature 10 days before AI

Mean °C	Total AI	CR %	P value	Odd ratio	Confidence interval 95%	
					Lower	Upper
< 7°C	393	***33.6^{ab}***	0.185	1.24	0.9	1.69
7-15°C	816	***38.5^{b}***	0.002	1.53	1.16	2.01
> 15°C	341	***29.0^{a}***	Reference			
chi. pr.	0.006					

B. Maximum temperature 10 days before AI

Maximum °C	Total AI	CR %	P value	Odd ratio	Confidence interval 95%	
					Lower	Upper
< 7°C	149	***27.5^{a}***	0.151	0.75	0.5	1.11
7-15°C	756	***38.0^{b}***	0.093	1.21	0.97	1.5
> 15°C	645	***33.6^{ab}***	Reference			
chi. pr.	0.027					

2.3.6.2 Effect of temperature on the day of insemination

Average temperature on the day of insemination showed no significant effect on conception rate (Table 2.7).

***Table 2.7** Effect of the temperature on the day of insemination on the outcome of AI. Temperatures were grouped according to 3 temperature ranges; <7°C, 7 to 15°C and >15°C (reference).*

Average temperature at AI

Average	Total AI	CR %	P value	Odd ratio	Confidence interval 95%	
					Lower	Upper
< 7 °C	431	***34.8***	0.57	1.09	0.80	1.49
7-15 °C	808	***36.3***	0.28	1.66	0.88	1.54
> 15 °C	311	***32.8***	Reference			
chi. pr.	0.54					

2.3.6.3 Effect of temperature 21 days after insemination

The effect of average temperature during the 21 day period after insemination on conception rate did not differ significantly among the different groups (Table 2.8A). However, there was an overall effect (P<0.031) of maximum temperature. The highest conception rate was recorded at 7-

15°C. This followed by higher temperature group, whereas the lowest conception rate occurred in the days had less than 7°C (Table 2.8B).

Table 2.8 *Effect of the temperature during the 21 days after AI on the outcome of AI. Temperatures were grouped according to 3 temperature ranges; <7°C, 7 to 15°C and >15°C (reference).*

A. Average temperature 21 days after AI

Mean °C	Total AI	CR %	P value	Odd ratio	Confidence interval 95%	
					Lower	Upper
< 7 °C	400	***33.75***	0.78	0.96	0.70	1.30
7-15 °C	819	***36.02***	0.68	1.06	0.81	1.38
> 15 °C	331	***34.74***	Reference			
chi. pr.	0.73					

B. Maximum temperature 21 days after AI

Maximum °C	Total AI	CR %	P value	Odd ratio	Confidence interval 95%	
					Lower	Upper
< 7 °C	97	***24.74^{a}***	0.06	0.63	0.39	1.02
7-15 °C	680	***37.65^{b}***	0.18	1.16	0.93	1.44
> 15 °C	773	***34.28^{ab}***	Reference			
chi. pr.	0.031					

2.3.7 Impact of temperature humidity index at physiologically important time points relative to insemination

When assessing the impact of THI, both low and high THI were associated with negative effects on reproductive performance. This study showed that the conception rate was reduced when THI was greater than 60 units (Table 2.9). The THI threshold influence conception rate in dairy cattle and the most favorable THI for dairy cattle in this study ranged from 40 to 60 THI regardless of AI time related to THI.

The conception rate in dairy cattle reduced when the THI was greater than 60 units during the 10 day period preceding insemination. However, lower THI (<40) tended (P=0.083) to reduced conception rate compared with those inseminated under a THI range of 40 to 49. In the present study, there was no effect of THI on the day of insemination on insemination outcome. However, the conception rate was reduced when the THI was lower than 40 compared to 40 to 49 during the 21 day period following insemination (Table 2.9).

Table 2.9 *Pregnancy outcome for artificial inseminations carried out at specific daily THI during the 10 days before, at the day of and during the 21 days after AI was performed. P values compare the frequency of conception rate in the THI groups against that found in the 40 to 49 THI (reference; ab=P<0.05).*

Time of THI related to AI	Total AI	Failed AI	Successful AI	CR%	P value	Odd ratio	Confidence interval 95%		Chi pr
							Lower	Upper	
Overall	**1550**	**1005**	**545**	**35.2**					
During the 10 days before AI									
Mean THI <40	164	114	50	***30.5***^{ab}	0.083	0.72	0.49	1.05	
Mean THI (40-49)	546	339	207	***37.9***^{b}	Reference				**0.039**
Mean THI (50-59)	548	346	202	***36.9***^{b}	0.719	0.96	0.75	1.22	
Mean THI ≥60	292	206	86	***29.5***^{a}	**0.015**	0.68	0.51	0.93	
On the day of AI									
Mean THI <40	181	125	56	***30.9***	0.195	0.79	0.55	1.13	
Mean THI (40-49)	493	314	179	***36.3***	Reference				0.277
Mean THI (50-59)	615	388	227	***36.9***	0.836	1.03	0.80	1.31	
Mean THI ≥60	261	178	83	***31.8***	0.217	0.82	0.59	1.13	
During the 21 days after AI									
Mean THI <40	144	106	38	***26.4***^{a}	**0.006**	0.57	0.38	0.85	
Mean THI (40-49)	602	369	233	***38.7***^{b}	Reference				**0.029**
Mean THI (50-59)	537	357	180	***33.5***^{ab}	0.069	0.799	0.63	1.02	
Mean THI ≥60	267	173	94	***35.2***^{ab}	0.326	0.861	0.64	1.16	

THI mean temperature humidity index, AI mean artificial insemination, CR mean conception rate.

2.4 Discussion

This study was undertaken to clarify the effect of season on dairy cattle fertility in the temperate climate of the UK. The results within this study showed that the season not only appear to influence time to mating but also it affected conception rate. The artificial insemination outcome was dependant on daily air temperature and THI before and after insemination.

Seasonal effect on birth and calving date

The results indicated that cows born in autumn and winter (short daylight) were younger at first AI and first conception than born in spring and summer (long daylight). Similarly, seasonal variations were observed by several researchers (Little et al., 1981, Schillo et al., 1983, El Abdein and Makkawi, 2006, Day et al., 2015). It is likely that dairy heifers exposed to long daylight hastened puberty (Hansen et al., 1983, Rius et al., 2005, Youngquist and Threlfall, 2006), reaching first oestrus and ovulation earlier (Hansen et al., 1983).

Environmental changes during both the first and second 6-month post-natal periods of life could influence the maturity of heifers. This may be due to growth rate, hormonal changes and body weight prior to puberty (Schillo et al., 1983). Calves during the second 6 month of their life exposed to spring and summer photoperiod and temperature showed a decreased age at puberty (Schillo et al., 1992). During the growth phase, calves raised under long photoperiod reached larger bodyweight at maturity (Parkunan et al., 2015). Holstein heifers exposed to long photoperiod with elevated diet hastened growth and puberty (Rius et al., 2005). In subtropical studies, it was shown that animals exposed to long photoperiod during last month stimulated skeletal muscle growth and induced puberty (Valenzuela-Jiménez et al., 2015). Previous results from Schillo et al. (1983) demonstrated that the plasma concentration of LH from 6 to 7 month age was greater in September born heifers and they had larger ovaries at 7 month age than March born heifers and reached puberty earlier as well.

Photoperiod could play an important role on puberty through the secretion of prolactin and growth factors as well as melatonin. This is could be related to the increased plasma IGF-1 in calves grown under long day photoperiod than the short day photoperiod (Spicer et al., 2007). Furthermore, treating heifers with exogenous melatonin during early summer during the first 6 months increased the incidence of those attaining puberty (Tortonese and Inskeep, 1992). The above results suggest that short day length during the first 6 months of life and long day length during second 6 months may result in earlier age at first insemination and first conception. Heifers born in the autumn will grow during the first 6 months post-natal under short photoperiod and the second 6 months will grow during the long photoperiod.

In contrast to the results of the current study dairy heifers (Menge et al., 1960, Roy et al., 1980) and beef heifers (Arije and Wiltbank, 1971, Swierstra et al., 1977) born in spring reached puberty at younger ages. However, seasonal influence fails to promote puberty in beef heifer as reported by (Greer, 1984). Inconsistent outcomes among research studies concerning age at puberty could be due to several factors. For example, the seasons during which each stage of development occurs could influences animal development (Hansen, 1985). Seasonal changes in nutrition may interact with season to effect the age at puberty (Little et al., 1981). Additionally, the conflicting finding in literature could be due to the different in temperature that prevailed in the results as this can affect the mechanism of follicular development of heifers (Wilson et al., 1998a). It is also possible that, the accelerated puberty in long photoperiod season did not result in heifer conceive earlier (Youngquist and Threlfall, 2006).

It has been reported that fertility is higher during cold periods, especially in hot weather areas such as Florida in USA (Bell, 2008, De Vries and Risco, 2005) and North Eastern Spain (Lopez-Gatius, 2003). It seems plausible that in hot regions, the summer season reduced fertility of cow and it may be advantageous to delay breeding during heat stress or to remove the effect of thermal stress on dairy cattle. Several studies have been done on this to minimise the effect of summer heat on reproductive function of dairy cattle (Worley, 2009, Shearer et al., 1999, Lucy, 2002).

Melatonin can improve reproductive performance during the warm season. Garcia-Ispierto et al. (2012) reported that cows treated with melatonin during the early dry period (after 220 days of gestation) not only decreased the number of open days, but also reduced the incidence of pregnancy loss and repeated breeding syndrome compare with the non-treated cow during warm season. In this study, season did not have significant effects on the reproductive parameters investigated. This finding could be due to management issues such as increased number of inseminations during summer and autumn, seasonal changes in cow management and variations in the severity of seasonal climate differences. It should be noted that the dairy cattle in the present study would not be heat stressed, because the THI was less than 75. Furthermore, the temperatures are often cooler throughout the year especially during the night, this should help animal during hot days in summer to withstand heat stress. It is true that the temperature degree increased with increased day length in summer. In these concepts, perhaps long photoperiod had a negative outcome on cattle fertility and increased temperature increased further seasonal outcome.

Seasonal effect on conception rate

In the present chapter, the highest conception rates were recorded in spring. However, the conception within 90 days post calving according to calving season was higher in autumn. Additionally, the conception rate within 90 days post calving according to the insemination season was greater in winter. The study by Al-Katanani et al. (1999) reported that 90 days non-return rate to first service was lower during summer. In another study the pregnancy rate at 90 and 135 days postpartum was higher in winter than summer (Rensis et al., 2002). It is possible that the different air temperature in different regions could lead to the variance of conception rates throughout the year. In north central Florida, the conception rate was higher from October to December (Momcilovic et al., 1998). However, in some studies, the investigators underestimated reproductive fertility by suggesting it was higher in the summer (Rensis et al., 2002). In Norway, reported that the reproductive performance was lower in winter compared with summer (Refsdal, 2007).

Compromised folliculogenesis may result from the effect of hot environments on endocrine imbalance as well as the fact that high producing dairy cows frequently suffer from metabolic stress (De Rensis and Scaramuzzi, 2003). It seems that the poor oestrous detection during summer may lead to reduction in fertility. The use of fixed time insemination without detection of oestrus may reduce the effect of poor oestrous detection in summer and subsequently may improve summer infertility (De Rensis and Scaramuzzi, 2003). It seems plausible that the reduction of LH during summer lead to increased fertility problem such as failure of ovulation (De Rensis and Scaramuzzi, 2003).

This study suggested that daily temperature pre and post breeding could affect dairy cattle fertility. This does not mean other factors are not involved in affecting cattle fertility. Our findings pointed out that the highest conception rate occurred when the ambient temperature ranged from 7 to 15°C. The results demonstrated that higher temperature (>15°C) before breeding reduced conception, whereas lower temperature (<7°C) reduced conception after breeding. In Spain, the highest conception rate resulted only when the maximum temperature was <20°C and high maximum temperature 1 day after insemination reduced the conception rate of dairy cattle (García-Ispierto et al., 2007).

Further finding in the current study indicated that greater THI (>60) before breeding reduced conception. The results within this chapter are in line with the research done in the moderate climates of Germany. They identified that increased THI before, at and after breeding reduced the conception rate of lactating dairy cows and the most sensitive period to heat stress was 21 days before breeding (Schüller et al., 2014). The conception rate in this study was higher when the average THI ranged from 40 to 60 in the period before and after breeding. However, in this study not only THI >60 but also THI <40 tended to reduce conception rate after breeding. Conception rate decreased from 30.6 to 23.0% when the maximum THI before insemination was higher than 80 (García-Ispierto et al., 2007). Recently similar results in buffalo showed that the pregnancy rate was higher at >50 THI and <75 THI, while the pregnancy rate reached highest at around 60 THI (Dash et al., 2015).

The above findings may correspond to the study by Roth et al. (2000) which established that the heat stressed cows had both immediate and delayed effects on fertility. FSH was increased and plasma inhibin concentration was decreased in heat stressed cows compared with cooled cows. They suggested that the granulosa and theca cells of previously heat stressed cows had a lower steroid production capacity. Cows exposed to heat either before or after insemination had significant effect on conception rate (Jordan, 2003, Morton et al., 2007, Schüller et al., 2014). The effect of heat stress on oocytes in pre-antral follicle during summer may be carried over to the next cooler months in autumn (Roth et al., 2001b, Roth et al., 2001a). Thus heat stress may affect conception rates 50 days before breeding and up to 45 days after breeding (Chebel et al., 2004). It is possible to consider that high temperature after insemination could affect the sperm within the genital tract of cows and the interaction of the two cell types during fertilization. Heat stress could affect uterine prostaglandin secretion leading to embryonic loss due to the function of this hormone which leads to luteolysis (Putney et al., 1988a). The prostaglandin concentration in postpartum cows was increased in heat stressed cows and the corpus luteum diameter after parturition was only about one half in unshaded cows (Lewis et al., 1984). It is likely that the high ambient temperature had influences on the physiological processes of ovaries; return to its normal activity may depend on the specific effect of elevated temperature on the follicular growth from primordial follicles to the dominant follicles and continue up to regression of corpus luteum.

The number of follicles 2-5 mm and >9 mm increased under heat stress, whereas dominant follicles size decreased (Guzeloglu et al., 2001). The mechanism may be due to the fact that the oestrous cycle had more follicular waves and smaller dominate follicles during thermal stress, which in turn led to longer luteal phases. Plausibly, the animals during hot temperature may have less reproductive activity. While the exact causes for the lower fertility and reduced conception rates are not yet clear, seasons might affect follicular and corpus luteum development prior and after ovulation and might contribute to the low fertility of the cows during the warm and long day light seasons. Using ovarian characteristics to study

the effects of season on the follicular and corpus luteum development would help to determine the mechanism by which season exert its effects?

2.5 Conclusion

Even though cows are generally regarded as non-seasonal breeders certain aspects of their reproductive performance was influenced by season in the present study. To the best of our knowledge, this is the first detailed study of seasonal influence on cattle fertility in the temperate climate of the UK. The results showed that the age at first insemination might be influenced via the season of the year. The finding revealed that short photoperiod born heifers had fewer days to first conception and calving. Environmental factors (temperature and THI) during the periods before and after insemination also affected conception rates in the present results. The highest conception rate occurred at a temperature range from 7 to 15°C and a THI range from 40 to 60 units. While cows were not classically "heat stressed" in the current study, it is possible to suggest that slightly increased temperature during summer months could have a negative effect on fertility.

The consequences of fertility changes associated with the impact of seasonal variation are unclear and more investigations are needed to identify the subfertility problem of dairy cattle in the United Kingdom. In the present research, we have analysed the effect of seasons on puberty, fertility and insemination outcomes. This model has been valuable in analysing reproductive function throughout the year. Subsequent research is essential to determine how the season exerts its effect on fertility of dairy cattle. The next study will focus on analysis of the reproductive tissues of dairy cattle collected throughout the annual cycle. Determination of ovarian follicle populations and characterization of follicular function will be analysed to address seasonal fluctuations. Furthermore, the seasonal influence ovulation rate will be considered to determine the proportion of multiple births in dairy cows throughout the seasons of the year.

Chapter 3: Seasonal effects on ovarian follicle development and incidence of multiple ovulations in dairy cows

3.1 Introduction

The reduction of fertility related to season is multifactorial; temperature and photoperiod can affect cellular function of various tissues in the reproductive system. Seasonal effects on dairy cattle fertility have been reported in temperate climate UK (Chapter 2). However, there is little or no data available in the literature to determine how season affects ovarian characteristic in temperate region. Seasonal factors alter the dynamics of ovarian follicles and hormonal secretions (Badinga et al., 1994), and influence the incidence of multiple ovulations (Fitzgerald et al., 2014). Recently, antral follicle counts have been of interest as it can influence reproductive performance (Silva-Santos et al., 2014, Morotti et al., 2015).

There are circumstances in which the frequency of multiple ovulations and seasonal infertility increase in dairy cattle. These could be explained by genetic manipulation for increased milk production and the involvement of gonadotrophins and factors produced locally. However, the underlying mechanisms of follicular dominance are not yet fully understood (Wiltbank et al., 2000, Webb et al., 2003, Echternkamp et al., 2004b, Webb and Campbell, 2007). The majority of multiple births in cattle are due to multiple follicles being selected and continuing to ovulate (Echternkamp, 1992). Multiple ovulations and subsequent multiple births in cattle historically have been considered as an unfortunate event. They can reduce cow reproductive efficiency, productivity and thus the profitability of enterprises. People with experience in dairy and beef programs identify that many problems can arise with multiple births (Nielen et al., 1989, Wiltbank et al., 2000, Çobanoğlu et al., 2010), though profitability in a beef herd may increase (De Rose and Wilton, 1991, Echternkamp and Gregory, 2002). Multiple births can lead to increases in the incidence of health problems such as subsequent lower conception rates, increased perinatal mortality, retained placenta, dystocia, and metritis (Kinsel et al., 1998, Gregory et al., 1990, Çobanoğlu et al., 2010).

Seasonal influences possibly alter the mechanisms of follicular development and selection resulting in multiple ovulations and/or poor fertility. While the follicular dynamics of the ovary and multiple ovulations are generally understood, little is known about the seasonal contribution to differences in ovarian characteristics, such as antral follicle count, ovulation rate, follicular cysts, and luteal tissue characteristics.

Season alters cattle fertility in high producing dairy cows through its effects on ovarian follicular development. These in turn affect the incidence of multiple ovulations, follicular cysts and subsequent CL developed in ovaries, and their viability and propensity to produce P_4. It has been proposed that melatonin protects GCs undergoing luteinisation and increasing P_4 secretion (Taketani et al., 2011). Progesterone is one of the factors responsible for low fertility before and after insemination in dairy cattle (Sartori et al., 2002).

Hypothesis

Follicle development, incidence of ovarian cysts and CL number and competency vary in different seasons. To test this hypothesis pairs of ovaries were collected around the time of the summer and winter solstices, and during the spring and autumn equinoxes during two consecutive years. Specifically, the following endpoints were assessed in abattoir derived ovaries from culled dairy cows:

1. Number of ovarian follicles.
2. Incidence of multiple ovulations and ovarian cysts, as indicator of ovulation rate and healthy follicles.
3. Corpus luteum development and luteal P_4 content.
4. Melatonin receptor expression in bovine ovarian tissue.

3.2 Materials and Methods

3.2.1 Sample collection

3.2.1.1 Ovarian collection

Ovaries of Holstein cows slaughtered during each season of each of two years were collected from a local abattoir (Telford, England; 52.72° North latitude and 2.47° West longitude). Each pair of ovaries was placed into polythene bags and transported on ice to the lab. The ovaries were collected during winter and summer solstices and during spring and autumn equinoxes for two consecutive years. The ovaries from 100 cows were collected per visit, with two visits per season (i.e. 200 cows) during two years (400 cows / season, 800 cows / year). Total number was 1600 cows over two years (Figure 3.1).

3.2.1.2 Evaluation of ovulation rate

The presence of more than one CL at same stage on a pair of ovaries indicated that more than one follicle ovulated at the previous oestrus cycle. Animals with multiple CLs were selected for further analysis. Additionally, an equivalent number of ovaries with single CLs were used as a control. For each ovarian collection, 20 pairs of ovaries with single ovulations were selected. Selection of both single and multiple ovulations were classified according to the stage of the oestrous cycle. The 20 pairs of single ovulation ovaries collected per each visit were about 25% early, 50% mid and 25% late stage of oestrous cycle.

The stage of the oestrous cycle for individual cows was determined based on follicle development and CL stage (Ireland et al., 1980, Miyamoto et al., 2000). All ovaries were divided into three stages of the oestrous cycle according to the appearance of CL development. Briefly, the early stage of the oestrous cycle (about 1 to 5 days) has a CL that from the time of ovulation to when the epithelium grows over the ruptured point, apex of a new CL is formed and it is red in colour and soft in consistency. The mid stage of the oestrous cycle (6 to 15 days) was characterized by a CL completely formed with vasculature at its periphery, red brown to orange in colour and mostly compact in consistency. The late stage of the oestrous

cycle (CL 16 to 20 days) was characterised by a CL being wholly orange to yellow/pale in colour and regressed with no surface vasculature and firm in consistency (Table 3.1).

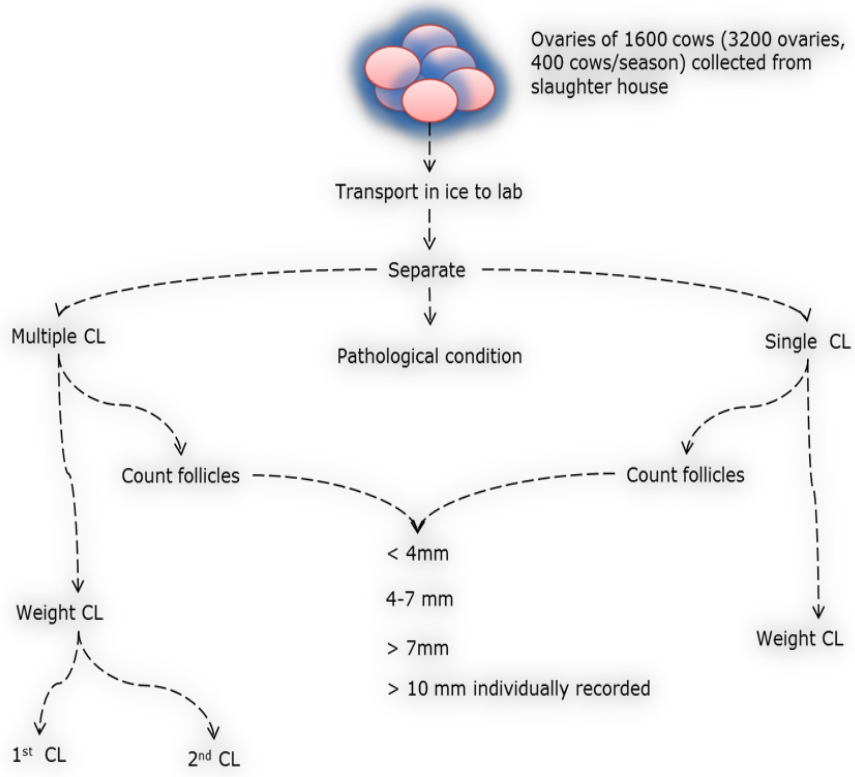


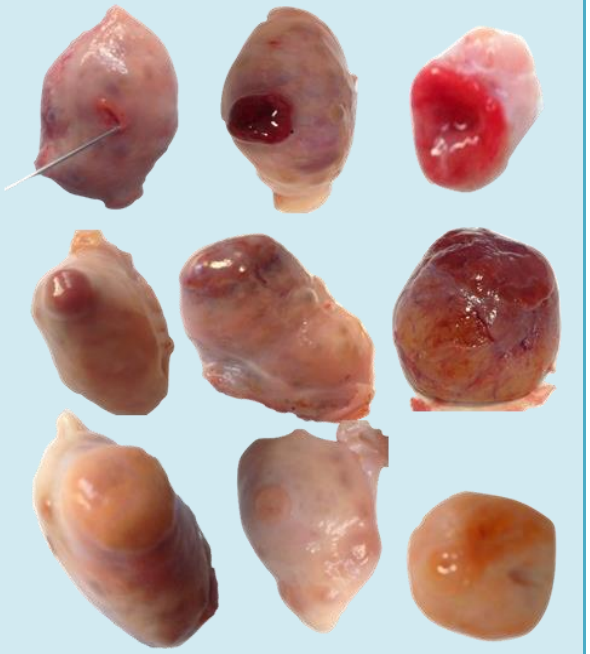
Figure 3.1 *Schematic for bovine ovarian collection from a local slaughterhouse.*

3.2.1.3 Antral follicle count

Follicle populations on the ovarian surface were recorded in all ovaries. The number of antral follicles, small (<4 mm), medium (4-7mm) and large (>7 mm) on each pair of ovaries were counted. Follicles were categorised as such for the following reasons:

- Small follicles, these follicles grow without FSH (Garverick et al., 2002) and appearance of follicles >4 mm indicates emerge of follicular wave (Rajmon et al., 2012).
- Medium follicles, several days after emergence of a wave of follicles about 7 to 8 mm in diameter one follicle continues to grow (Fortune et al., 1990, Ginther et al., 1996).

Table 3.1 *Classification of corpora lutea according to the stage of development during the oestrous cycle of cattle.*

Stage	External appearance		Colour		Internal	Vasculature	Picture
Around	From	To	From	To			
Early stage (1–5)	Recently ovulated follicles (rupture point)	Rupture point with luteal tissue	Bloody	Red	Cells loosely organized filled with blood or soft tissue	Not visible	
Mid stage (6–15)	Point of rupture (luteal tissue formed)	Complete luteal tissue, start to change in colour and consistency	Red / brown	Tan / orange	The apex is red or brown and the bottom is orange	Presence of a vascular network on surface of CL	
Late stage (16-20)	Pale tissue light yellow to white		Bright orange	Yellow/ pale	Bright orange to yellow	Not visible (disappearance of vascular network). No bleeding with bisected CL	

Day 1= day of ovulation and day 20= days of oestrous cycle

- Large follicles, the selection of the dominant follicle estimated to occur around 7 mm in diameter (Ginther, 2000) and the development of follicles (7 - 9 mm) is dependent on LH (Gong et al., 1995).
- Follicles greater than 10 mm were recorded individually presumptively as a dominant follicle.

3.2.1.4 Follicular fluid aspiration

The follicular fluid was aspirated from pairs of ovaries of individual cows using a syringe and 21 G needle. The fluid was aspirated from animals with single and multiple CLs from small follicles (≤6 mm) and from the largest follicle (≥7 mm) during each of the four seasons. The follicular fluid was then transferred into clean Eppendorf tubes and centrifuged at 800 X g for 10 min at 4°C. The supernatants were then transferred into another tube and stored on dry ice prior to storage at -80°C until further analysis. The GC pellets from individual cows were kept on dry ice and stored at – 80°C.

3.2.1.5 Luteal collection

Corpus luteum were gently dissected out from the ovarian matrix and sliced using scissors and forceps. All connective tissue was trimmed away from the CL which was then weighed using a digital balance. Each CL was divided into three pieces; two pieces of the CL were frozen on dry ice immediately after collection and stored at -80°C for further analysis. The other pieces were fixed in Bouin's solution for 8 h then transferred into ethanol (70%) for immunohistochemistry.

3.2.1.6 Ovarian pathology

All ovarian pathological conditions were recorded during each season. Ovarian cysts, which are follicles larger than 20 mm in diameter with thick wall and contain red coloured fluid, were recorded to be a follicular cyst, and follicles converted to luteal cyst counted as luteinizing follicular cyst. Large CL like structure filled with fluid are reported as non-ovulatory luteal cysts. Any cases suspected to be abnormal cases were also recorded.

3.2.2 Sample analysis

3.2.2.1 Progesterone extraction from corpus luteum tissue

Individual CL segments were homogenized by polytron homogenizer. Briefly, about 50 mg of CL was taken from ovaries of cattle from single and multiple CLs during each of the four seasons. CL slices were rinsed with 1 X PBS then homogenised using polytron homogeniser at maximum speed for 2 min in one ml of PBS. The resulting suspension was subjected to two freeze-thaw cycles to further breakdown cell membranes. The homogenates were centrifuged at 1000 X g for 5 min at 4°C to remove cell debris. The supernatants were aspirated and transferred to a fresh 1.5 ml Eppendorf tube and stored at -80°C until P_4 assay by ELISA. Luteal P_4 content were measured only in samples collected during the first year and excluded late stage (regressing CL stage) of oestrous cycle. Sample collection are shown in Figure 3.2.

3.2.2.2 Progesterone assay

Analysis of P_4 in CLs was done by ELISA (Ridgeway Research Ltd, Gloucesershire, UK). Samples were processed according to the manufacturer's instructions. Briefly, all reagents and specimens were brought to room temperature. In order to get the concentration range of P_4, samples were diluted in PBS 1:400 fold for high P_4 content CL and 1:800 dilutions for low P_4 content CL. After thawing, each sample was mixed by vortex and centrifuged before assay.

The standards, blank, samples and quality controls (QC) (10 µl volume of each) were run in duplicate, followed by additional 200 µl of progesterone enzyme label to each well. The enzyme label was replaced by wash buffer in blank wells. The plates were mixed thoroughly using a plate shaker for 10 min then incubated at room temperature for 2.25 h. After incubation, the contents of the wells were briskly shaken out and rinsed three times with 200 µl of wash buffer. After washing, plate tapped onto absorbent paper to remove the residual droplets; this step was repeated after each wash. Alkaline phosphatase substrate (200 µl) was added to each well and the plate was mixed thoroughly and incubated at room temperature for 30 min. The absorbance (optical density) was determined at 570 nm with a

plate reader using Thermo Lab-system Multi-Scan Ascent plat reader (Thermos Fisher, Loughborough, UK). At the end, sample concentrations were calculated using a four parameter logistic standard curve. In order to avoid seasonal variation among plates, summer *vs* winter and spring *vs* autumn were run together. Samples outside of the linear standard curve and the coefficient variations (CV) of any sample greater than 20% were repeated. Additionally, the variation among standard curve was assessed depending on the variation of quality control and the curve correction was performed if necessary by estimating the QC variation then each sample was corrected by the variation of QC between plates. Inter- and intra-assay CV were (5.6%) and (6.0%) respectively.

3.2.3 Melatonin assay in follicular fluid

Analysis of melatonin was done by bovine melatonin ELISA kit (DRG, GmbH, Germany. MBS743340). Samples were processed according to the manufacturer's instruction. Briefly, all kit components and samples were brought into room temperature before use. 100 µl of the standards, samples and blank (PBS) were added into appropriated wells, followed by adding 50 µl of conjugate to each well except blank. Samples then mixed thoroughly and incubated for 1 h. Then plate was washed five times with wash solution, it was blotted into absorbent paper until no moisture. Next, 50 µl of substrate A and 50 µl substrate B add to each well and incubated the plate in dark place for 15 min at room temperature. To stop the reaction, 50 µl of stop solution were added into wells. Finally, optical densities were measured by microplate reader at 450 nm using parameter logistic curve fit to determine the melatonin concentration.

3.2.4 Tissue processing of fixated ovarian tissues

Ovaries of normal appearance containing follicles were cut vertically and horizontally into several pieces. Each segment was fixed in Bouins solution for 8 h at room temperature. Following fixation, the tissues were preserved in ethanol (70%) until use.

Tissue preparation for histological staining was processed through a series of increasing concentrations of methylated spirit industrial (IMS), to

dehydrate, followed by sectioning in histoclear II and embedded in paraffin wax using standard procedures in an automated tissue processor (Histokinette automated system) (Appendix A.6). The wax blocks were stored at room temperature. The block was cut at 6 µm using a Leica Microm microtome and mounted onto Superfrost plus slides (positively charged slides). After being baked overnight at 40°C, the slides were processed for immunohistochemistry.

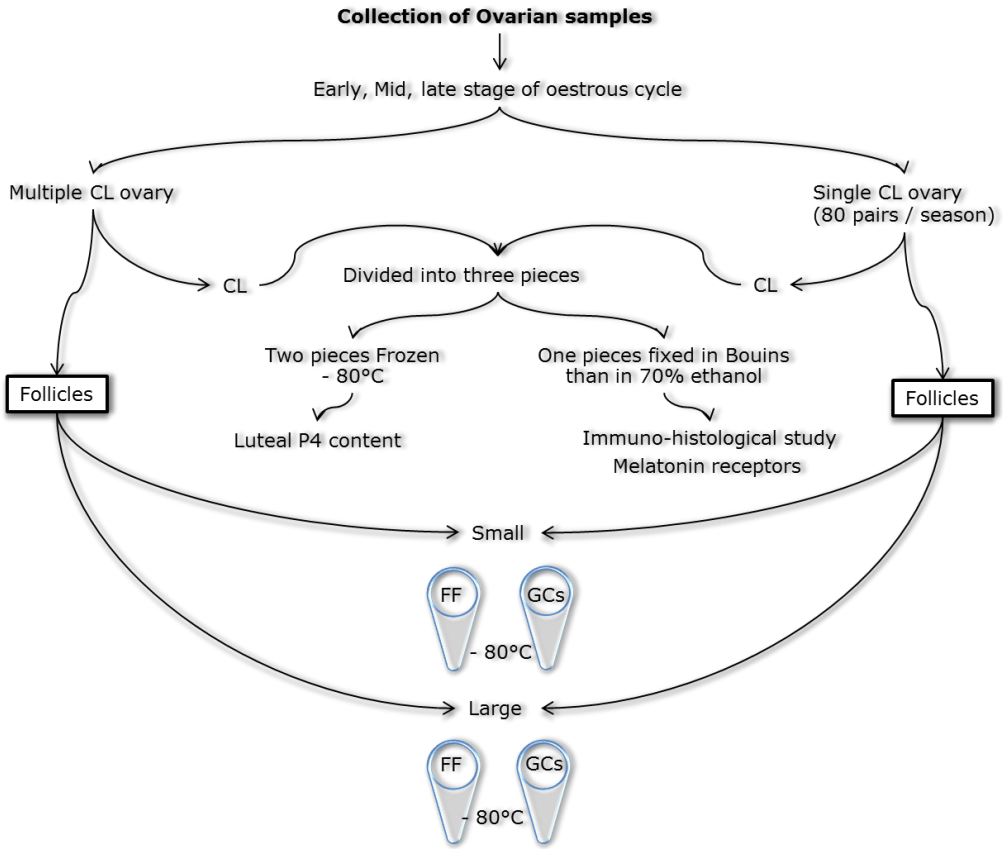


Figure 3.2 *Experimental protocol for sample collection of multiple and single ovulations in cows. FF = follicular fluid, GCs = Granulosa cells.*

3.2.5 Immunohistochemistry of melatonin receptor

Prepared slides were dewaxed by histoclear (2 X 5 min) then rehydrated through decreasing ethanol concentrations 100%, 95%, and 70% (v/v). After washing the section with 1 X PBS (2 X 5 min), sections were boiled in 10 mM citrate buffer at pH 6.0 (2 X 5 min) for antigen retrieval in a 900W microwave, to breakdown the molecular cross links formed during the previous fixation process. The sections were then allowed to cool for 30 min and washed with PBS (2 X 5 min). Then sections were blocked with

endogenous peroxidase (3% H_2O_2 in methanol) for 5 min to reduce background staining, and then washed with PBS (2 X 5 min), before incubation with primary antibody to prevent nonspecific binding, slides were blocked with 4% normal goat serum at room temperature for 30 min.

Melatonin receptors (MTN1AR (R18): sc-13177 and MTN1BR (T18): sc-13177) primary antibody (goat polyclonal anti-rat) were purchased from Santa Cruz Biotechnology 200 µg/ml. The section was incubated with primary antibodies diluted 100 fold in humidified chamber at 4°C overnight. Goat IgG (1 mg/ml) diluted 500 fold in PBS was used as negative control. Bovine hypothalamus sections were used as positive tissue control. On the next morning, slides were washed with PBS (2 X 5 min) and incubated with conjugated secondary antibody (bovine anti goat IgG: sc-2347) for 30 min at room temperature. Following washing with PBS (2 X 5 min), slides were incubated for 30 min with AB complex (Avidin and Biotin complex) prepared 30 min before use. The slides were then washed with PBS (2 X 5 min) and visualisation was performed with diaminobenzidine (DAB) for 5 min under microscope. The sections were counterstained with haematoxylin before mounting with coverslips using DPX. Sections were then dehydrated and cleaned with IMS (70%, 95% and 100%) and Xylene respectively (Appendix A.6). At the end, slides were allowed to air dry in the fume hood. Photo of section were taken by microscope (Olympus BH-2, with camera Leica MZ16).

3.2.6 Immunofluorescence microscopy for granulosa cell culture

Granulosa cell collection and cultured as described by detail in the next Section 4.2.4; Chapter 4. Briefly, cells were cultured on pre-coated coverslips with fibronectin in 12 well plates for up to 144 h. After incubation, the culture media was removed and cells were gently washed by pre warmed PBS. After fixation by adding 4% paraformaldehyde for 20 min, cells were washed with buffer (PBS and 1% BSA) for 5 min then washed with blocking buffer (PBS, 4% BSA and 0.3% Triton) and incubated with fresh blocking buffer for 10 min.

Granulosa cells fixed on coverslips were incubated with specific primary melatonin receptors antibodies (Goat polyclonal anti rat; MTNR1A, sc-

13186; MTNR1A, sc-13177; diluted 1:75) overnight. Next cells were washed by wash buffer and then incubated with secondary antibody (Monkey anti goat diluted 1.250) for 60 min in dark. After incubation, cells were washed three times with PBS. A drop of vectashield was added on to clean slide and the cells attached to the coverslips was faced onto the mountant DAPI and seal the coverslip with nail vanish and stored in dark. Photographs were taken using florescent microscope (Leica DMP).

3.2.7 Statistical analysis

The frequency of single and multiple ovulations depending on the season of the year were analysed by a logistic regression model (Binomial distribution) using GenStat software. Logistic regression was also used to analysis the effect of season on ovarian cysts. The other data were analysed by linear mixed model to determine the seasonal effect and its interaction with multiple ovulations and stage of oestrous cycle in follicular population (total antral follicle count, <4, 4 to 7, >7, > 10 mm), first and second dominant follicles diameters, weight of CL tissue and P_4 content in luteal tissue. Season, type of ovulation and stage of oestrous cycle were considered as fixed effects and season within year (year/season) as random effects. Antral follicle count, diameter of first and second dominant follicles, CL weight and P_4 content luteal tissue were the variables analysed. For CL weight, the stage of the cycle by season of the year interaction was non-significant (P=0.61) and therefore the data was pooled across the three stages of the cycle for analysis. Least significant differences (LSD) were used as a difference between means. The data were presented as mean with SED.

3.3 Results

Out of the 1600 cows which ovaries were evaluated, 9 cows were excluded from the analyses due to pathologies or incompleteness of the reproductive organs. The remaining 1591 cows were classified according to the stage of CL development, in the early (n=179), mid (n=1008) or late oestrous cycle (n=404).

3.3.1 Antral follicle count

The overall number of antral follicles in the ovary did not differ among seasons of the year (P>0.10), nor were there difference if the number of follicles in each of the size categories (Table 3.2).

Table 3.2 *Number of bovine ovarian follicles present in the ovaries of Holstein cows culled in different seasons over two consecutive years.*

AFC	Winter	Spring	Summer	Autumn	SED	P value
Small	48.09	51.36	59.12	49.99	10.33	0.71
Medium	2.83	3.23	3.80	2.84	0.68	0.54
Large	1.87	2.04	2.64	2.03	0.43	0.49
>10 mm	0.84	0.76	1.05	0.91	0.24	0.67
Total follicle	52.79	56.63	65.56	54.86	10.9	0.68

The number of small (P<0.001), medium-size (P<0.001) and the total number of follicles (P<0.001) were greater during the middle of the oestrous cycle, coinciding with a fully developed CL. However, the mean number of large follicles (>7mm) was greater (P=0.009) during later phase of the cycle (Table 3.3). Follicles larger than 10 mm were more abundant during the later phase of the cycle, when a regressing CL was present in the ovaries (P<0.001), but were often absent in the early stage of the cycle. There was no interaction between season and the stage of the oestrous cycle on the number of ovarian follicles (P>0.10).

Table 3.3 *Number of ovarian follicles present in the ovaries of Holstein cows culled whilst in the early, mid- and late stages of the oestrous cycle.*

AFC	Early	Mid	Late	SED	P value
Small	51.42[a]	57.06[b]	47.94[a]	2.36	<0.001
Medium	3.57[a]	3.26[a]	2.69[b]	0.22	<0.001
Large	1.89[a]	2.23[b]	2.32[b]	0.13	0.009
>10 mm	0.43[a]	1.06[b]	1.18[c]	0.07	<0.001
Total follicles	56.89[a]	62.56[b]	52.93[a]	2.37	<0.001

a,b,c= differ between cows with different stage of oestrous cycle

The number of small (P=0.06), medium-sized (P=0.33) follicles did not differ between animals with single or multiple CLs on the ovarian surface. However, the number of large follicles (>7mm) was higher (P=0.02) in cows with multiple CLs than in cows with a single CL, although this difference was not present in follicles >10 mm (Table 3.4).

Table 3.4 *Number of ovarian follicles present in the ovaries of Holstein cows with single and multiple ovulations.*

AFC	Single ovulation	Multiple ovulations	SED	P value
Small	54.62	50.07	2.67	0.06
Medium	3.13	3.40	0.24	0.33
Large	2.18 [a]	2.53 [b]	0.14	0.02
>10 mm	1.02	1.02	0.08	0.87
Total follicles	59.93	56.00	2.678	0.10

ab= differ between cows with different ovulation rate

In this study, the diameter of follicles greater than 10 mm was recorded individually to determine the effect of season on the size of the first and second largest follicle. There was no significant effect of season on the diameter of the dominant and subordinate follicles (P=0.39; Table 3.5). The diameter of the larger and second larger follicles was greater (P<0.001) during the later stage of the oestrous cycle (Figure 3.3 and Figure 3.4). In addition, there was a season by stage of the cycle interaction for the diameter of the largest follicle. The dominant follicle present in the later stage of the cycle was bigger during autumn and winter compared to the spring follicle (P=0.05; Figure 3.3).

Table 3.5 *Diameter of the largest and second largest antral follicles in ovary of cows slaughtered during the four seasons of the year.*

Presumptive dominant follicles	Winter	Spring	Summer	Autumn	SED	P value
Largest dominant follicles	15.91	15.17	15.75	15.95	0.66	0.39
Second largest follicles	14.51	13.20	12.87	13.32	1.71	0.39

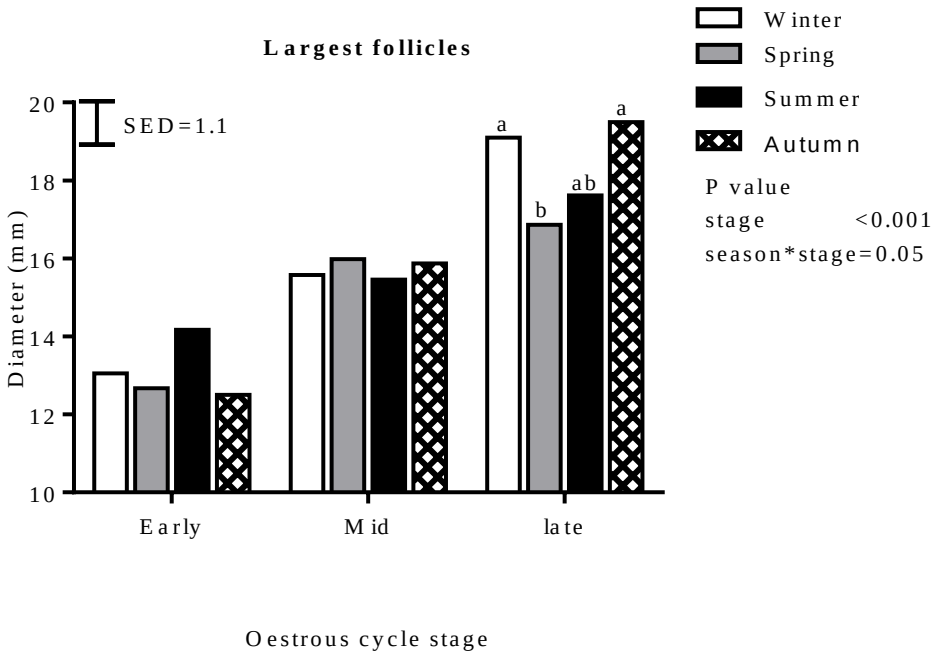


Figure 3.3 *Diameter of the first Presumptive dominant follicles present in ovaries during the early, mid or late stage of the oestrus cycle of cows slaughtered in the four seasons. There was a stage of cycle by season interaction approached significance (P=0.05), where follicles in the late stage of the cycle were largest during the autumn and winter seasons than those observed during spring. a, b mean different between seasons.*

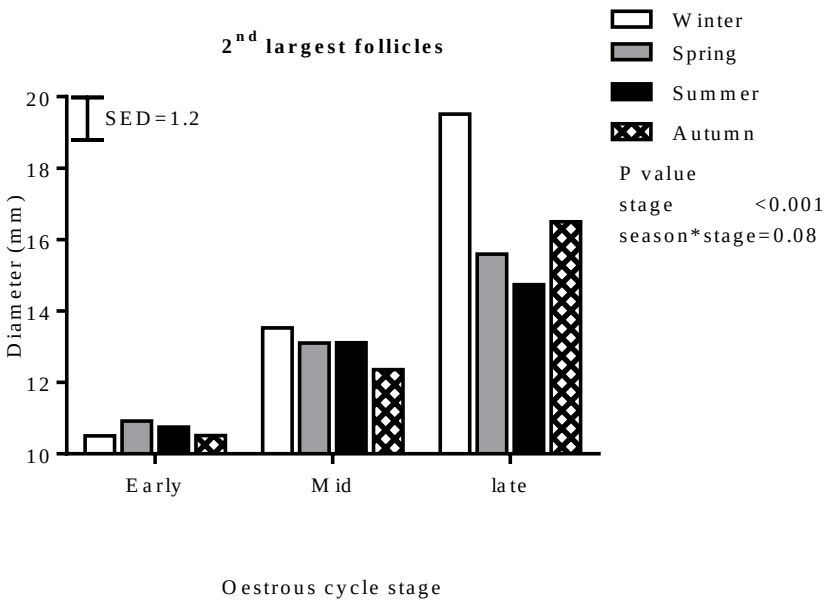


Figure 3.4 *Diameter of the second Presumptive dominant follicle during four seasons of the year interact (P=0.08) with the stage of cycle on the bovine ovaries.*

3.3.2 Ovulation rate

The incidence of multiple ovulations was 9.6%. The incidence of multiple ovulations was greater (P<0.05) in the summer and autumn than in winter. Interestingly, multiple ovulations were found more often arising from the same ovary. Out of the 1591 cows studied it was found that two cows had three CLs and one cow had four CLs (Figure 3.5 and Table 3.6).

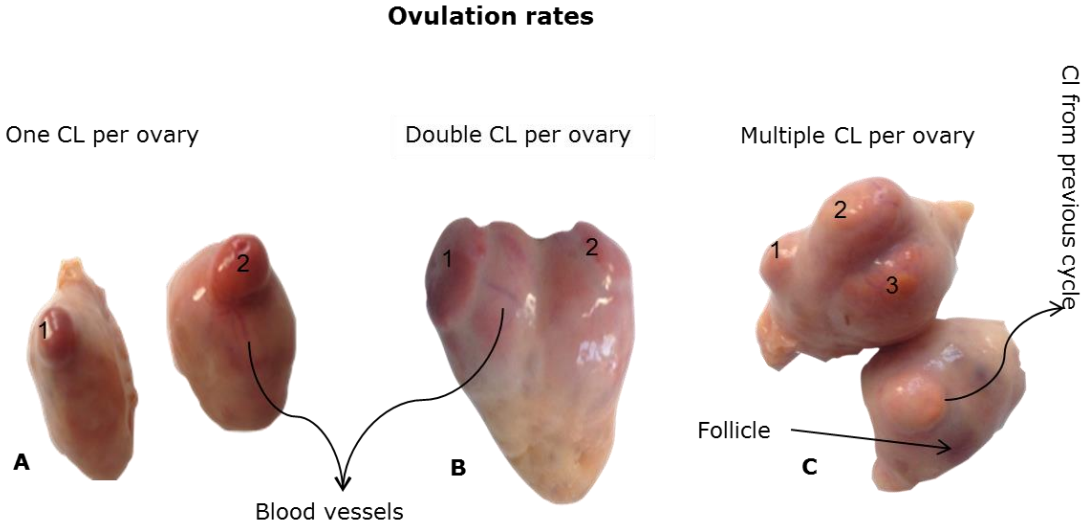


Figure 3.5 *Ovaries of Holstein cows showing multiple ovulations.* ***A****) double ovulations arising from both ovaries,* ***B****) double ovulations from a single ovary and* ***C****) triple ovulations from a single ovary.*

Table 3.6 *Effect of season of the year on the ovulation rate of Holstein cattle in the Midlands, UK. P values compare the frequency of multiple ovulations (M.O) in the spring, summer and autumn against that found in the winter season.*

Season	Animals	Multiple CL in		Total M.O	M.O %	P value	Odds ratio
		Unilateral ovary	Bilateral ovary				
Winter	393	16	11	27	6.9[a]	Reference	
Spring	395	19	14	33	8.4[ab]	0.321	1.24
Summer	401	30	19	49	12.2[b]	0.022	1.89
Autumn	402	26	18	44	10.9[b]	0.045	1.66
Total	1591	91	62	153	9.6		

3.3.3 Corpus luteum weight

The weight of individual and total luteal tissue was compared between single and multiple ovulations as well as among the four seasons. For CL weight, the season of the year by stage of cycle interaction (P=0.61) and season by type of ovulation interaction (P=0.68) were non-significant. Therefore, the data for CL weight was pooled across the stage of the cycle and type of ovulation. Regardless of the ovulation rate, season affected the weight of the CL. The weight of the luteal tissue, regardless of whether it came from single or multiple ovulations, was the lower in the spring and increased linearly reaching its maximum weight in the autumn (P<0.001; Figure 3.6 A and B). Corpora lutea was heavier during the mid-cycle stage (P<0.001; Figure 3.7 A and B).

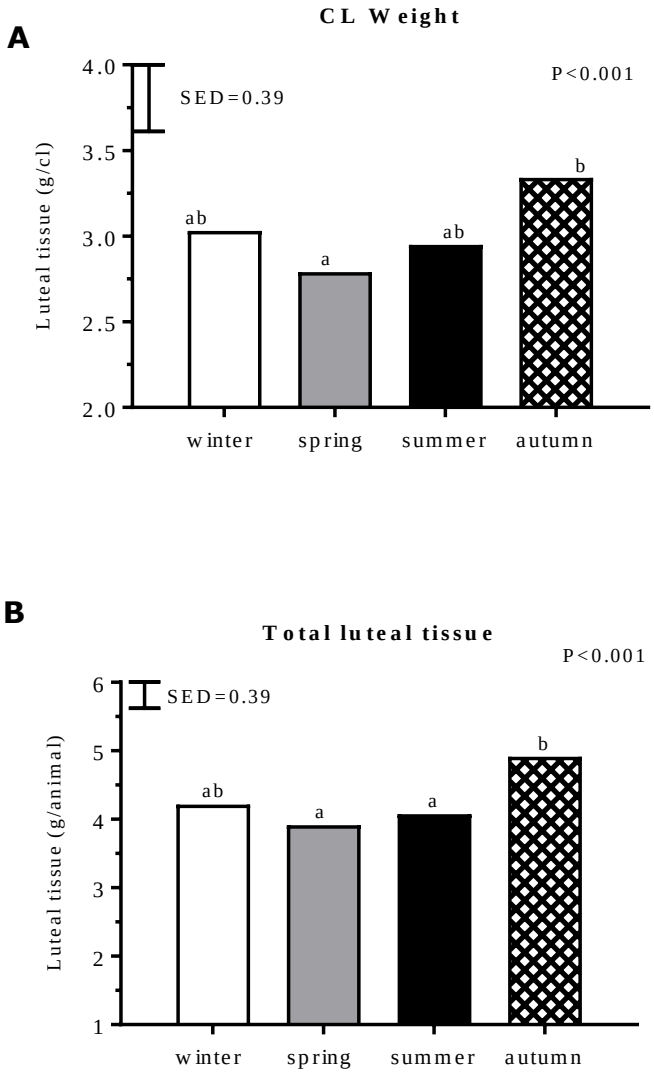


Figure 3.6 *Effect of season on CL weight regardless of luteal stage and related to the CL stage. Seasonal effect on luteal tissue (g/CL) (**A**) and total luteal tissue (g/animal) (**B**). a, b mean different within the season (winter; n=220, spring; n=219, summer; n=292 and autumn; n=326) of the year.*

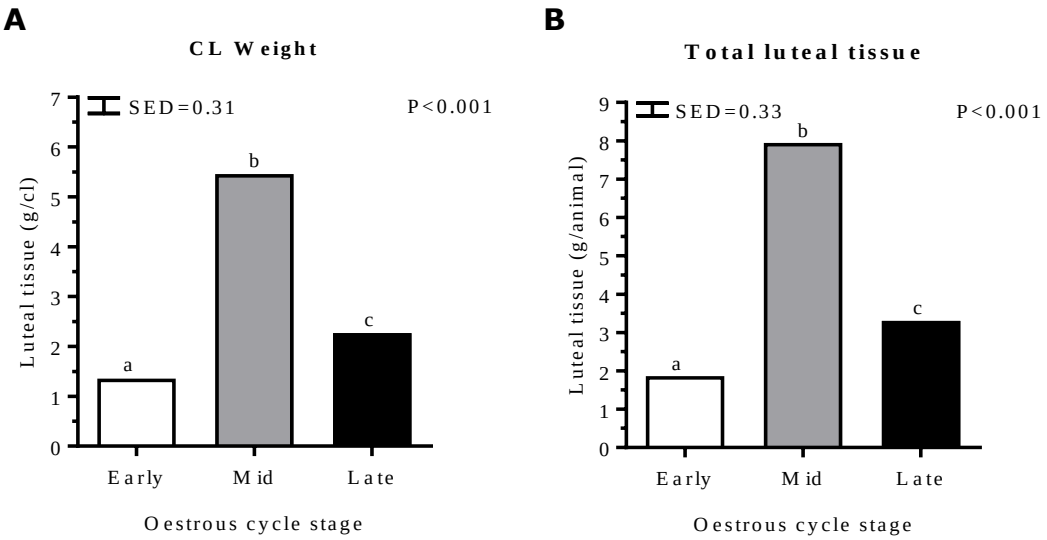


Figure 3.7 *Weight of single CL (**A**) or total luteal tissue (**B**) during the early, mid or late stages of the oestrus cycle in cows with single or multiple ovulations respectively. Luteal tissue (g/CL) total luteal tissue per animal (g/animal) a, b, c= significant different within the CL stage (Early; n=141, Mid; n=779 and Late; n=137).*

The weight of individual CLs, in the mid stage of the cycle, was significantly (P<0.001) heavier in single ovulation cows compared to the CLs from cows with multiple ovulations. However, the weight of total luteal tissue in multiple ovulations was significantly (P<0.001) heavier than that of single ovulation cows. This difference in total luteal tissue was not observed in the early stage of the cycle (Figure 3.8).

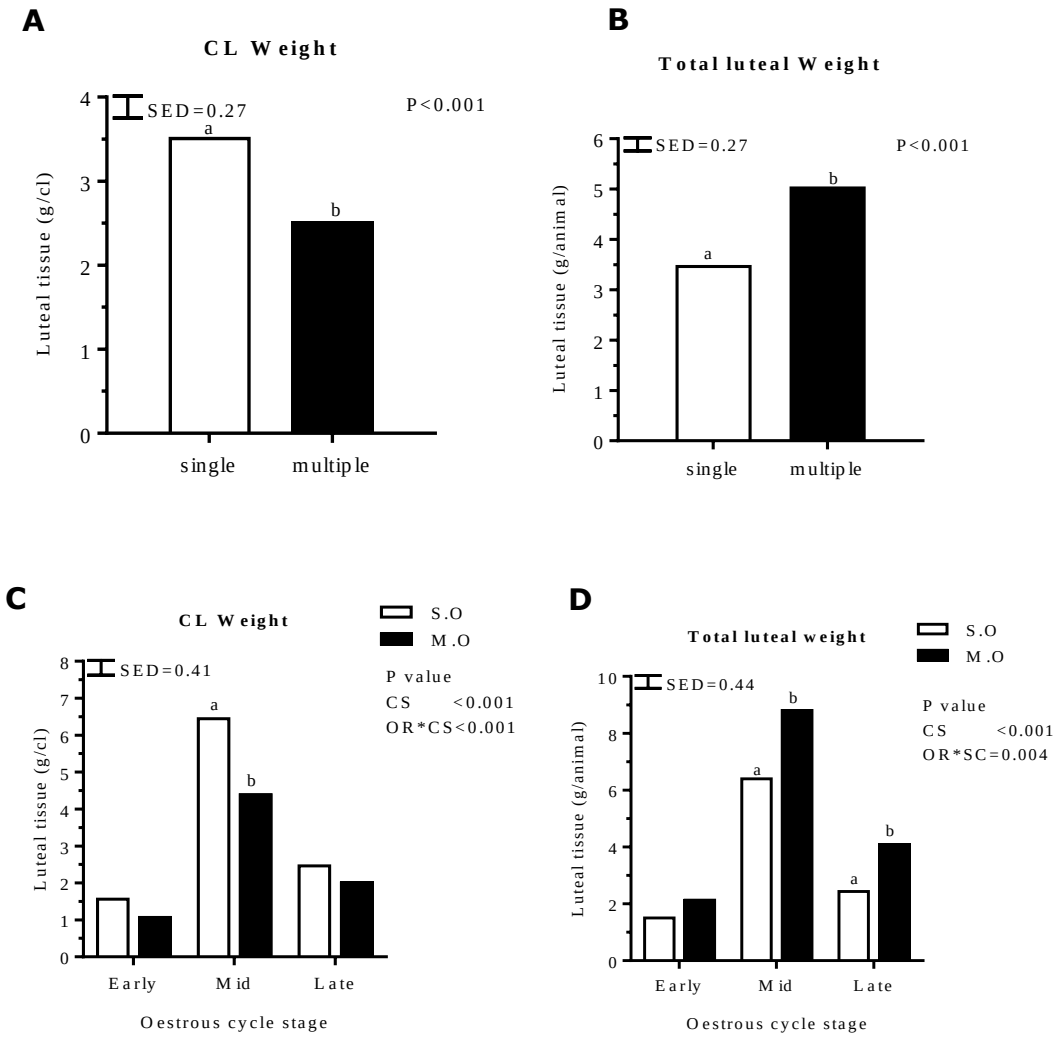


Figure 3.8 *Weight of single or total luteal tissue for cows with single or multiple ovulations (S.O; n=907; M.O; n=150 respectively), pooled across seasons of the year (Interaction P=0.67). Single CL (g/CL) (**A**) considered oestrous cycle stage (g/CL) (**C**). Total luteal tissue (g/animal) (**B**) Total luteal tissue (g/animal) considering oestrous cycle stage (**D**). (n=120, 672 and 115 for single ovulation and 21, 107 and 22 for multiple ovulation at early, mid and late stage of cycle respectively). CS= oestrous cycle stage and *= interaction a, b mean different between groups.*

3.3.4 Corpus luteum progesterone content

The progesterone content of the CL tissue (P_4 ng/mg) was similar in winter and summer collections but higher (P<0.001) than the observed for spring and autumn. However, the P_4 content in the whole luteal tissue per animal was similar (P=0.09) across all seasons (Table 3.7).

There was no difference in the CL P_4 content (ng/mg) between single (67.07) and multiple (70.96) ovulations (P=0.53; SED=4.8ng/mg). Nonetheless, the total P_4 content (µg/animal) was higher in cows with

multiple (574.9) than in cows with single (404.6) ovulation (P<0.001; SED=49.08 µg/animal).

As expected, P_4 content per milligram of CL tissue (49.59 vs. 69.02; SED=5.8 ng/mg; P<0.004) and per total luteal tissue (90.8 *vs.* 489.8; SED=49.8 µg/total luteal tissue; P<0.001) was lower in the early than in the mid-stage of the oestrous cycle. Additionally, the P_4 per total luteal tissue tended to differ between multiple and single ovulation cows during the early and mid-stages of the cycle (stage of CL interact with ovulation; P=0.08; Figure 3.9). The P_4 content luteal tissue in early stage of cycle was similar in both single and multiple ovulations. However, P_4 content per total tissue tended to be higher in multiple ovulations cows than single ovulation during the mid-luteal phase.

Table 3.7 *Progesterone concentration in luteal tissue at mid cycle of cows slaughtered over four seasons (winter; n=41, spring; n=41, summer; n=48 and autumn; n=44) in two consecutive years.*

Season	Progesterone		progesterone	
	ng/mg CL	SED	µg/animal	SED
Winter	78.47[a]	6.8 P<0.001	597.0	59.9 P=0.088
Spring	61.14[b]		430.7	
Summer	78.54[a]		508.8	
Autumn	57.92[b]		422.5	

[ab]= *different among seasons*

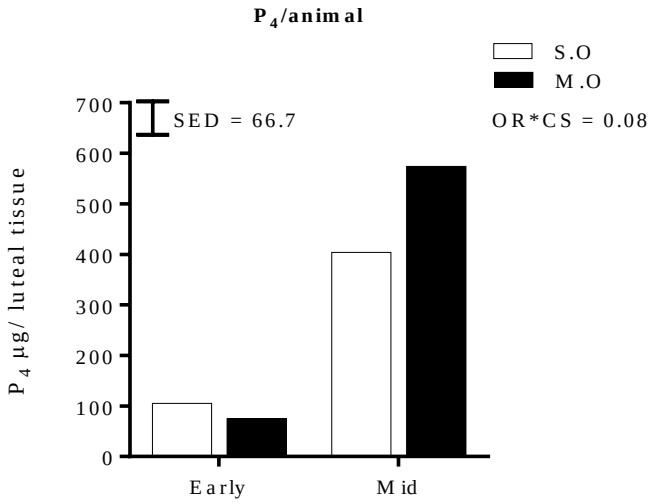


Figure 3.9 *Progesterone content in total luteal tissue of Holstein cows with single or multiple CLs on the ovaries. Ovulation rate (OR) tended (P=0.08) to interact (*) with stage of cycle (CS). Single ovulation (S.O) Early; n=26 and Mid; n=92 and multiple ovulation (M.O) Early; n=11 and Mid; n=45).*

3.3.5 Pathological conditions

Ovarian cysts in the current study were classified as follicular cysts, luteinized follicular cysts and non-ovulatory luteal cysts (Figure 3.10). The incidence of ovarian cysts was 5.8% with a greater incidence in summer and autumn (Table 3.8). Another abnormal condition observed was inactive ovaries in 5 cows. The overall frequency of ovarian adhesion was 0.5 %.

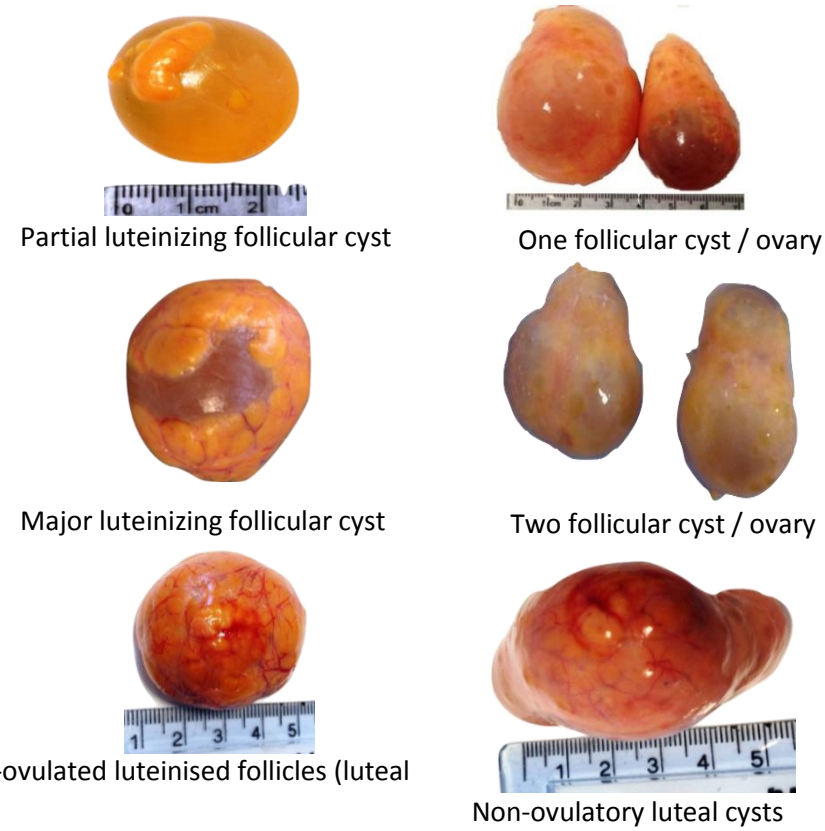


Figure 3.10 *Types of ovarian cyst in dairy cattle collected from slaughter house.*

Table 3.8 *Seasonal distribution of ovarian cysts in dairy cows slaughtered across the year.*

Season	Number of cows	Number of cows with cyst	Type of cyst		Cyst %	Odds	P value
			Follicular Cyst	Luteal Cyst			
Winter	393	20	18	2	5.1[ab]	Reference	
Spring	395	15	14	1	3.8[a]	0.73	0.38
Summer	401	24	18	6	6.2[ab]	1.24	0.58
Autumn	402	33	24	9	8.2[b]	1.67	0.08
Total	1591	92	74	18	5.8		

3.3.6 Localization of melatonin and its receptors in bovine ovarian tissue

Melatonin receptor A (MTNR1A) was expressed in the oocyte, GCs, luteal cells and endothelial cells of capillaries of the ovary. The expression of MTNR1A in theca cells was faint. In contrast, melatonin receptor B (MTNR1B) had weak or no expression in the ovarian tissue (Figure 3.11). The expression of both melatonin receptors was confirmed in cultured bovine GCs by immunofluorescence (Figure 3.12). In addition, large concentrations of melatonin were found in the follicular fluid of dominant follicles ranging from 50 to above 2000 pg/ml.

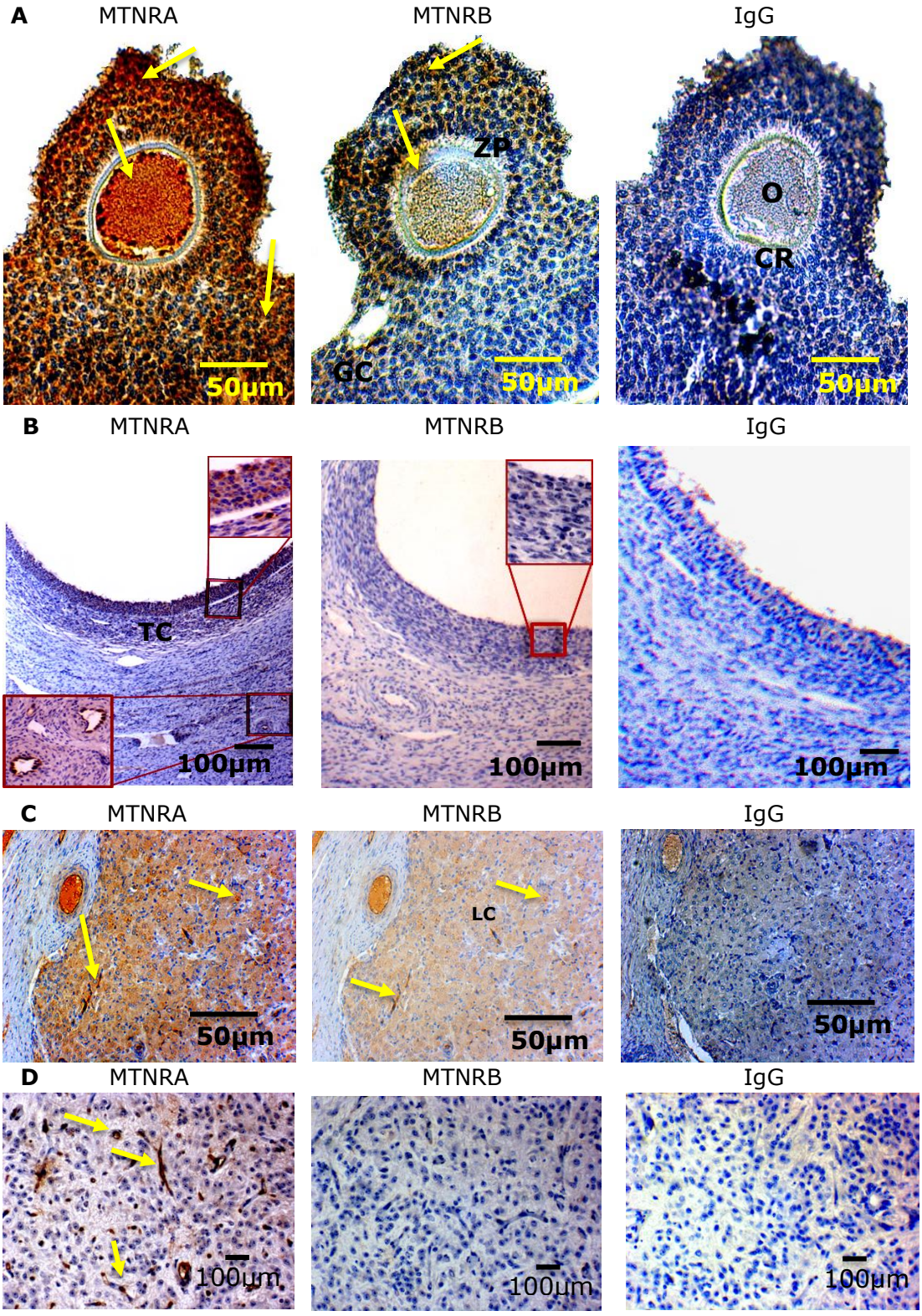


Figure 3.11 *Localization and distribution of melatonin receptor in the bovine ovary. A positive staining (brown) MTNR1A was strongly expressed in oocyte, cumulus cells (→ **A**), granulosa cells (→ **B**) and endothelial cells (→**C**). However, theca cells (TC) and luteal cells (LC) had weak expression. Positive immunostaining was observed in cytoplasm and most abundant on oocyte membrane. However, MTNR1B has weak or no expression. Negative control section (IGG) was incubated with goat IgG instead of primary antibody (IgG) and hypothalamus was used as a positive control (**D**). O = oocyte, ZP = zona pellucida, CC = cumulus cell and CR = corona radiate.*

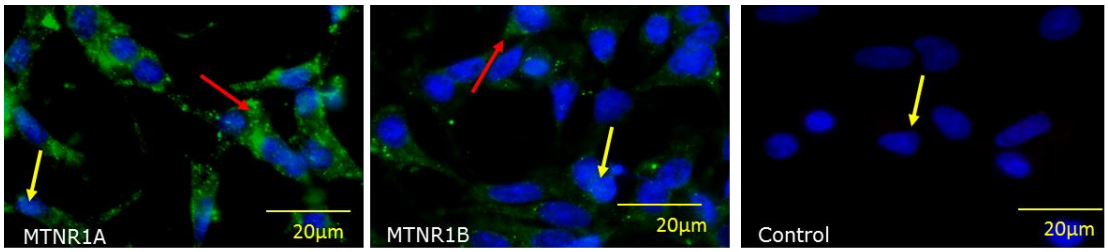


Figure 3.12 *Immunofluorescent detection of melatonin receptor on cultured bovine GCs. Cells were cultured as described in Section 4.2.3. Granulosa cells have dominantly MTNR1A expression (green colour; red arrow in MTNR1A). MTNR1B was also expressed in bovine GCs (green colour; red arrow in MTNR1B). Most abundant were expressed in cytoplasm. Control= negative control, GCs were incubated with PBS instead of primary antibody showed only DABI staining (blue colour; yellow arrow).*

3.4 Discussion

This study undertaken in a temperate climate provides evidence that seasonal factors can affect follicle development in dairy cattle. The frequency of multiple ovulations was higher in summer and autumn, and the incidence for ovarian cysts was also higher in summer and autumn. Additionally, the season affected CL weight and propensity to produce P_4. However, the numbers of antral ovarian follicles were not significantly affected by season. Possibly, the previous results may be the reasons for the variation of multiple ovulations during the warm and long daylight periods.

Follicular population

Previous studies indicate that antral follicle count is positively associated with cattle fertility (Ireland et al., 2010, Morotti et al., 2015). Therefore, we postulated that season of the year can lead to altered follicular populations in dairy cattle. In the current study, there was no significant effect of season on follicular population. On other hand, in hot regions antral follicle counts for 3 to 8 mm follicles decreased in summer (Zeron et al., 2001). However, large number of follicles sized 3-5 mm (Guzeloglu et al., 2001), 6-9 mm and ≥ 10 mm were observed in heat stressed cows (Roth et al., 2000). Generally, normal cows have an oestrous cycle with two (91%) to three (9%) follicular waves, whereas cows exposed to heat stress had 45%, 27% and 9% with three, four and five follicular waves respectively (Wilson et al., 1998b).

The diameter of ovarian follicles could alter during high environmental temperatures (Badinga et al., 1993, Wilson et al., 1998a, Wilson et al., 1998b, Trout et al., 1998, Zeron et al., 2001, Torres-Junior et al., 2008, Roth, 2015) and with multiple ovulations (Mann et al., 2007, Echternkamp et al., 2009). It has been reported that the number of large follicles were greater in heat stressed cows than control cows in the first follicular wave, and the dominant follicles in the second follicular wave emerged earlier in heat stressed cows (Wolfenson et al., 1995). In the current study, the diameter of the first presumptive dominant follicle possibly was greater in summer during early stage, whereas during late stage of cycle greater size was recorded in autumn and winter (season x stage P=0.05).

In a heat stress study, Torres-Junior et al. (2008) reported that the number of large follicles (>9mm) increased in heat stressed cows after removal from the environmental chamber. As previously reported by Roth et al. (2000) early antral follicle 0.5 to 1 mm could be sensitive to thermal stress. However, in a study performed in winter to identified the delay effect of heat stress on follicles, they reported that the diameter of medium sized and pre ovulatory follicles in previously heat stressed cows fail to reach significant level (Roth et al., 2001b).

Both inhibin and E_2 are an important factors to regulate FSH secretion (Mann et al., 1992, Kaneko et al., 1993). Impaired function of GCs under heat stress leads to increased concentrations of FSH (Roth et al., 2000). In an earlier study, the growth rate of pre-ovulatory follicles was greater and the largest subordinate follicle was recorded in summer (Badinga et al., 1994). Ineffective follicular selection mechanisms resulting more codominant follicles were related with P_4 reduction after cessation of heat stress (Torres-Junior et al., 2008).

Incidence of multiple ovulations

From above, increased multiple large follicles, prolonged dominance of ovulatory follicles and increased FSH might contribute to increased rates of multiple ovulations during summer. The incidence of multiple ovulations (Echternkamp et al., 2007, Echternkamp et al., 1990b), preantral follicle number (Cushman et al., 2000) and antral follicle number (Echternkamp et al., 2004a) were increased within the ovaries of cattle selected spontaneously for multiple ovulation. It has come to light that both large follicles and incidence of multiple ovulations increases during the summer. Regarding antral follicular counts and ovulation rate, the pairs of ovaries in multiple ovulation cows had greater numbers of large follicles (>7 mm) than cows with single ovulation. However, there was no different between single and multiple ovulations on follicles >10 mm.

It has been reported that follicle selection occurs around 7 mm and after this point only dominant follicles will continue to grow (Fortune et al., 1990, Ginther et al., 1996, Ginther, 2000). Total antral follicle count (>5mm) increased with increased multiple ovulations. While the diameter of individual ovulatory follicles was reduced with increased ovulation rate

(Echternkamp et al., 2009). They also reported that increased proportion on small ovulatory follicles related with multiple ovulations indicates that follicles at earlier stage of maturity were selected to ovulate or rescued follicles undergoing atresia.

The results of this study identified that the mean prevalence of multiple ovulations was 9.6 (ranging from 6.9 to 12.2%). In Ireland, the mean prevalence of multiple ovulations was 6.8 % (Fitzgerald et al., 2014). The highest incidence of multiple ovulations was recorded in summer and autumn, whereas the lowest rate was observed in winter. Thus, results of the present study are in accord with the previous studies performed in various regions, assuming a gestation length about 9 months, higher incidence of multiple ovulations in summer and autumn will increase the twinning rate in the next spring and summer. Similar outcomes were reported in the Poland in spring and summer (Sawa et al., 2015), Netherlands from April to September (Nielen et al., 1989), Saudi Arabia from May to June (Ryan and Boland, 1991), Midwest US in summer (Cady and Van Vleck, 1978, Sartori et al., 2002). The twinning rate was increased when conception were established from August to October (Del Río et al., 2007). The probability of multiple ovulations in Ireland increased between October and December, whereas greater prevalence of twin birth occurred in August and September (Fitzgerald et al., 2014). In a previous study with non-lactating dairy cows in the UK, animals synchronized on day 5 or 8 following oestrus prior to slaughter had high incidence of multiple ovulation 28.3% and there was no effect of season on ovulation rate (Mann et al., 2007). In contrast, a Spanish study with dairy cattle reported the highest multiple ovulations in winter, whereas the highest multiple birth rates were reported in summer (López-Gatius et al., 2005).

There is strong evidence suggesting an essential role of warmer periods increasing multiple ovulations (Sartori et al., 2002). Several factors may influence follicular development and multiple ovulations; 1. photoperiod stimulates milk production (Dahl and Petitclerc, 2003), 2. Heat increases codominant follicles (Torres-Junior et al., 2008) , 3. Increased FSH during summer (Khodaei-Motlagh et al., 2011). The first two factors are only seen during summer (hot and long photoperiod) and FSH is considered to be the

main factors increasing multiple ovulations (Glick et al., 2013). The proposed mechanisms are shown in (Figure 3.13).

Increased twinning rate when animals conceive during cooler, short daylight could be attributable to the reduction of direct and delayed effects of hot and long daylight on embryonic development and failure of implantation. Pregnancy loss and embryonic reduction during early period of gestation were higher in cows diagnosed with multiple pregnancies (Silva-del-Río et al., 2009). These could be supported by the current results that ovulation rate tended (P=0.08) to interact with the stage of oestrous cycle to affect P_4 content luteal tissue. The total luteal P_4 content per animal during early stage of cycle was similar or lower in animals with multiple CL compared with single CL. However, in mature CL stage P_4 concentration was higher in animals with multiple CL. One of the limitations of the above results, is that the stage of CL development has remarkable effect on P_4 production which is mean that P_4 content luteal tissue has strong correlation with CL development particular during early development. Therefore, it will difficult to compare the early CL stage between single and multiple CL as it may be in different stage of development.

Corpus luteum weight

This study proposes that season affected the development of CL and P_4 secretion. The individual weight of CL and total luteal weight has a tendency to be heavier in autumn and lighter in spring season. The results related to seasonal variation was in agreement with Edwards (1962) the heaviest CL weight was demonstrated in autumn season and lighter CL weight in spring season. The individual CL weight of cow that had multiple CL was significantly lighter than cow had single CL. However, the total luteal weight increased in cows with each extra CL. Similar outcomes demonstrated by (Mann et al., 2007). Another study reported that the diameter of individual CLs reduced and total volume of CL increased with each additional CL in ovarian surface (Echternkamp et al., 2009).

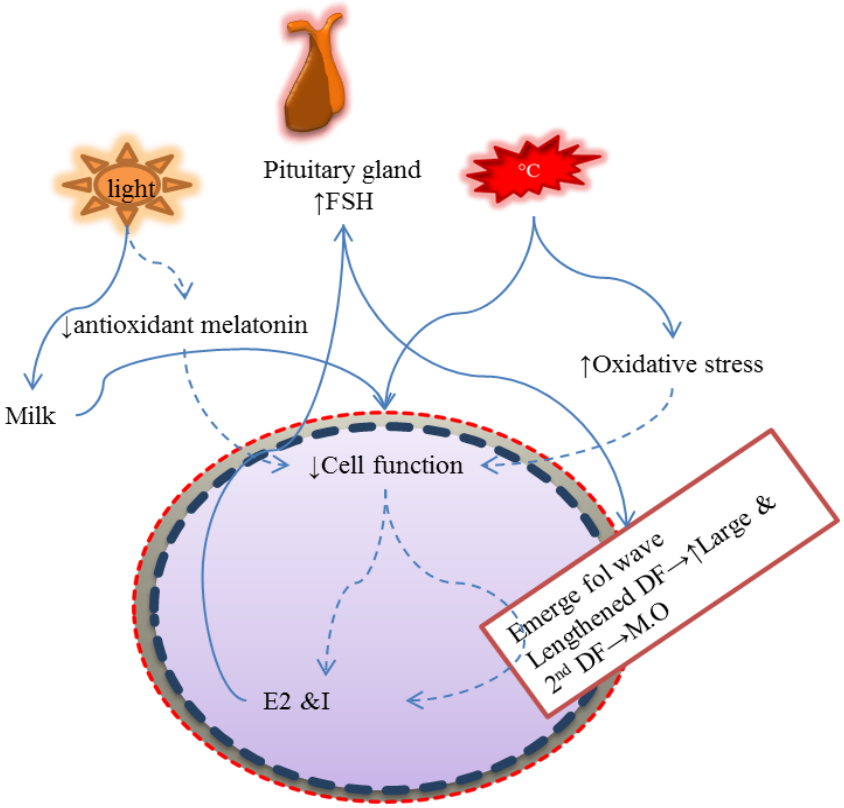


Figure 3.13 *Proposed effect of season on the ovarian follicle, influencing multiple ovulations through increased number of large follicles (i.e. more follicles could pass selection to become dominant follicles). This could be the effect of season on the factor responsible for suppression of FSH at time of selection.*

Progesterone content luteal tissue

The concentration of P_4 in luteal tissue per animal was similar to that reported by Hansen et al. (1987). The P_4 content in luteal tissue did not differ between winter and summer. Parallel findings were observed in serum and luteal tissue (Rhodes et al., 1982). In the current study, the P_4 content of corpora lutea was lower in spring, and the lowest plasma concentrations of P_4 was also reported in spring (Badinga et al., 1994). However, total P_4 concentration per animal was similar between seasons but higher concentrations tended to be reported in winter. An earlier study reported that plasma P_4 levels decreased in summer compared to winter (Washburn et al., 2002). Recently, reduced luteal activity in hot period leads to decrease plasma P_4 concentration (Satheshkumar et al., 2015). This could be supported by increased oxidative stress and reduced total antioxidant activities during heat stress (Ganaie et al., 2013). Additionally, melatonin reduction in long daylight as a potent antioxidant (Tan et al., 2015) could influence negative outcome of heat on granulosa and luteal

cell functions. These could subsequently impair gonadotropin receptor expression and inhibit steroid production (Vega et al., 1995) and prevent intracellular cholesterol transport to mitochondria and reduced P_4 production (Behrman and Aten, 1991). However, the inconsistence results about seasonal effect on P_4 concentration has been proposed to be related with several factors such as luteal blood flow (Lublin and Wolfenson, 1996), heat exposure, metabolism of P_4 in liver, volume of blood, nutrition, lactation stage, and age of animals (Jonsson et al., 1997, Trout et al., 1998). Therefore, the concentration of P_4 in blood not only depends on the P_4 production but also on the secretion into the circulation (Roth, 2015).

The progesterone content in corpora lutea in single and multiple ovulations did not differ in the current study. Similar results was demonstrated by (Mann et al., 2007). However, the total concentration of P_4 per animal was higher in animals with multiple CLs than animals with single CLs. Similar results were reported by Echternkamp et al. (2009).

Incidence of follicular cyst

Increased number of ovarian cyst and deficiency of CLs may increase the frequency of multiple ovulations (Silva-del-Río et al., 2009). The incidence of both follicular cysts and multiple ovulations in the current study was highest in summer and autumn. The highest ovarian cyst ratio was recorded in autumn, which confirms the pattern found in Sweden (Emanuelson and Bendixen, 1991) and in Norwegian dairy cattle (Nelson et al., 2010). The latter study concluded that ovarian cystic disease correlated with seasonal factors and multiple births. These results lead to the suggestion that not only high temperatures but also day length affects the mechanisms of follicular development.

Melatonin

Attention of melatonin secretion and its role in reproductive system have been undertaken by several groups (Tamura et al., 2008a, Reiter et al., 2009). Melatonin considered as an antioxidant (Tamura et al., 2012) and powerful free radical scavenger (Tamura et al., 2013), influence steroid secretion in luteinizing GCs (Webley and Luck, 1986, Taketani et al., 2011, Wang et al., 2012), protects DNA from damage (Takada et al., 2012), oocyte quality (Tsiligianni et al., 2009, El-Raey et al., 2011), embryo

development (Sampaio et al., 2012, Wang et al., 2014) and implantation. Also, the melatonin concentration in multiple pregnancy were higher during the first six months of gestation (Nakamura et al., 2001). Furthermore, high concentration of melatonin in follicular fluid has been reported in humans (Yie et al., 1995). The results from this study demonstrated high and variable concentrations of melatonin in bovine ovarian follicular fluid during the follicular phase in large follicles. Also, melatonin receptor has been expressed in bovine ovarian tissue, intensive expression was observed in GCs and oocyte. Also, melatonin receptor expression was revealed in cultured GCs by immunofluorescence. There are consensuses in the melatonin receptors expression in bovine GCs, cumulus cells, oocyte (El-Raey et al., 2011). Based on these results, expression of melatonin receptors and high level of melatonin in follicular fluid mean it could have an important role in the reproductive system in particularly follicular cells.

It has been reported that the percentage of pregnancy diagnosis was higher in female with multiple ovulation, whereas the frequency of pregnancy loss was greater in female bearing multiple foetus (Echternkamp et al., 2009). Perhaps more luteal cells formed from GCs which subsequently produce more P_4 during dark cool season, may help animal to maintain multiple fetus during implantation and early embryonic development. Perhaps, reduced CL function resulted from reduced GCs function and undergoing luteinisation.

3.5 Conclusion

This study is the first detailed research of seasonal effects on ovarian characteristics in temperate climates in dairy cattle. The results show that season has no significant effect on antral follicle count. Conceivably, two years-worth of data could be a small group to offer confident results, and a larger study could produce higher levels of confidence. Season could increase incidence of multiple ovulations and ovarian cysts in temperate climate which in turn affects CL and luteal P_4 content.

The different categories of follicular diameters were similar between seasons. However, follicle numbers were higher in the warmer and long-daylight periods could increase the incidence of multiple ovulations and cystic ovaries during the summer and autumn season. This study revealed the heaviest CL weight in autumn. However, total P_4 concentration in luteal tissue per animals tended to be greater in winter. Total CL weight and P_4 content were higher in multiple ovulations than in animals with single CL. The results from the current and previous chapter reveal that season can influence cattle fertility in temperate climates. Ovarian characteristics in cattle are not the only aspect to quantitatively interfere with seasonal infertility of cattle. It has been accepted that the pineal gland regulates reproduction in mammals. Subsequent research *in vitro* are essential to quantify how season (melatonin and temperature) exerts its effect on bovine GCs.

The presence of melatonin in the follicular fluid and melatonin receptor in follicular cells encourage next studies to focus on melatonin's role. The next chapter will focus on GC culture in the presence and absence of melatonin. We propose that season could affect cattle fertility through increasing oxidative stress and reduced steroidogenesis during high temperature and long day photoperiod. It would be better to develop new culture system mimic physiological O_2 levels, to address and separate the effects of melatonin and temperature on GCs.

Chapter 4: The effect of melatonin on bovine granulosa cells cultured under low or high oxygen tensions

4.1 Introduction

The biological functions of GCs are critical for normal ovarian function. Granulosa cells produce a variety of hormones essential for TC function and oocyte maturation (Rawan et al., 2015, Li et al., 2016). Granulosa cell produce factors that stimulate TC growth and differentiation (Yada et al., 1999). Culture of bovine GCs can serve to study physiological functions of GCs. However, GC luteinisation *in vitro* limits its utility. For many years, somatic cells derived from the ovarian follicle have been cultured under atmospheric conditions supplemented with carbon dioxide. However, cells within the body develop in a hypoxic environment and new applications for cell culture *in vitro* require conditions mimicking those in *vivo*.

Most cellular processes from cell metabolism to cell division require O_2. Oxygen concentrations within the body are lower than atmospheric O_2 (20%) levels (Ivanovic, 2009). Additionally, O_2 levels within tissues vary depending on the distance from arterial blood. Accordingly, most cells within tissues develop at lower O_2 concentrations than those in typical culture (Ivanovic, 2009). Therefore, atmospheric normoxia should be considered as tissue hyperoxia. It has been reported that high O_2 concentrations stimulate cell differentiation (Ivanovic, 2009). Therefore, GC differentiation and luteinisation may be reduced under low O_2, and GC production of E_2 may be extended.

Oxygen analyses in follicular fluid have been studied by several researchers (Van Blerkom et al., 1997, Clark and Stokes, 2011). Within the ovary, vascularization of follicles is restricted to the theca layer, and GCs and oocytes are avascular. Blood vessels of the theca layer increase both in number and size, when the follicle develops. However, the vessels do not penetrate the GC layer (Redmer and Reynolds, 1996, Tamanini and De Ambrogi, 2004). Ovarian blood flow in the follicle reduces towards ovulation, and regularly rises with luteal formation (Wise et al., 1982). Hence, lower concentrations of O_2 in large compared to small follicles have

been reported (Fischer et al., 1992). Haemoglobin has been discovered recently in human follicular cells (Brown et al., 2015). This could regulate O_2 levels in the ovarian follicle.

One of the factors responsible for reactive oxygen species (ROS) production is the steroidogenic enzyme cytochrome P450 (Hanukoglu, 2006). Also several physiological events of GCs are modulated by ROS formation (Griendling et al., 2000). In response to ROS, superoxide dismutase is one of the first compounds produced (Matés, 1999) and low O_2 concentrations increase SOD and peroxidase activity leading to a reduction of ROS production by GCs (Basini et al., 2004). The imbalance between ROS and it is antioxidant lead to oxidative stress. Increased oxidative stress and SOD activity in erythrocyte during the hot season has been reported in dairy cattle by Bernabucci et al. (2002).

Melatonin not only regulates circadian rhythms but also plays a crucial role in seasonal breeding. Additionally, the main function of this indole amine is as a free radical scavenger and broad spectrum antioxidant (Korkmaz et al., 2009). This potent antioxidant ability may treat the imbalance of ROS which have a decisive impact on ovarian function (Fernando and Rombauts, 2014). Melatonin can serve both as an oxygen scavenger and stimulus for antioxidant enzymes (Rodriguez et al., 2004). Interestingly, melatonin metabolites exhibit antioxidant capacity which makes melatonin differrent to other antioxidants (Reiter et al., 2007). Taken together, both high concentrations of melatonin (Nakamura et al., 2003) and low concentrations of O_2 (de Castro e Paula et al., 2008) in ovarian follicular fluid indicates that both factors could interact to regulate cell development and function. It has been reported that melatonin blocked hypoxic response by enhancing apoptotic factors in tumour cells (Lee et al., 2014), and melatonin affects O_2 transportation and enhances tissue oxygenation (Hlutkin and Zinchuk, 2008). Futhermore, melatonin as a 'dark hormone' could be the factor that induces oestrus in cows at night (Pinheiro et al., 1998). Therefore, melatonin could influence steroidogenesis.

This chapter considers the culture of GCs *in vitro* in order to study direct effects of O_2 and melatonin. Despite cells being removed from their natural

environment, cell culture has several potentially useful quantifiable applications. One such application is to address cell proliferation and steroid production. There is enough evidence from the literature that GCs within follicles develop under low O_2 (Thompson et al., 2015). Taken together melatonin and O_2, raises an intriguing question: do GCs growing under low O_2 differ from those cultured under atmospheric O_2? If so, what is the role of melatonin on GCs function? According to our knowledge, this is the first study to evaluate the mechanism of melatonin action on bovine GCs under physiological levels of O_2.

Hypotheses

1. Culturing GCs under physiological O_2 maintain near-normal physiological functions critical for fertility.
2. Melatonin treatment under low O_2 will enhance aromatase activity and steroid production by cultured GCs.

To test the first hypotheses, we used an *in vitro* model of bovine GCs. Bovine GCs were cultured for up to 144 h in either under 5% CO_2 and 5% O_2 or 5% CO_2 in atmospheric O_2. Cell proliferation, steroidogeneses and aromatase activity were evaluated.

To test the second hypotheses, GCs were cultured under low and high O_2 with different levels of melatonin. Cell proliferation, steroidogenesis, ROS generation, and molecular markers of oxidative stress, melatonin biosynthesis and haemoglobin expression were determined.

4.2 Materials and Methods

4.2.1 Experimental design

Bovine GCs were cultured in Exp. **1** in a 3 X 2 factorial arrangement (3 time points, 2 O_2 concentrations) and Exp. **2** and **3** as 4 X 3 X 2 factorial arrangements (3 time points, 2 O_2 concentrations and 4 melatonin doses). Melatonin was added at 0, 20, 200, and 2000 pg/ml (Table 4.1). Melatonin doses could be mimic the minimum and maximum physiological concentration in the body fluid according to the Table 1.4.

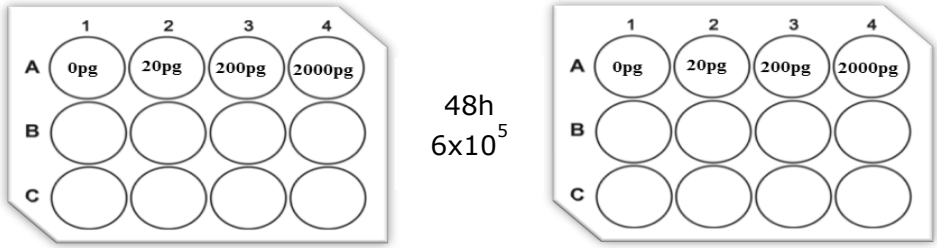
Table 4.1 *Granulosa cells cultured in different plates and seeded at different cell densities considering growth area. All experiments were incubated under low O_2 (5%) and air (20% O_2) for 144 h with collection, harvesting and refreshing culture media at 48, 96 and 144 h.*

Exp.	Plate	Cell density	Culture media	Replicated experiments	Within plate replicates	Growth area (cm^2)
1	6 wells	1.5 X 10^6	2 ml	5	2 wells	9.5
2	12 wells	0.6 X 10^6	1 ml	4	2 wells	3.8
3	96 wells	6 X 10^4	250 µl	3	5 wells	0.143

Granulosa cells were cultured in two separate incubators. One group was incubated under air (20% O_2) with 5% CO_2. The second group was incubated in 5% O_2 and 5% CO_2. The low O_2 concentration was performed in humidified incubator. Only two gases were supplied carbon dioxide (CO_2) and nitrogen to reduce the O_2 level.

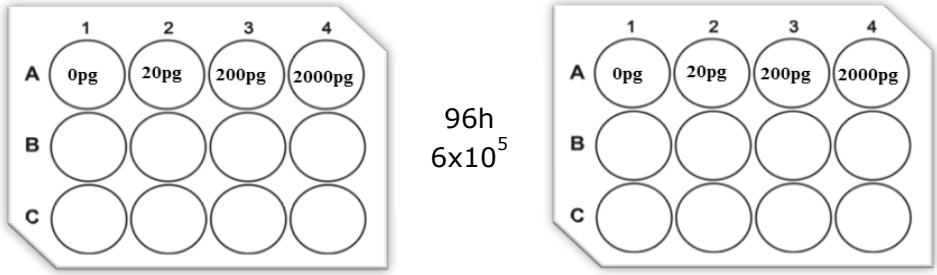
Oxygen level was controlled by incubator sensor. The concentration of O_2 and CO_2 were monitored by incubator reading and further reading for CO_2 and O_2 level were analysed by O_2/CO_2 analyser (Servomex survey mix 1440 analyser) before culturing GCs. Temperature of incubators were also checked by three thermometers; incubator digital screen, external electronic thermometer and external probe thermometer (Temperature loggers, EL-USB-1, Lascar Electronics, Salisbury, UK). To evaluate the effects of melatonin on GCs in Exp. **2** and **3**, cells were cultured in the presence of different melatonin doses (0, 20, 200 and 2000 pg/ml) for 48, 96 and 144 h (Figure 4.1).

48 h **5% O_2** **Air**



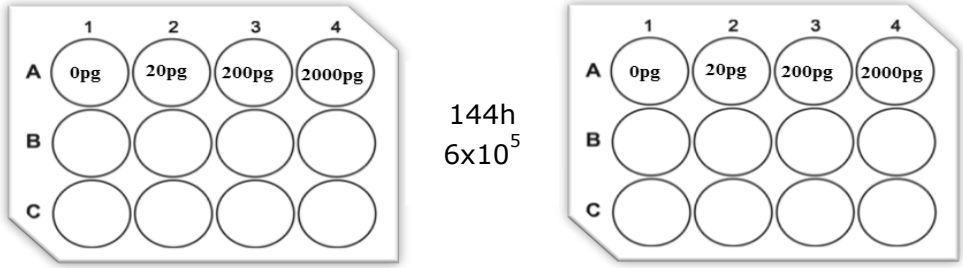
- cells and culture media from both plates (20% and 5% O_2) (duplicate well / each treatment) were harvested at 48 h and media replaced for 4 remaining plates with freshly added melatonin doses.

96h



- cells and culture media from boths plates (20% and 5% O_2) (duplicate well / each treatment) were harvested at 96 h and media replaced for 2 remainng plates with freshly added melatonin doses.

144h



- cells and culture media from both plates (20% and 5% O_2) (duplicate well / each treatment) were harvested at 144 h.

Figure 4.1 *Experimental design for GCs cultured under atmospheric and low O_2 in the presence and absence of melatonin for Exp. 2.*

4.2.2 Materials and reagents

All chemicals were obtained from Sigma (Sigma Aldrich, company Ltd, Gillingham, Dorset), if not otherwise stated. Granulosa cell culture media contained Medium 199, penicillin (50 IU/ml), streptomycin (50 µg/ml), bovine serum albumin (1 mg/ml) free fatty acid, testosterone (100 ng/ml), bovine FSH (1 ng/ml), insulin (10 ng/ml) from (ITS), transferrin (2.5 µg/ml), sodium selenite (4 ng/ml) and L-glutamine (3654 µg/ml). Trypsin/EDTA (0.05%), fibronectin (6 µg/ml), trypan blue dye and

Dulbecco's PBS without calcium and magnesium (DPBS). Nicotinamide adenine dinucleotide phosphate (NADPH; Santa Cruz), TES, EDTA, KCL and protein inhibitor.

4.2.3 Ovarian collection

Bovine ovaries were collected post mortem from a local slaughter house. Typically, 120 ovaries were collected per biological replicate and placed in a thermally insulated flask containing pre-warmed (38°C) phosphate buffered saline (PBS). The ovaries were washed by sterile pre-warmed PBS to remove debris and blood. Ovaries were rinsed with 70% ethanol briefly than washed again with pre-warmed PBS.

4.2.4 Granulosa cell culture

Antral follicles (4-6 mm in diameter) were aspirated using a 21 G sterile needle. Follicular fluids were centrifuged for 2.5 min at 1000g. Pellets re-suspended with 2 ml pre warmed 1 X dPBS to each tube. The cell suspension was combined into a 15 ml tube, centrifuge again at 1000g for 2.5 min, and the supernatant removed. Red blood cells were lysed by adding 4.5 ml pre-warmed dH_2O followed immediately by adding 500 µl of pre-warmed 10 X dPBS to avoid GC death. Cells were centrifuged at 1000g for 2.5 min and the supernatant removed and re-suspend in 2 ml pre-warmed 1 X dPBS.

Cell suspensions were then filtered through the cell strainer 70 µm (Fisher Scientific UK Ltd., Loughborough, Leicestershire), and placed in a 50 ml falcon tube to remove any debris, clumps and oocytes. The cells were then centrifuged at 1000g for 2.5 min and the supernatant removed. Re-suspended cell pellet in 1 ml of M199 culture medium incubated for at least 2 h before using. According to previous research, M199 is the common medium using for GC culture (Li, 2012). The cell pellet was dispersed by 1 ml pipette. Finally, the cell viability was assessed by transfer of 10 µl of cell suspension to 290 µl dPBS and 50 µl of trypan blue in 1 ml Eppendorf tube and mix well by pipetting. 10 µl of cell suspension was transferred and counted with a haemocytometer. Fibronectin coated wells (Nunclon

microtiter) were seeded at the rate of viable cells per culture medium as shown in (Table 4.1).

Three plates were incubated at atmospheric O_2 (20% O_2, 5% CO_2) and the remaining three plates were cultured under 5% O_2 and 5% CO_2; both at 37.5°C (Van Blerkom et al., 1997). The temperature was chosen to be the optimal temperature according to previous research (Grøndahl et al., 1996, Hunter et al., 2000). Cells were cultured for 144 h in the absence of melatonin in the first experiment. The second and third experiments included melatonin (0, 20, 200 and 2000 pg/ml). Cells and supernatant were harvested at three time point 48, 96 and 144 h post seeding. For each time point, 80% of cell supernatant was removed and replaced by 80% of fresh equilibrated culture media (incubated culture media under humidified atmosphere for at least 2 h before use) to each well.

4.2.5 Cell number and viability

Culture media from each well were transferred to Eppendorf tubes. After centrifugation at 10000g for 1 min, spent media were placed into fresh tubes and snap frozen in liquid nitrogen before being kept at -80°C until hormone analysis.

4.2.5.1 Trypan blue

To harvest GCs, 1000 (Exp. **1**) or 500 (Exp. **2**) µl of pre-warmed trypsin-EDTA were added to each well and incubated at 37.5°C for 5 min. The attached cells were dislodged from wells by pipetting and 200 (Exp. **1**) or 100 (Exp. **2**) µl of heat inactivated fetal calf serum (HI FCS) were added to each well and mixed to cancel the trypsin effect. Next, cell suspensions were transferred into Eppendorf tubes and well rinsed with 1000 (Exp. **1**) or 500 (Exp. **2**) µl of pre warm dPBS and combined with previous cell suspensions. Cell suspensions were then centrifuged at 10000g for 2 min and the supernatant removed and pellet re-suspended in 500 (Exp. **1**) or 250 (Exp. **2**) µl pre-warmed 1 X dPBS. Before counting cell number tubes were manually agitated by the hand "finger flick" and mixed well by gentle pipetting. An aliquot of 10 µl cell suspension was used for counting GCs. The remaining cells were centrifuged at 10000g for 2 min and the

supernatant removed. The GCs pellets were then snap frozen in liquid nitrogen and stored at -80°C until protein for Exp. **1** and RNA extraction Exp. **2.** The number of the GCs was determined by adding 10 µl of trypan blue to 10 µl of cell suspension and counted with a haemocytometer.

4.2.5.2 Crystal violet

Granulosa cells were cultured as described in Section 4.2; Chapter 4. Cells were seeded at a density of 6 X 10^4 live cells per well in 96 well plates with 250 µl of medium (Exp. **3**). After 48 and 96 h of culture 75% of culture media was replaced by fresh culture media.

For the *in vitro* proliferation assay, at each time point (48, 96, and 144 h of culture) all culture media were removed. Cell proliferation was assessed and changes in cell number quantified by crystal violet (Chiba et al., 1998) with slight modification. Crystal violet is a colorimetric reagent which only stains the nuclei of attached cells (Siddiqui et al., 2006). Briefly, live GCs were fixed by adding 100 µl of methanol for 5 min. After methanol was removed from wells, 100 µl of 2% Crystal violet solution was added to each well and incubated for 15 min. After penetration of crystal violet dye, cells were washed three times with distilled water and air dried. Finally, 70% ethanol was added to each well and shaken for 5 min to solubilise the crystal violet and the absorbance was read on spectrophotometer at 570 nm wavelength.

The viability of cells was measured based on the intensity of relative dye. The optical densities for blanks (containing culture media) were subtracted from each sample including standard curve. To quantify cell viability by standard curves, the optical densities were plotted against known cell numbers using linear standard curves. Cell numbers were adjusted by interpolating from a standard curve using prism software (coefficient of determination R^2=0.98).

4.2.5.3 Immunofluorescent detection of proliferation marker Ki67 and aromatase CYP19A

The proliferation rate of GCs was determined by immunofluorescent detection of Ki67. Ki67 is a nuclear protein which is strictly associated with

the cell proliferation (Schlüter et al., 1993). Specifically, Ki67 is a cell cycle protein expressed during active phases (G1, S, G2 and mitosis) (Scholzen and Gerdes, 2000). Granulosa cells were cultured as described in Section 4.2.4; and immunofluorescent as described in Section 3.2.6; primary CYP19 antibody (polyclonal Goat anti-human Sc-14244; diluted 1:200) with secondary antibody bovine anti-goat IgG (diluted 1:500; Sc-2347), and primary Ki67 antibody (rabbit polyclonal to human Ki67; ab15580; diluted 1:150) with secondary antibody goat anti-rabbit IgG (diluted 1.250; ab6717). Immunofluorescent staining for the expression of Ki67 differed slightly from previously described (Manoir et al., 1991). Cells were fixed with methanol: acetone (1:1) for 20 min at -20°C then washed 3 times with wash buffer (1 X PBS with 1% BSA) for 5 min. Next, cells were incubated with primary Ki67 antibody and incubated for 90 min at room temperature. After washing 3 times for 10 min each, coverslips were incubated with secondary antibody for 60 min then washed 3 times for 10 min. Finally, mount the coverslip on slide with Vectorshield and DAPI then sealed the slides and kept in dark and cold.

4.2.6 Hormone analyses

4.2.6.1 Progesterone

Analysis of P_4 in GC supernatant was done by ELISA. The assay was performed as described in Section 3.2.2.2. Progesterone concentrations were analysed in spent culture media for cells cultured for 48, 96 and 144 h in the presence of melatonin 0, 20, 200 and 2000 pg/ml under both 5% O_2 and air. Samples were diluted 100 fold in PBS and analysed in duplicate alongside standard (0, 0.5, 1, 2, 5, 10 and 20 ng/ml), blank and quality control. To reduce variation among plates, all samples were run together. Inter- and intra-assay CV were 11.1% and 5.1% respectively.

4.2.6.2 Oestradiol

Oestradiol concentrations in the GCs media were measured by ELISA kits (DRG GmbH, Germany. EIA-2693). Based on the competitive binding assay with the detection range of 9.7- 2000 pg/ml. The samples were run according to the manufacturer procedure. Briefly, all reagents and required

strips of the plate were brought to room temperature. 25 µl of (diluted 20 fold samples with 1 X PBS) were dispensed in duplicate along with the standard curve, blank and quality controls (low 41-86 and high 242-504 pg/ml). Followed by the addition of 200 µl E_2 conjugated to horsedish peroxidase enzyme. The plate was mixed thoroughly on the plate vortex for 10 s and incubated for 2 h at room temperature. After shaking out the contents of each well, the plates were washed three times with 400 µl of wash buffer. At each wash, the plates were struck sharply on tissue paper to remove residual droplets. Then 100 µl of substrate solution (tetramethylbenzidine) was added to each well and incubated for 15 min at room temperature. To stop the enzymatic reaction 50 µl of stop solution were added to each. Finally, the absorbance (optical density) was determined at 450 nm with a microtiter plate reader using Thermo Labsystems Multiscan Ascent plate reader (Thermo Fisher, Loughborough, UK). The concentration of E_2 were analysed from standard curve using four parameter curve fit. Inter- and intra-assay CV were 8.6% and 6.8% respectively.

4.2.7 Aromatase assay

4.2.7.1 Protein extraction

Granulosa cells (~1 X 10^6 cells/tube) were homogenized in 200 µl of aromatase buffer solution pH 7.4 (20 mM TES, 10 mM EDTA, 150 mM KCl, Protease inhibitor Cocktail (cOmplete ULTRA Tablets)) using tissue and cell homogenizer (Fast Prep-24, model 6004-500) for 30 s. The protein was then extracted by centrifugation at 1000g for 5 min. Then the supernatant was transferred into another tube and centrifuged again to remove the pellets and debris. All steps were performed in ice or at 4°C and samples were stored at – 80°C until protein assay and aromatase activity assay.

4.2.7.2 Optimization of protein assay

Both Bradford and BCA protein assays were used to identify the most appropriate protein assay, considering the compatibility of assay with working solution and appropriate optical observation and/or to select the more accurate standard curve. The standard curve was prepared by using

bovine serum albumin (BSA; A8806) (62.5, 125, 250, 500, 750 and 1000 µg/ml) standard curves were included in each run.

Protein assay in the GC homogenates were used according to the manufacturer protocol. Briefly, for Bradford assay, 5 µl of each standard and blank were added to 96 well plates in triplicate. After added 100 µl of BioRad solution to each wells, plates were incubated for 30 min at room temperature. Finally, the plates were shaken for 10 s and the optical density was measured at 595 nm using a microplate reader.

For BCA assay, 25 µl of standard and blank were added into 96 well plates in triplicate. Next, 200 µl of protein working solution (BCA solution and 4% cupric sulphate) were added to each well. Protein solutions were prepared before starting the assay by mixing 200 µl of BCA solution with 4 µl of cupric sulphate. The plate was then shaken for 30 s and incubated for 30 min at 37°C. After incubation, the plates were left at room temperature to cool and the optical densities were detected by plate reader using 562 nm wavelength. Figure 4.2 reveals that the BCA assay obtained more accurate protein concentrations in samples than the Bradford assay. However, BCA required more protein.

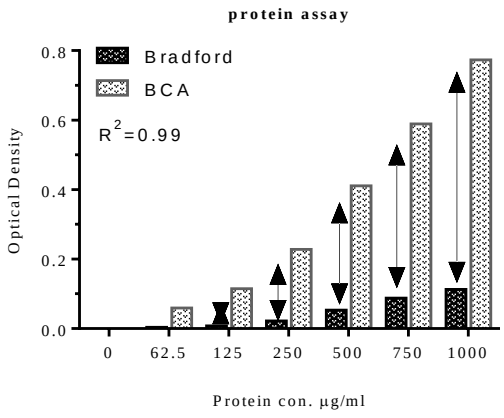


Figure 4.2 *The optimization of protein assay performed by BCA and Bradford assay. The concentration of protein extracted from bovine GCs.*

4.2.7.3 Detection of aromatase activity by measuring oestrogen

Aromatase activity was determined by incubating GC protein with testosterone and NADPH (Nicotinamide adenine dinucleotide phosphate, Santa Cruz, sc 202725). According to the previous studies described in rat ovary (Satoh et al., 2008, Tinwell et al., 2011), aromatase activity were detected in bovine GCs protein with slight modification. Briefly, 60 µg of GCs protein per tube was mixed with 100 nM testosterone and 10 mM NADPH, added aromatase working buffer to a final reaction volume of 200 µl/tube (pH 7.5). Then the tubes were incubated for 25 min at 37°C. After incubation, enzyme activity was terminated by heating the tube at 100°C (block with water using; DRI-BLOCK, DB3) for 5 min. Samples then kept on ice and stored at -80°C until measurement of E_2 by ELISA as described in Section 4.2.6.2.

The data spanned 5 biological replicates with duplicate wells in Exp **1**. However, for the aromatase assay, total protein for duplicate wells was mixed and run as duplicate wells for (**A**) aromatase activity and (**B**) inactivate aromatase (to determined intracellular E_2 content). Each sample was divided into two groups before measuring aromatase activity. The first group was kept on ice and the second group was heated at 100°C for 10 min to stop aromatase reaction than kept in ice. After cooling, the first tube (active aromatase reaction) and the second (inactivated aromatase reaction) were used for aromatase assay (Figure 4.3).

A series of experiments were done to optimize the aromatase method. Three protein concentration (30, 60 and 90 µg/tube) and five incubation times (10, 20, 30, 40 and 60 min) were assessed to obtain optimum aromatase activity conditions. The results indicated selecting 60 µg/tube. After combining the two wells at cell density ~1.5 million cells, the protein concentration was enough to run 60 µg / tube. In terms of incubation time 25 to 30 min of incubation was found to be the optimum time for aromatase activity because longer incubations plateaued (Figure 4.4).

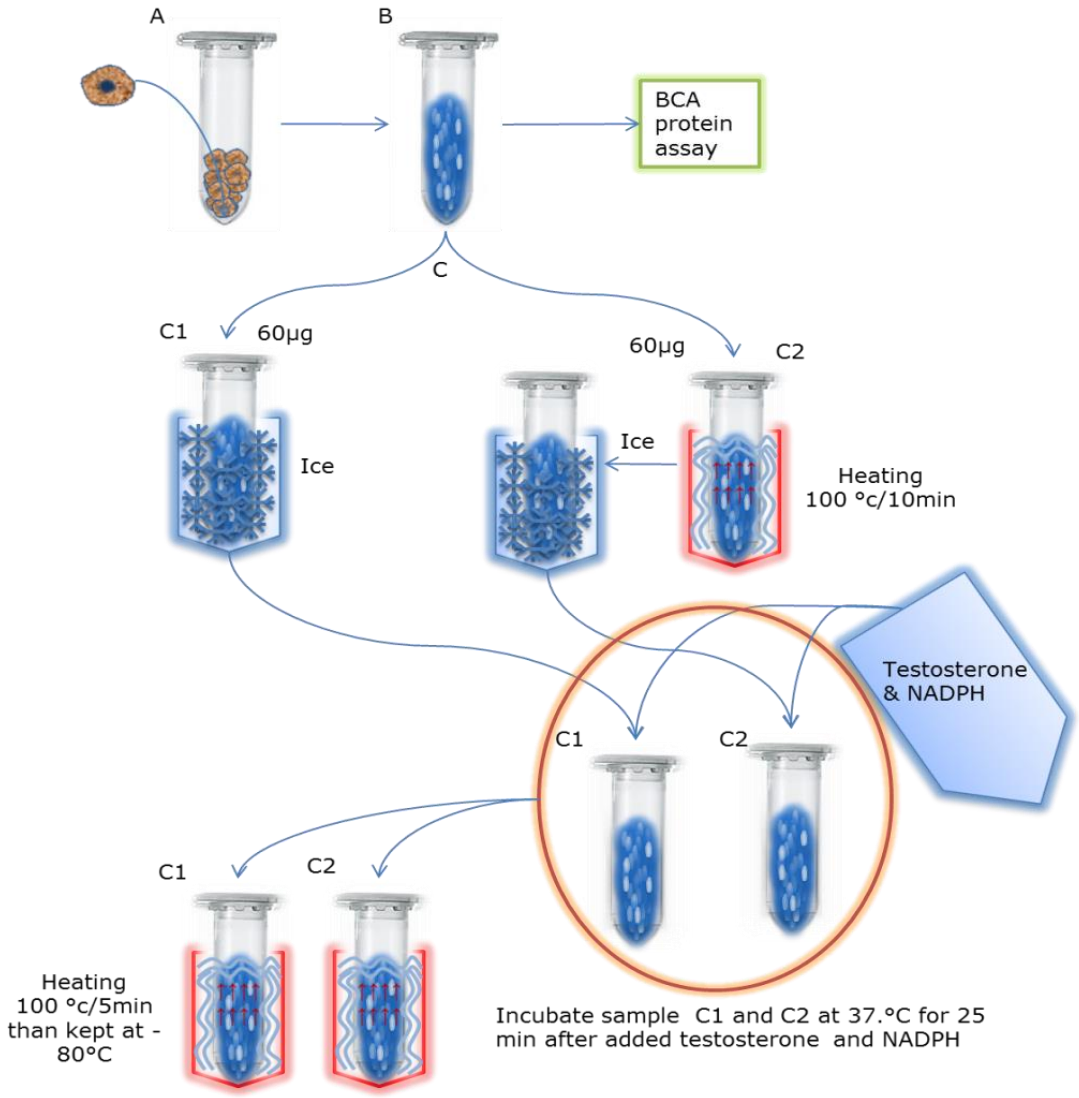


Figure 4.3 *Experimental design for aromatase assay.* ***A****) GCs (at density~3 X* 10^6 *cells / tube) harvested under low (5%) and high (20%) oxygen incubated for 48, 96 and 144 h,* ***B****) means homogenise cells,* ***C****) cell lysates were divided into two tubes containing 60 µg,* ***C1****) kept on ice and* ***C2****) heated at 100°C for 10 min then kept on ice, then both* ***C1****) and* ***C2****) were incubated for 25 min at 37°C after adding NADPH and testosterone. Finally, the reactions were terminated by heating (****C1*** *and* ***C2****) for 5 min and kept at -80°C.*

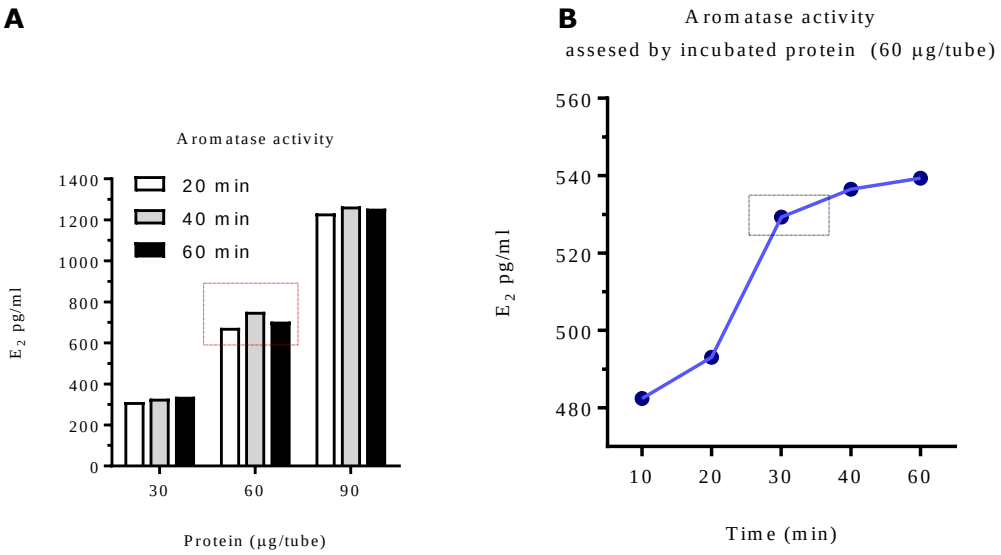


Figure 4.4 *Effect of protein concentration (**A**) and incubation time (**B**) on aromatase activity by measuring E_2 production in the presence of 100nM testosterone and 10 mM NADPH, at a final validation volume of 200 µl/tube. Box mean selected protein concentration (**A**) and incubation time (**B**).*

4.2.8 RNA extraction and reverse transcription

4.2.8.1 Extraction of nucleic acids

Total RNA was extracted from cultured GCs using RNeasy Mini Kit (Qiagen Ltd., West Sussex, UK) according to the manufacturer protocol. Briefly, cells were homogenised on ice by adding 600 µl of RLT buffer containing 1% β-mercaptoethanol. Lysis was performed by agitating the tube manually then vortexing cells in RLT buffer. Next, an equal volume of 70% ethanol was added to all samples and mixed by pipetting. The mixture was then loaded into the RNeasy spin column (Qiagen Ltd., West Sussex, UK) into 2 ml tube by centrifuge for 30 s at 8000g. Next, the column was washed with 700 µl RW1 buffer and two washes with 500 µl RPE buffer. The flow through was discarded after each step. Before eluting total RNA from column by RNase free water, the column was transferred to a new tube and centrifuged at full speed for one minute to eliminate RPE residue. At the end, 2 µl of RNA was taken for quantification using Nanodrop Spectrophotometer (ND-1000; UK) and the remaining RNA was stored at -80°C for cDNA synthesis (Figure 4.5).

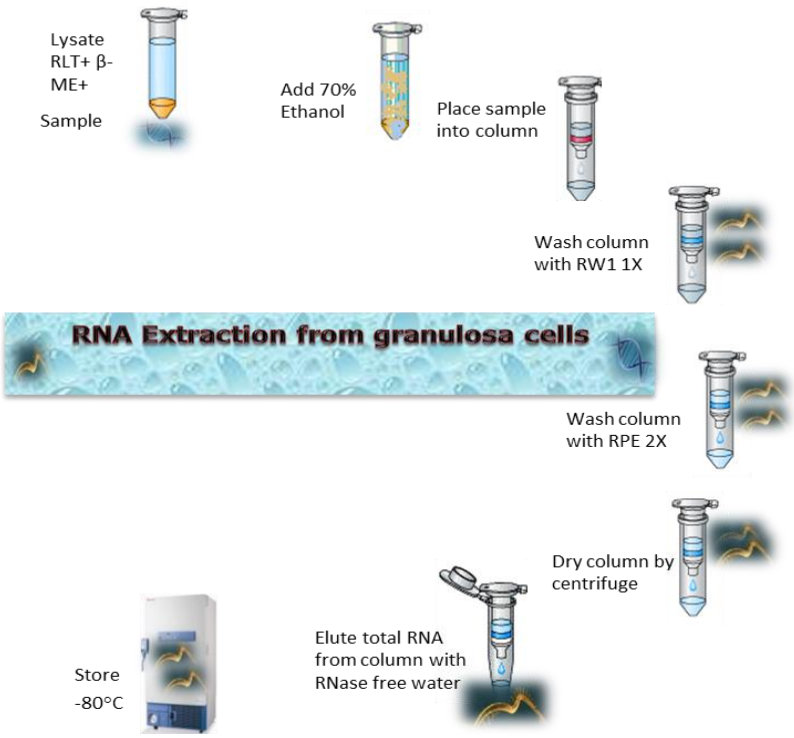


Figure 4.5 *Diagram of RNA extraction from GCs and storage at – 80°C.*

4.2.8.2 cDNA Synthesis

cDNA was synthesised from treated 900 ng/8 µl RNA with 1 µl 10 X reaction buffer and 1 µl RQ1 DNase (1 unit/µl: Promega, Southampton, Hampshire, UK) and mixed well by pipetting, then spun briefly. After the total volume of 10 µl incubated at 37°C for 30 min, the tubes were transferred to ice and 1 µl of DNase stop solution (Promega, Southampton, Hampshire, UK) added. Mixing was by flicking to terminate the reaction and the mixture was incubated further at 65°C for 10 min to inactivate DNase. The resulted DNase treated RNA was used for further analysis.

Typically, reverse transcription (RT) included both minus reverse transcriptase (–RT) control (no amplification control) and plus reverse transcriptase (+RT). For each RT, 5 µl of DNase treated RNA was used for analysis. The Omniscript cDNA synthesis kit (Qiagen Ltd., West Sussex, UK) was used for RT. According to the manufacturer's protocol, for +RT 2 µl 10 X buffer, 2 µl dNTP (10 mM each dNTP), 1 µl OligodT (0.5 µg/µl), 1 µl random hexamers (0.5 µg/ µl), 0.25 µl of RNase inhibitor (40 unit/ µl), 1 µl Omniscript enzyme and 7.75 µl RNase free water were added to 5 µl

DNase treated RNA. For –RT, 2 µl of 10 X buffer and 13 µl of RNase free water were added to 5 µl DNase treated RNA. The total final volume 20 µl per reaction was incubated for 60 min at 37°C. Once the RT was completed, the cDNA was used as the template for amplification. The products were stored at -20°C for polymerase chain reaction (PCR) and quantitative real time polymerase chain reaction (qRT PCR).

4.2.9 Quantitative real time polymerase chain reaction (qRT PCR)

The relative quantification of gene expression for control and extreme melatonin (2000 pg/ml) treated GCs groups cultured under air (20% O_2 and 5% CO_2) and low O_2 tension (5% O_2 and 5% CO_2) at 37.5°C during 48, 96 and 144 h were analysed by qRT-PCR. To increase the specificity of the target gene, TaqMan technology was used, as TaqMan probes can detect only target product (Uyar et al., 2013).

4.2.9.1 Primer and TaqMan probe design

Transcripts for specific genes were searched in the commercial available sequence databases (NCBI and Ensemble homepage). The exon, variant, codon region and location of each gene were considered to choose the best primers and TaqMan probes (Appendix Table A.3). The primers and TaqMan probes of specific genes were designed by Primer Express software (Applied Biosystems, Warrington, UK). Briefly, the sequences of specific transcripts was taken from the Ensemble homepage and entered into the Prime Express. After selecting Taq quantification, each exon junction was highlighted in order to design the primer and probe to ensure that more than one exon was spanned. The best primers and probes were selected according to the location of products within the sequence and penalty criteria. Primers and probes were then BLAST to ensure the specificity of target gene using (NCBI). TaqMan probes for all target genes were labelled with the 6-carboxyfluorescein (FAM) and tetramethylrhodamine (TAMRA) at 5’ and 3’ ends respectively. They were purchased from (Eurofins Genomics, Ebersberg, Munich, German). The primers and probes of each gene were diluted using RNase free water in order to prepare 10 pmol/µl.

4.2.9.2 Conventional PCR and primer test

Conventional PCR used to test primers before ordering the TaqMan probes. PCR was used for DNA synthesis in order to replicate the sequences of target DNA, within a few hours, millions of target DNA copies are produced by thermal cycling instrument. The thermal cycle was performed through the three temperature steps to get PCR endpoint. Denaturation of double stranded DNA at 95°C for 10 s, followed by lower annealing temperature 60°C for 10 s, extension at 72°C for 10 s and finally, cooling at 40°C for 2 s. The three stages (denaturation, annealing and extension) were repeated 40 times by the thermal cycler. To perfume conventional PCR, 10 µl of Light Cycle 480 Probes Master (Roche Diagnostics Ltd, CHEM062, Penzberg, Germany), 0.6 µl of forward and reverse primers, 2 µl of GCs cDNA and 6.8 µl of PCR water were used.

The conventional PCR endpoint products were visualized by gel electrophoresis. The PCR products were mixed by 6 X dye and loaded on 2.5% agarose gel stained with ethidium bromide along with an appropriate molecular marker. The size of the products was determined by marker size using ultraviolet (UV) light (UVP GelDoc-IT-imaging system). A negative reagent control (using water instate cDNA) and –RT were run along with each tested primer.

4.2.9.3 Validation of housekeeping genes

Four HKGs were selected based on previous studies (O'Shaughnessy et al., 2011, Baddela et al., 2014). The level of mRNA of four bovine genes, β-2 Microglobulin (*B2M*), TATTA box binding protein (*TBP*), Ribosomal protein large, P0 (*RPLP0*) and ribosomal protein L19 (*RPL19*) were analysed using cycle threshold values. We explored if O_2, melatonin and culture duration changed the expression of HKG. First, the Cp value for each sample was analysed by general ANOVA. Secondly, NormFinder software package was used to analyse the data. Further ranking of the most stable HKG transcripts confirmed by analysing the CV of each HKG. Finally, the stability of HKGs was evaluated by RefFinder. RefFinder is comprehensive method for monitoring and assessing HKGs from datasets (Xie et al., 2012). It

compares and ranks the HKGs by the currently available computational programs such as GeNorm, NormFinder, BestKeeper, Delta CP value and comprehensive gene stability (Appendix A.1).

4.2.9.4 Relative transcript expression

qRT-PCR were used to provide reliable quantification of target gene expression. Quantification was performed in a total volume 20 µl, including 10 µl of Light Cycle 480 Probes Master, 0.6 µl of forward and reverse primers, 6.4 µl of PCR water, 0.4 µl of probe and 2 µl of two fold diluted GCs cDNA. All samples, -RT reaction control and reagent control (replaced cDNA by water) were run in duplicate. Controls were included to check for contamination of master mix, water and DNA. The amplification cycle included three steps; (i) denaturation of double strands at 95°C for 10 s, (ii) primer annealing 60°C for 50 s (except *P53* 58°C), and (iii) primer extension at 72°C for 15 s followed by cooling at 40°C for 10 s.

Before running samples, PCR efficiency for each gene were run to determine the efficiency. The efficiencies (E) were calculated, according to the following formula ($E = 10^{[-1/slope]}$) the slope is the change in Cp for each sample dilution. To ensure standard assay linearity, and the concentration of primer and probe were optimal, a series 5 fold dilution of GCs cDNA were used. The acceptable efficiency ranged from 1.8 to 2.0 (Appendix Table A.3). Four biological replicates were conducted per experiment and individual replicates for each sample were analysed in duplicate in the same run for particular target gene.

4.2.9.5 Presentation of data

The relative quantification of target gene was calculated by threshold cycle method with the correction of efficiency built on Pfaffl method (Pfaffl, 2001). The slope $\log_{10}$ of the target and reference serial dilution lines were used to calculate the relative quantification based on (Hughes et al., 2007) as follow:

$$Corrected\ Cp = Cp_{\,target} + \frac{\left(Cp_{\,mean\,reference} - Cp_{\;reference}\right) * Slop_{\,taget}}{Slop_{\,reference}}.$$

All transcripts were analysed relative to the best housekeeping gene (*TBP*) under O_2 tension, culture duration, melatonin treatments (validation of housekeeping genes; Appendix A.1).

4.2.10 Measurement of reactive oxygen species

Granulosa cells were cultured as described in Section 4.2.4; Exp.**3**. ROS generated from cells were measured by Nitroblue tetrazolium (NBT; N6876, Sigma-Aldrich) assay according to the Sharma et al. (2013), Amarasekara et al. (2014), Huang and Li (2014) with some modifications. Briefly, Nitroblue tetrazolium was prepared by dissolving 1 mg of NBT powder to 1 ml of PBS and stirred for 1 h at room temperature in dark. The solution then filtered with a 0.2 µm filter (Allegiance Health Care). At various time points (48, 96 and 144 h) cell supernatants were removed and 50 µl of 1 mg/ml NBT added to each well from the edge in order avoid disturbing the cells. To stop the reaction, 100 µl of 1 M hydrochloric acid (HCl) was added to each well and the plates were mixed for 10 min at room temperature. After incubation, the solutions were removed completely and the wells were washed three times with PBS and mixed for 5 min. Next, formazan deposits were solubilized by re-suspending cells in 150 µl of dimethyl sulfoxide (DMSO) and to develop the colour, 10 µl of 1 M NaOH were added to each well and shaken for 5 min. The optical density was read at 630 nm wavelength using a plate reader (Thermo Fisher, Loughborough, UK) at 20 min after adding NaOH. Finally, optical density was adjusted by cell viability assessed by crystal violet (Section 4.2.5.2).

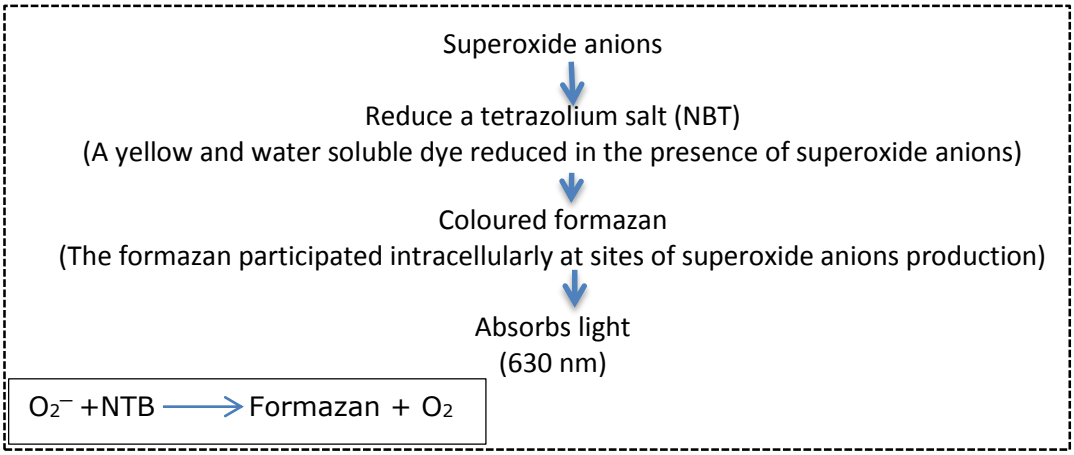


Figure 4.6 *The mechanism of detection of ROS by Nitro blue tetrazolium assay.*

4.2.11 Statistical analyses

Results were analysed by ANOVA using GenStat. Terms fitted to this 2 X 4 X 3 factorial model were O_2 (Low vs High), melatonin (0, 20, 200 and 2000 pg/ml) and time in culture (48, 96 and 144 h). Oxygen level, melatonin and culture duration were considered as fixed effects and blocked by culture replicate, incubator and plate. Oestrogen and progesterone production were expressed per 10^5 cells. Natural log transformations of the data were used to correct for heteroscedasticity of the residuals. The data are shown as the natural log of the mean with SED.

4.3 Results

4.3.1 Effect of oxygen tension and melatonin on reactive oxygen species generated by cultured granulosa cells

Oxygen concentration did not affect (P=0.15) ROS production by GC. However, the inclusion of melatonin significantly reduced (P<0.001) ROS production under both 5% O_2 and 20% O_2 concentrations. Melatonin at all three doses tended to reduced ROS production under atmospheric O_2, whereas melatonin at high doses tended to reduce ROS at 5% O_2 (Oxygen x melatonin interaction; P=0.08).

Time in culture increased ROS production at 96 h of culture (P<0.001), but at 144 h of culture ROS level reduced in neither of the O_2 concentrations tested (Figure 4.7).

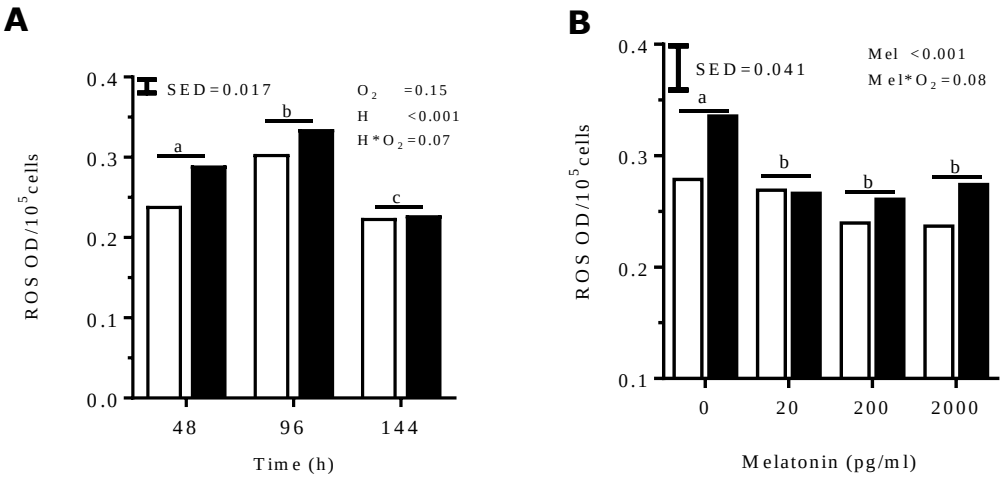


Figure 4.7 *Effect of O_2 tension and melatonin on ROS generation by bovine GCs after 48, 96 and 144 h of culture. Low O_2 environment (□) was a mixture of 5% CO_2, 5% O_2 and 90% N_2, whilst the high O_2 environment (■) contained 5% CO_2, and air having an O_2 concentration of 20%. Reactive oxygen species was significantly reduced at 144 h (**A**) and by melatonin (**B**). The data are presented as ROS/10^5 cells with SED and LSD used to estimate significant differences between means. a, b, c highlight differences between time point (**A**) and melatonin (**B**). Data represented three replicate cultures and five wells within each replicate.*

4.3.2 Effect of melatonin on granulosa cells proliferation and steroid production under 5% or 20% oxygen tension

4.3.2.1 Number of viable cells

Oxygen tension did not affect the number of GCs after 48, 96 or 144 h of culture (P=0.18). Cell number declined at 96 h of culture but increased at 144 h to numbers similar to those found at 48 h of culture. In contrast, 20 pg/ml of melatonin increased cell number (~9%) across all time points and in both O_2 tension (p<0.001). However, melatonin at concentrations of 200 pg/ml and above did not affect cell number (Figure 4.8).

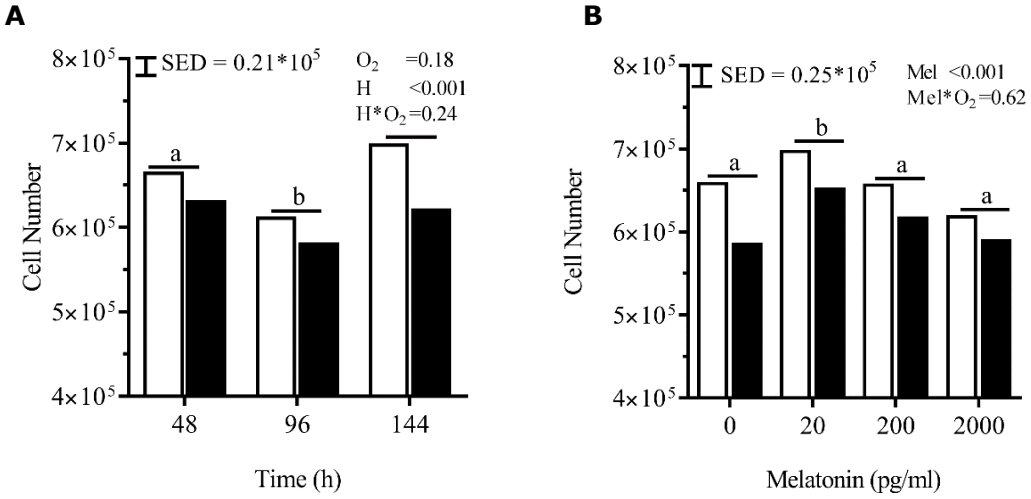


Figure 4.8 *Effect O_2 tension and melatonin on GCs number after 48, 96 and 144 h of cultured in pre coated 12 well plates. Granulosa cells were seeded at a cell density of 0.6 million/ml. Cells were incubated under low (□; 5% O_2, 5% CO_2 and 90% N_2) or high O_2 tension (■; 5 % CO_2, 20% O_2) in the presence of melatonin. Viable cells were counted at 48, 96 or 144 h of culture after trypan blue exclusion (**A**). There was no interaction (P>0.10) between O_2 and melatonin, and between the above treatments and time of culture, thus the data presented are the means and LSD of the treatments pooled across the levels of the non-significant variables. Melatonin at 20 pg/ml increased GCs number under both conditions (**B**). a, b highlight differences between time point (**A**) and melatonin (**B**). Data represented four replicate cultures and duplicate wells within each replicate.*

4.3.2.2 Progesterone production

Progesterone concentration in spent media increased with time in culture ($P<0.001$) from 48 to 96 and to 144 h. In addition, P_4 production of cells cultured under high O_2 tension was higher ($P<0.02$) than for cells cultured with 5% O_2, and these differences became larger as time in culture progressed (time by O_2 concentration interaction, $P=0.04$; Figure 4.9 A).

Melatonin did not ($P=0.15$) affect P_4 production by cultured GCs under neither O_2 concentration nor with time in culture (Figure 4.9 B).

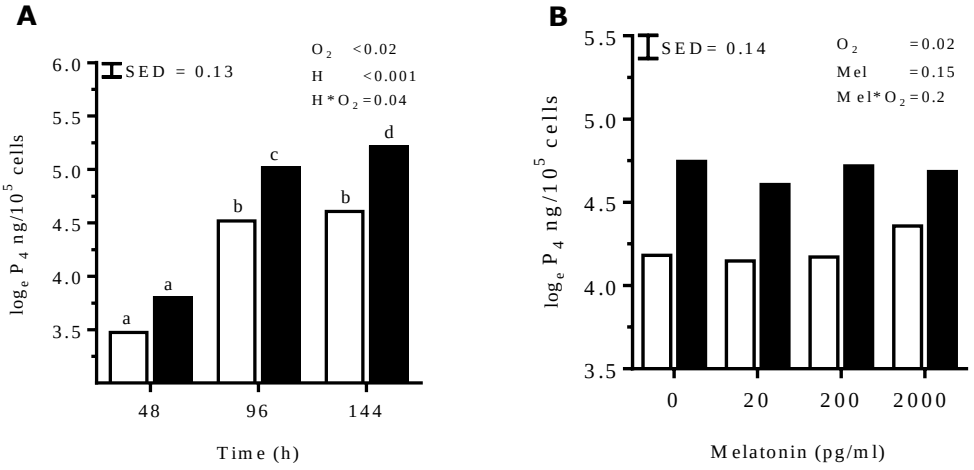


Figure 4.9 *Effect of O_2 tension and melatonin on GCs P_4 production in vitro after 48, 96 and 144 h of culture. Low O_2 (□) environment was a mixture of 5% CO_2, 5% O_2 and 90% N_2, whilst the high O_2 (■) environment contained 5% CO_2, and air having an O_2 concentration of 20% O_2. Progesterone production increased under high O_2 compared with low O_2 additionally these differences was increased dependent on the culture time (**A**). Data was analysed after transformation to the natural log of ng/ml media for 1 X 10^5 cells. The data are presented as mean with SED and LSD used to estimate significant differences between means. a, b, c, d highlight differences between O_2 x time point (**A**) and melatonin (**B**). Data represented four replicate cultures and duplicate wells within each replicate.*

4.3.2.3 Oestrogen production

Oestrogen concentration was not affected by O_2 tension (P=0.6). However, E_2 production decreased (P<0.001) between 48 to 96 h of culture under high and low O_2. At 48 h, E_2 production of cells cultured with 20% O_2 was higher than for cells cultured at 5% O_2. Oestrogen production declined at 96 h in both treatments and did not differ between them. However, at 144 h of culture E_2 production increased in cells exposed to 5% O_2, without changes to the E_2 output of cells cultured with atmospheric O_2 concentration (time by O_2 interaction P<0.001; Figure 4.10 A).

Melatonin at 20 pg/ml had lowest E_2 production during culture (main effect across all time frames and O_2 treatments; P=0.02), whereas melatonin at higher concentrations (200 and 2000 pg/ml) did not affect E_2 production. There was no melatonin interaction with O_2 and time point (Figure 4.10 B).

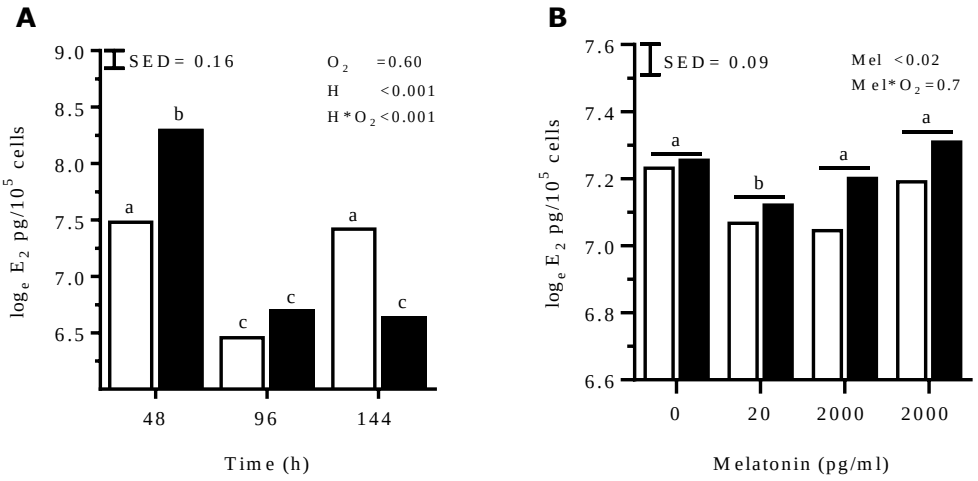


Figure 4.10 *Effect of O_2 tension and melatonin on GCs E_2 production in vitro after 48, 96 and 144 h of culture. Low O_2 (□) environment was a mixture of 5% CO_2, 5% O_2 and 90% N_2, whilst the high oxygen (■) environment contained 5% CO_2, and air having an O_2 concentration of 20% O_2. Oestrogen concentration was significantly lower after 48h of culture under air, whereas increased at 144 h at low O_2 level (**A**). Data was analysed after transformation to the natural log of pg /ml media for 1 X 10^5 cells. The data are presented as mean with SED and LSD used to estimate significant differences between means. a, b, c highlight differences between O_2 x time point (**A**) and melatonin (**B**). Data represented four replicate cultures and duplicate wells within each replicate.*

4.3.2.4 Oestrogen to progesterone ratio

The ratio between the E_2 and P_4 production was greater ($P<0.015$) in spent media at 5% O_2 than atmospheric O_2 (20% O_2). Oestrogen to progesterone ratio reduced ($P<0.001$) between 48 and 96 h under both O_2 concentration and further reduction was noted at 144 h of culture time for cells cultured at 20% O_2, whereas the E_2:P_4 ratio at 144 h of culture time increased under 5% O_2 (time x O_2 interaction $P<0.001$; Figure 4.11 A).

Granulosa cells treated with melatonin under both O_2 levels had no significant effect on E_2:P_4 ratio (Figure 4.11 B). Additionally, there was no significant interaction between O_2 concentration and melatonin treatments on E_2:P_4 ratio production.

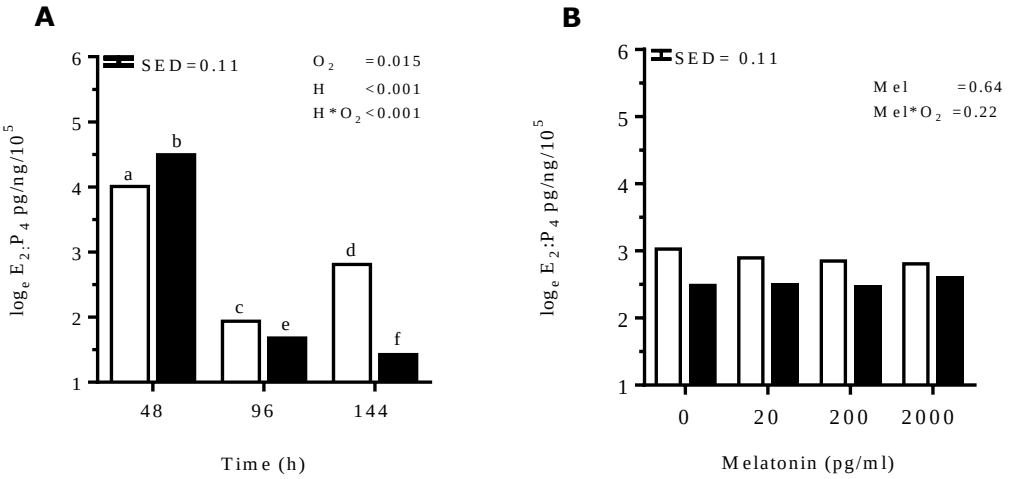


Figure 4.11 *Effect of O_2 tension and melatonin on GCs E_2:P_4 ratio production in vitro after 48, 96 and 144 h of culture. Low O_2 (□) environment was a mixture of 5% CO_2, 5% O_2 and 90% N_2, whilst the high O_2 (■) environment contained 5% CO_2, and air having an O_2 concentration of 20% O_2. Oestrogen to progesterone ration was significantly lower after 48 and 96 h of culture under atmospheric O_2, whereas at low O_2 level E_2:P_4 ratio increased at 144 h (**A**). Data was analysed after transformation to the natural log of pg/ng in 1 ml media for 1 X 10^5 cells. The data are presented as mean with SED and LSD used to estimate significant differences between means. Different letter highlight differences between O_2 x time point (**A**) and melatonin (**B**). Data represented four replicate cultures and duplicate wells within each replicate.*

4.3.3 Gene expression on granulosa cells cultured under different oxygen concentrations in the presence or absence of melatonin

4.3.3.1 Housekeeping genes

The gene expression of 4 housekeeping genes was tested under the same conditions used for cell culture, with the O_2 and hormonal treatments as the GCs and the same time points. Expression of TATTA box binding protein (*TBP*) mRNA was the more stable among the genes tested and as its Cp values were not affected by O_2, culture time, melatonin treatment or their interactions (Table 4.2). We further confirm this result by analysis of Cp values with NormFinder, % CV and RefFinder where *TBP* in all occasions was the most stable reference gene followed by *RPLP0* (Appendix A.1). In consequence, all target genes in this study were normalized with *TBP*.

Table 4.2 *The variable and interact (P values) of HKG in GCs showed the effect of O_2, melatonin and culture duration on the stability of HKG in GCs.*

HKG	O_2	Mel	H	Mel*H	Mel*O_2	H*O_2	Mel*H*O_2
B2M	0.64	0.51	**0.004**	0.31	0.50	0.17	0.49
RPL19	**0.02**	0.28	0.48	0.94	0.36	0.58	0.73
RPLP0	0.15	0.97	0.51	0.37	0.23	**0.06**	0.86
TBP	0.42	0.45	0.17	0.794	0.62	0.31	0.68

*O_2=Oxygen, Mel=melatonin, H=culture duration hour, *= interaction. Using ANOVA test.*

4.3.3.2 Apoptotic genes (*BAX* and *P53*)

Oxygen concentration and melatonin treatment did not influence the expression of apoptotic genes *BAX* and *P53* (P>0.10) in cultured bovine GCs (Figure 4.12). However, the level of *BAX* mRNA was significantly reduced (P=0.02) at 96 h and 144 h compared with 48 h of culture (Figure 4.12 A1).

Expression for *Bcl2* transcript was low throughout culture and could not be quantified despite the use of primers and probes varying in their concentrations, design and annealing temperatures.

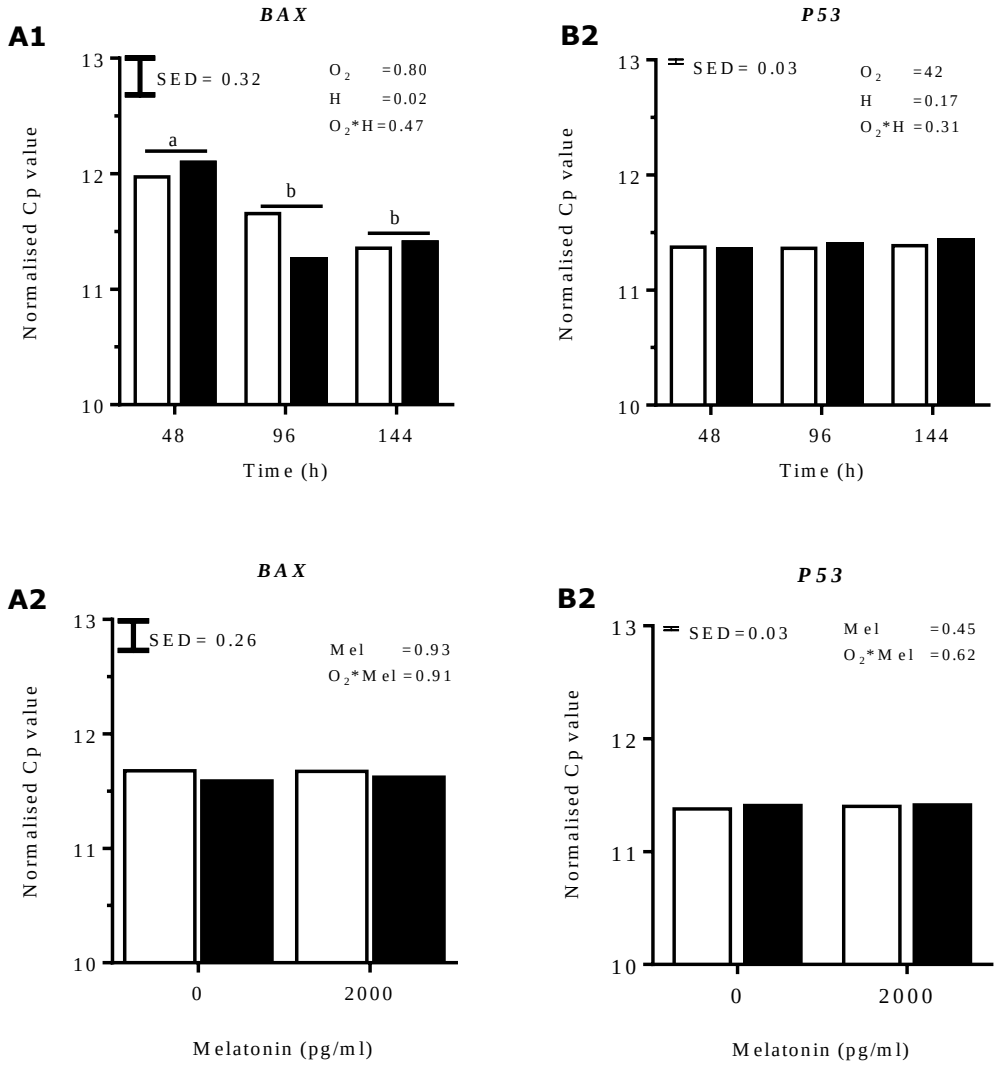


Figure 4.12 *Effect of O_2 tension (**1**) and melatonin (**2**) on transcript expression of BAX (**A**) and P53 (**B**) in bovine GCs after 48, 96 and 144 h of culture. Low O_2 (□) environment was a mixture of 5% CO_2, 5% O_2 and 90% N_2, whilst the high O_2 (■) environment contained 5% CO_2, and air with an O_2 concentration of 20 %. Mean expression of BAX mRNA decreased at 96 h and 144 h compared with 48 h of culture. The data are presented as mean with SED and LSD was used to estimate differences between means. a, b highlight differences between time in culture. Data represented four replicate cultures.*

4.3.3.3 Steroidogenic enzymes (*HSD3B1* and *CYP19A*)

Transcript expression of *HSD3B1* was significantly increased by high O_2 concentration (P=0.028). Atmospheric O_2 concentration augmented *HSD3B1* mRNA expression in GCs from 48 to 96 and again from 96 to 144 h of culture. However, *HSD3B1* mRNA level with low O_2 was unchanged

throughout the culture time (O_2 x time interaction P=0.003; Figure 4.12A). There was no significant effect of melatonin on the expression of *HSD3B1* (Figure 4.12B).

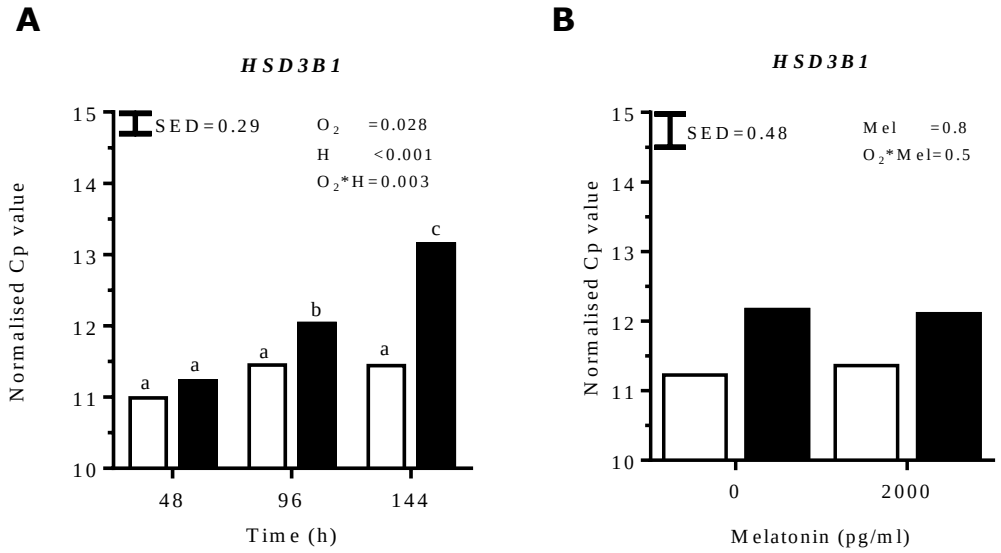


Figure 4.13 *Effect of O_2 tension (**A**) and melatonin (**B**) on HSD3B1 mRNA expression in bovine GCs after 48, 96 and 144 h of culture. Low O_2 (□) environment was a mixture of 5% CO_2, 5% O_2 and 90% N_2, whilst the high O_2 (■) environment contained 5% CO_2 and air, with an O_2 concentration of 20%. The expression of HSD3B1 mRNA increased with culture time at 20% O_2 and was unchanged with 5% O_2 (P<0.003). Data are presented as mean with SED and LSD was used to estimate significant differences between means. a, b, c differ between O_2 x time point. Data represented four replicate cultures.*

Expression of *CPY19A1* transcript in freshly collected GCs directly from follicles was high (Cp=21.5 cycles). However, after culture the expression of *CPY19A1* mRNA was in very low amount and nearly undetectable. To explore whether the low expression was due to primers-probes design or culture system, different primers and probes from different coding and non-coding regions were checked and are presented in appendix (Table A.3). Further analysis for CYP19A by immunofluorescence showed CYP19A expression in GCs cultured under 5% O_2 at 24, 96 and 144 h (Figure 4.14).

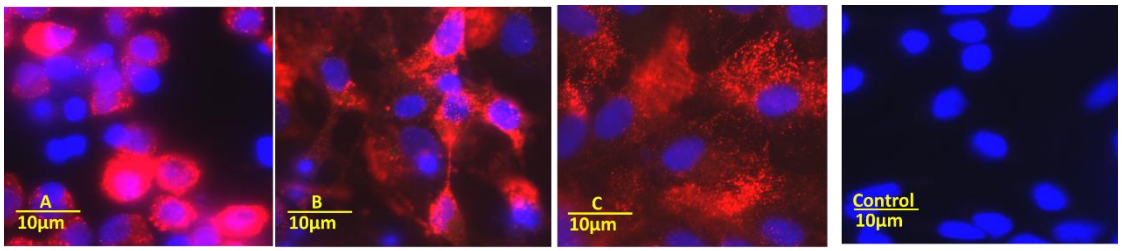


Figure 4.14 *Immunolocalization of CYP19A (red colour) in cultured GCS. Cells were cultured in non-coated plate during 24 h (A), GCs cultured in pre coated plate with fibronectin for 96 h (B), 144 h (C) and negative control (Control; blue colour; DAPI) counterstained with DAPI (blue colour) which was present in cell nucleus.*

4.3.3.4 Heat shock 70 KD protein 1A (*HSPA1A*)

The *HSPA1A* mRNA was expressed in bovine GCs and the O_2 level and melatonin treatments did not affect its level of expression. However, time in culture decreased (P=0.02) the expression of *HSPA1A* mRNA after 48 h of culture. Melatonin treatment did not affect *HSPA1A* mRNA expression (Figure 4.15).

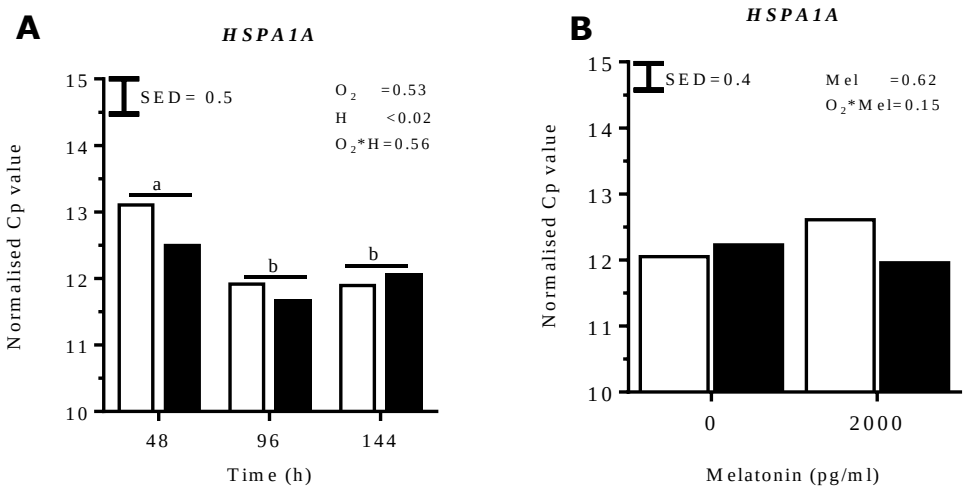


Figure 4.15 *Effect of O_2 tension (**A**) and melatonin (**B**) on expression of HSPA1A in bovine GCs after 48, 96 and 144 h of culture. Low O_2 (□) environment was a mixture of 5% CO_2, 5% O_2 and 90% N_2, whilst the high O_2 (■) environment contained 5% CO_2, and air having an O_2 concentration of 20%. The data are presented as mean with SED and LSD used to estimate significant differences between means. a, b highlight differences between time in cultures.*

4.3.3.5 Melatonin biosynthesis

Enzymes responsible for melatonin synthesis aralkylamine N-acetyltransferase (*AANAT)* and acetyl serotonin O-methyltransferase (*ASMT)* were expressed in cultured bovine GCs. *ASMT* mRNA expression was present in cultured GCs throughout the culture and its level of expression was not affected by O_2 tension or melatonin treatment (Figure 4.16). In contrast, *AANAT* mRNA was in very low amount and nearly undetectable. Therefore, further analysis for *AANAT* was ignored.

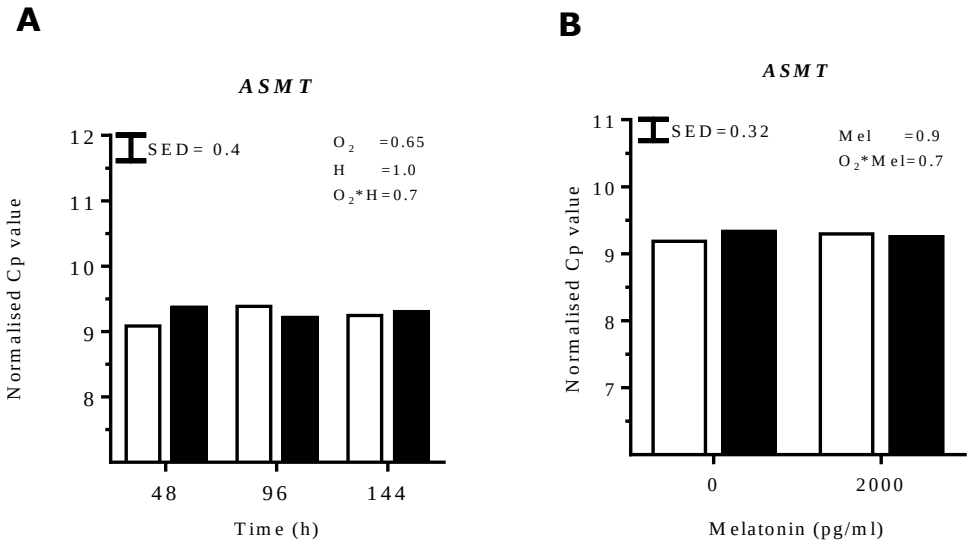


Figure 4.16 *Effect of O_2 tension (**A**) and melatonin treatment (**B**) on transcript expression of ASMT mRNA level in bovine GCs after 48, 96 and 144 h of culture. Low O_2 (□) environment was a mixture of 5% CO_2, 5% O_2 and 90% N_2, whilst the high O_2 (■) environment contained 5% CO_2, and air having an O_2 concentration of 20%. The data are presented as mean with SED and LSD used to estimate significant differences between means. Data represented four replicate cultures.*

4.3.3.6 Haemoglobin (*HB*)

Haemoglobin (*HBA* and *HBB*) transcripts were present in GCs of follicles 4 to 6 mm in diameter. The expression of *HBA* mRNA was significantly higher ($P=0.015$) in cells culture under 5% O_2 than cultured under 20% O_2 at all time points studied. Haemoglobin *A* mRNA expression after 48 h of culture at 5% O_2 was similar ($P>0.10$) to that found in fresh GCs (Cp value 26.5) but declined steadily ($P<0.05$) thereafter. In contrast, high O_2 tension

caused a decline in *HBA* mRNA expression after 48 h of culture and remained low throughout the culture (time x O_2 interaction P=0.006; Figure 4.17A). Haemoglobin *B* mRNA expression levels were very low and only detectable at the endpoint of a 40-cycle PCR. Melatonin treatment did not affect *HBA* level in bovine GCs cultured (P=0.42; Figure 4.17B) irrespective of O_2 conditions.

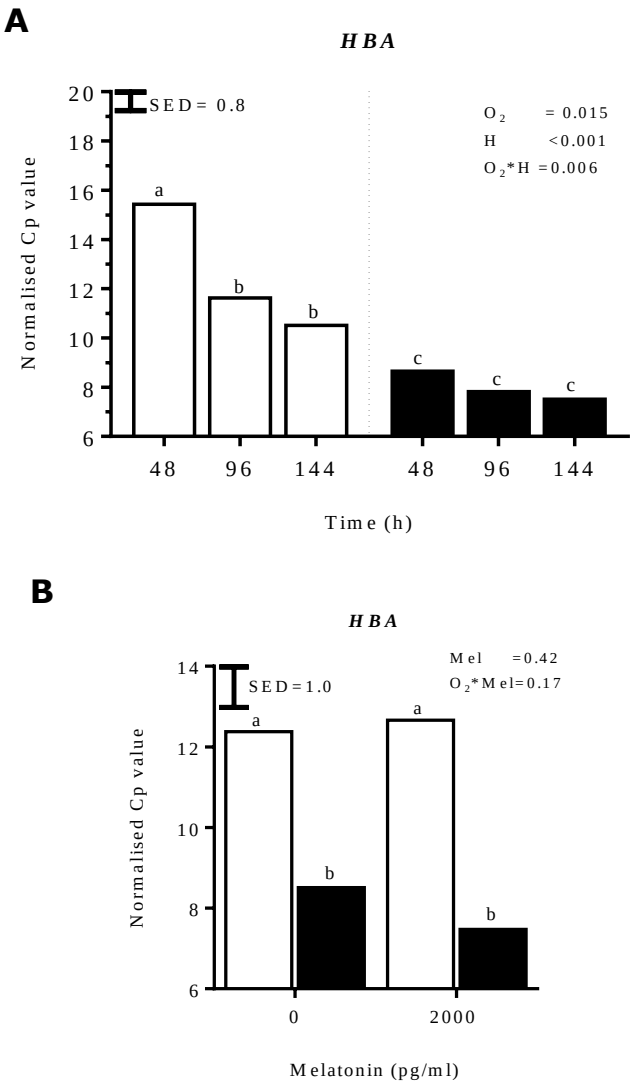


Figure 4.17 *Effect of O_2 tension (**A**) and melatonin (**B**) on expression of HBA mRNA level in bovine GCs after 48, 96 and 144 h of culture. Low O_2 (□) environment was a mixture of 5% CO_2, 5% O_2 and 90% N_2, whilst the high O_2 (■) environment contained 5% CO_2, and air having an O_2 concentration of 20%. The expression level of HBA was reduced after 48 h of culture at 5% O_2 and unchanged with 20% O_2. The data are presented as mean with SED and LSD used to estimate significant differences between means. a, b, c highlight differences between groups. Data represented four replicate cultures.*

4.3.3.7 Superoxide dismutase (*SOD1* and *SOD2*)

Superoxide dismutase (*SOD1* and *SOD2*) mRNA expression was not affected (P>0.10) by O_2 tension. Expression of *SOD2* mRNA was high at 48 h of culture and decline at 96 and 144 h of culture (P<0.001). The expression of *SOD1* was lower than that of *SOD2*. Expression of SOD1 did not change between O_2 treatments or with time in culture. Melatonin treatments did not affect the mRNA level of superoxide dismutase mRNA (*SOD1* and *SOD2*) in cultured bovine GCs for 144 h (Figure 4.18).

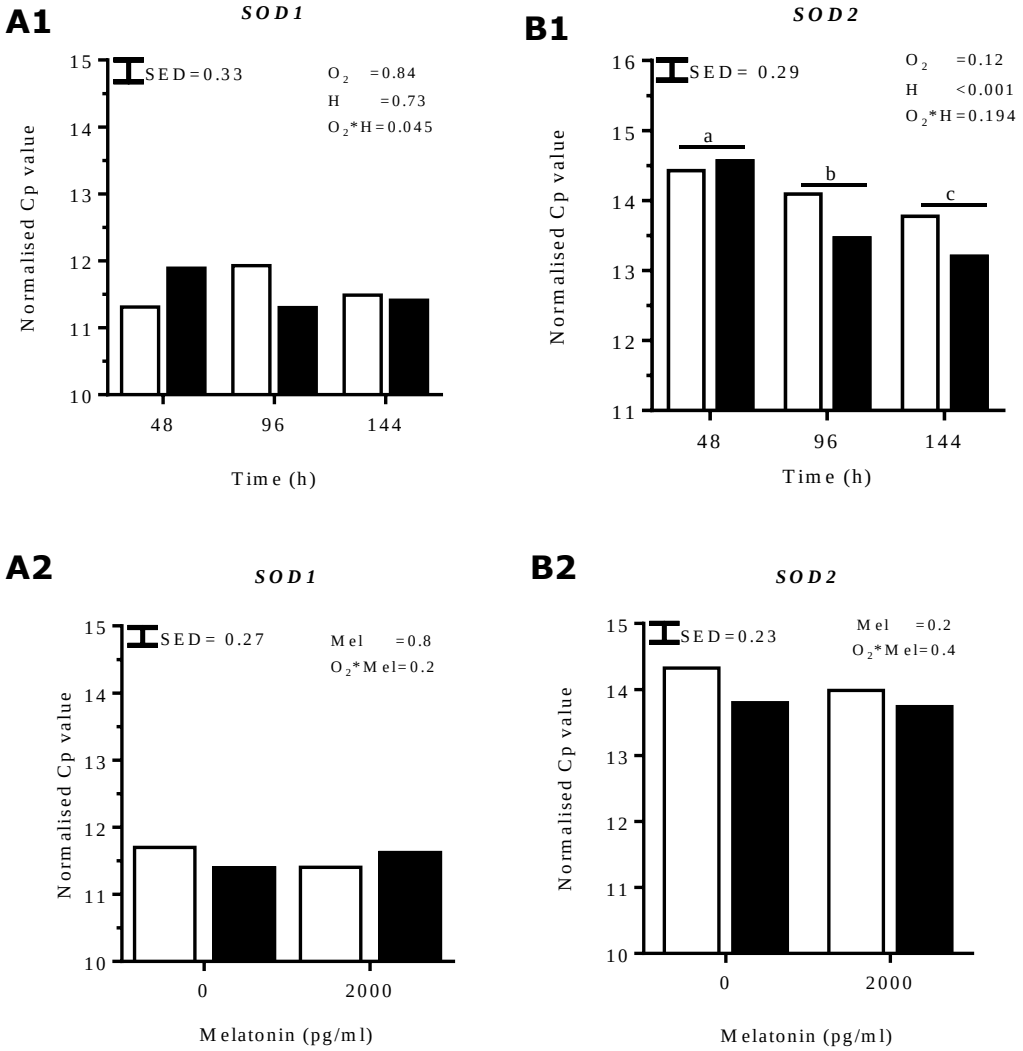


Figure 4.18 *Effect of O_2 tension (**1**) and melatonin (**2**) on expression of SOD1 (**A**) and SOD2 (**B**) mRNA level in bovine GCs after 48, 96 and 144 h of culture. Low O_2 (□) environment was a mixture of 5% CO_2, 5% O_2 and 90% N_2, whilst the high O_2 (■) environment contained 5% CO_2, and air having an O_2 concentration of 20%. The expression level of SOD2 was reduced by culture time. The data are presented as mean with SED and LSD used to estimate significant differences between means. a, b, c mean differ between culture time. Data represented four replicate cultures.*

4.3.4 Effect of oxygen tension on long term granulosa cells culture under oxygen tension

4.3.4.1 Granulosa cell number and proliferation after culture under low and high oxygen tension

Culture of GCs number was lower at 48 h after plating and increased with time in culture (P=0.001) and at the end of culture cell density was similar to that initially seeded (1.5 x 10^6 cells/ well). Cells cultured with low O_2 concentration had higher cell number than those under atmospheric O_2 concentration (P=0.004). There was no significant interaction (P>0.10) between O_2 tension and time in culture on GCs number (Figure 4.19). In addition, GCs expressed the cell proliferation marker Ki67 in their nuclei at a low rate in both treatments (Figure 4.20).

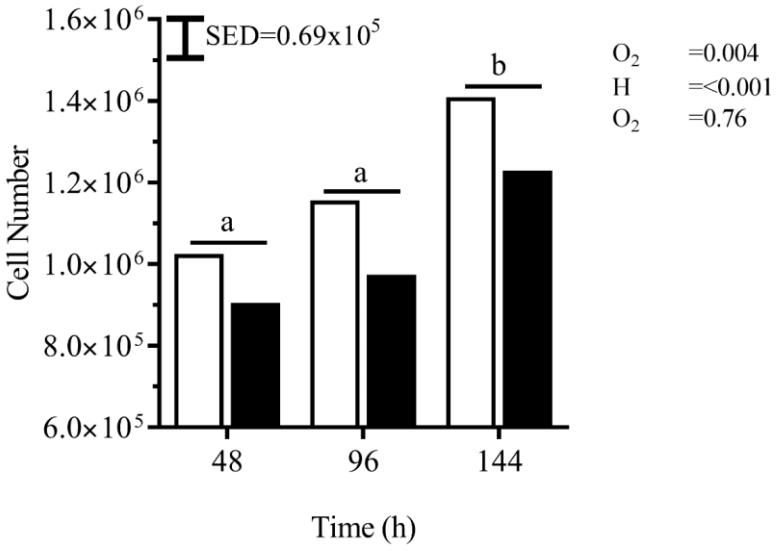


Figure 4.19 *Granulosa cell number after 48, 96 and 144 h of cultured in pre coated 6 well plates. Granulosa cells were seeded at a cell density of 1.5 million / 2 ml. Cells were incubated under low (□) and high O_2 (■) tension (5% O_2, 5% CO_2 and 90% N_2 vs 5 % CO_2, 20% O_2) and viable cells counted after 48, 96 or 144 h of culture after trypan blue exclusion. a, b highlight differences between time in culture. Data represented five replicate cultures with duplicates wells within plate replicate.*

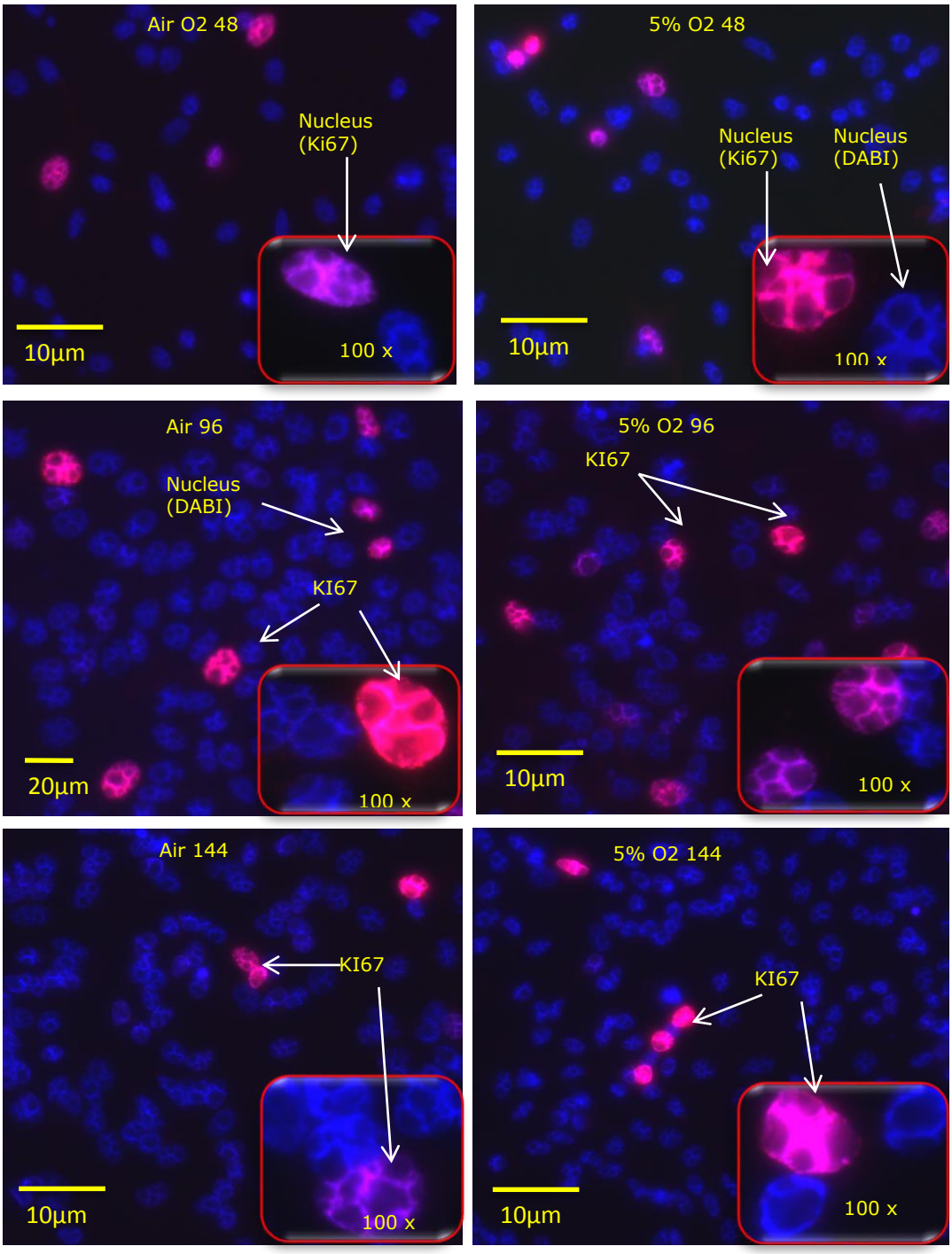


Figure 4.20 *Immunofluorescent expression of nuclear antigen Ki67 in bovine GCs cultured under low (5%) and high (20%) O_2 for 48, 96 and 144 h. Cells were stained with a rabbit anti-Ki67 IgG and counterstained with DAPI (blue colour) which was present in cell nucleus and red colours (Ki67) shows cells are in active phase.*

4.3.4.2 Effect of oxygen tension on steroid hormone production and aromatase enzyme activity

4.3.4.2.1 Progesterone production

Overall, GCs P_4 production were similar under both low and high oxygen tension (P=0.10). Progesterone production at 48 h of culture was similar between the two O_2 treatments. However, P_4 production after 96 h of culture was higher in cells cultured under high O_2 tension than for cells under low O_2 tension (P=0.014; Figure 4.21). At 144 h of culture P_4 production declined slightly in high O_2 tension (P<0.001) and no difference was observed between both treatments.

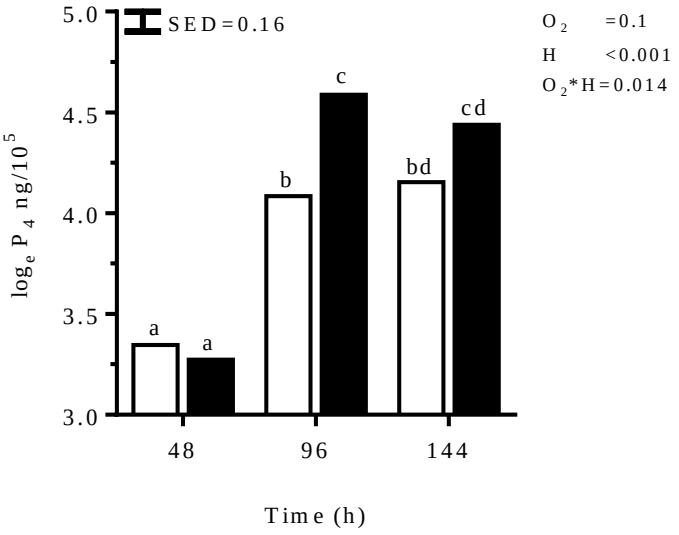


Figure 4.21 *Effect of O_2 tension on GCs P_4 production in vitro after 48, 96 and 144 h of culture. Low O_2 (□) environment was a mixture of 5% CO_2, 5% O_2 and 90% N_2, whilst the high O_2 (■) environment contained 5% CO_2, and air having an O_2 concentration of 20%. Progesterone production was significantly higher after 96 of culture. Data was analysed after transformation to the natural log of ng /1 ml media for 1 X 10^5 cells. The data are presented as mean with SED and LSD used to estimate significant differences between means. a, b, c, d highlight differences between groups. Data represented five replicate cultures with duplicates wells within plate replicate.*

4.3.4.2.2 Oestrogen production and aromatase activity

Granulosa cell E_2 production declined ($P<0.001$) from 48 to 96 h of culture. At 144 h of culture GC cultured with 20% O_2 were producing E_2 at concentrations similar to those observed at 96 h. However, for cells cultured under low O_2 tension (5% O_2), E_2 production increased at 144 h of culture (time by O_2 interaction: $P<0.001$; Figure 4.22).

Further confirmation of the above fluctuation of E_2 production under low O_2 was recorded by the aromatase assay of the proteins extracted from the GCs. Testosterone conversion to E_2 in the presence of NADPH revealed aromatase activity in the cells similar to the E_2 production mentioned above. Aromatase activity was present in cells at 48 h of culture. Enzyme activity of cells cultured at 20% declined continuously throughout the culture period ($P<0.05$). In contrast, aromatase activity for cells cultured under low O_2 remained unchanged and similar to the activity found at 48 h, throughout the culture period ($P> 0.05$; Figure 4.23).

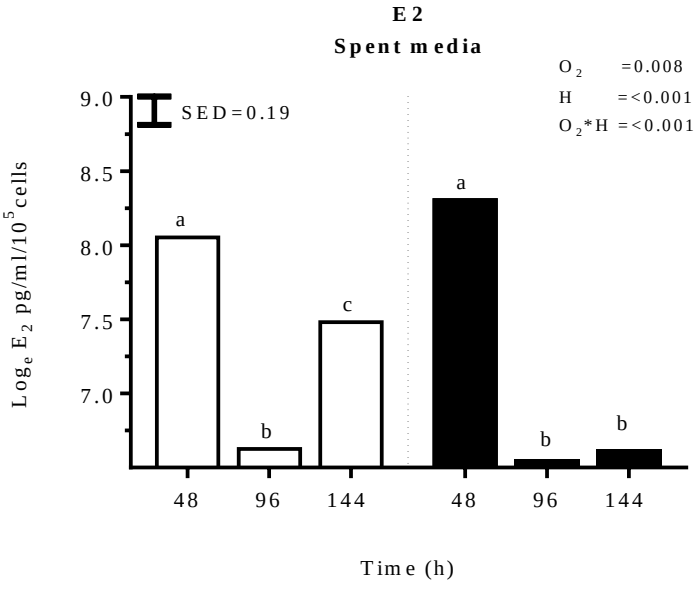


Figure 4.22 *Effect of O_2 tension on granulosa cell E_2 production in vitro after 48, 96 and 144 h of culture. Low O_2 (□) environment was a mixture of 5% CO_2, 5% O_2 and 90%N_2, whilst the high O_2 environment contained 5% CO_2, and air (■) having an O_2 concentration of 20%. Oestrogen concentration in spent media was significantly lower after 48h of culture under air, whereas increased at 144 h at low O_2 level. Data was analysed after transformation to the natural log of pg /1 ml media for 1 X 10^5 cells. The data are presented as mean with SED and LSD used to estimate significant differences between means. a, b, c highlight differences between groups. Data represented five replicate cultures with duplicates wells within plate replicate.*

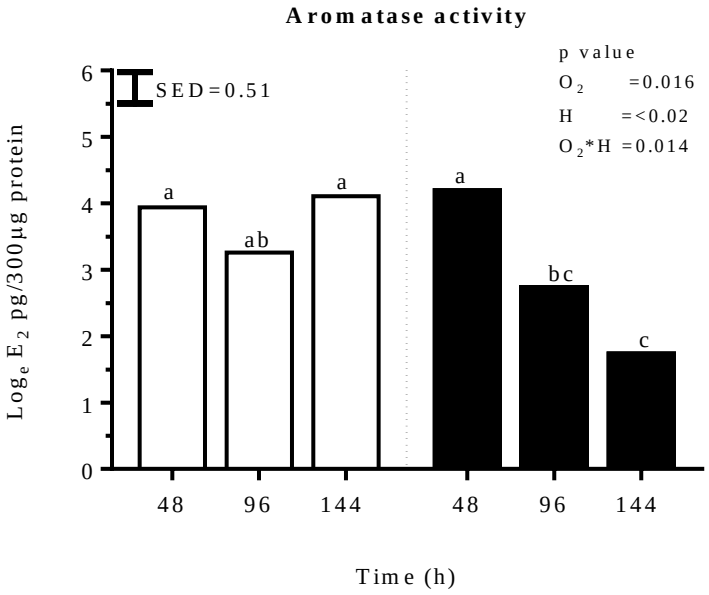


Figure 4.23 *Effect of O_2 tension on aromatase activity in GCs cultured after 48, 96 and 144 h of culture. Low O_2 (□) environment was a mixture of 5% CO_2, 5% O_2 and 90% N_2, whilst the high O_2 (■) environment contained 5% CO_2, and air having an O_2 concentration of 20%. Aromatase activity also decreased after 48 h under air and maintained throughout the culture duration under low O_2. Data was analysed after transformation to the natural log of pg/300µg GCs protein. The data are presented as mean with SED and LSD used to estimate significant differences between means. a, b differ between groups. Data represented five replicate cultures with duplicates wells within plate replicate.*

4.3.4.2.3 Oestrogen to progesterone ratio

The relationship between the E_2 and P_4 production in GCs was greater (P=0.008) at more physiological O_2 level (5% O_2) than at the atmospheric O_2 (20% O_2). Oestrogen and progesterone ratio at both O_2 concentration (20% O_2 and 5% O_2) reduced between 48 and 96 h of culture, whereas at 144 h of culture E_2:P_4 production for cells cultured at 20% O_2, was similar to those observed at 96 h (P<0.001). However, the E_2:P_4 ratio for cells cultured under low O_2 tension (5% O_2) at the end of culture increased at 144 h post seeding (Figure 4.24).

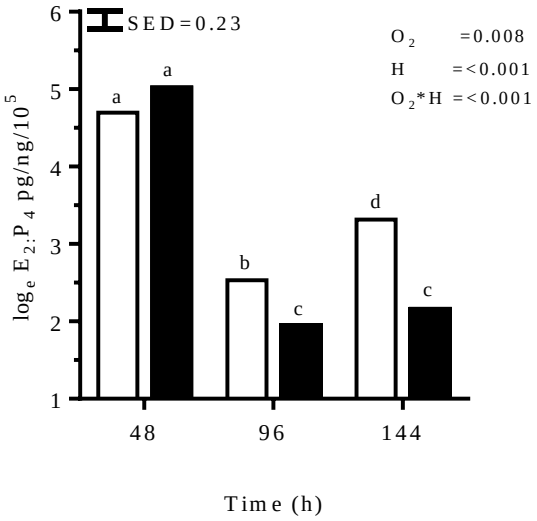


Figure 4.24 *Effect of O_2 tension on GCs E_2:P_4 ratio in vitro after 48, 96 and 144 h of culture. Low O_2 (□) environment was a mixture of 5% CO_2, 5% O_2 and 90% N_2, whilst the high O_2 (■) environment contained 5% CO_2, and air having an O_2 concentration of 20%. Oestrogen to progesterone ratio in spent media was reduced from 48 to 96 and to 144 h of culture under high O_2 tension. However, at 144 h E_2:P_4 ratio increased at low O_2 level. Data was the ratio of E_2 (pg) to P_4 (ng). The data are presented as mean with SED and LSD used to estimate significant differences between means. a, b, c, d differ between groups. Data represented five replicate cultures with duplicates wells within plate replicate.*

4.4 Discussion

A key indicator of GC differentiation and luteinisation is altered steroid hormone production. This chapter provides evidence that O_2 affected the differentiation and luteinisation of bovine GCs, as evidenced by changes in aromatase activity and E_2 production during 144 h culture. In terms of luteinisation, in contrast to low O_2, both P_4 production and *HSD3B1* expression were increased under atmospheric O_2. These differences are not likely due to cell viability as cell number, *BAX* and *P53* expression were not affected. This chapter reports, for the first time, the expression of high *HBA* and low *HBB* mRNA in bovine GCs. It also confirms expression of *AANAT* and *ASMT* in these cells. Furthermore, *HBA* expression increased in GCs cultured under low O_2, providing a putative mechanism by which O_2 delivery to GCs can be enhanced under physiological conditions. This is the first study to evaluate the effects of melatonin on GCs cultured under physiological O_2, and to simultaneously relate these effects to culture under atmospheric O_2. Melatonin, operating as an antioxidant, slightly reduced ROS production under both 5% and 20% O_2. However, there did not appear to be any additional consistent effect of melatonin on cultured GCs at either O_2 level. Collectively, these results indicate that culture of GCs under low O_2 is likely to better recapitulates physiological conditions within the growing ovarian follicle.

Cultured granulosa cells under low and high oxygen tension

Cell number and viability

Mean number of viable GCs was greater under 5% O_2 than under atmospheric O_2. Low O_2 could induce cell proliferation by upregulation proliferating cell nuclear antigen (*PCNA*) under hypoxia (Jiang et al., 2011). Oxygen levels in GCs declines as ovulation approaches (de Castro e Paula et al., 2008). Granulosa cells cultured under 5% O_2 proliferate more and differentiate less compared to those cultured under atmospheric conditions (20% O_2). It has been reported that metabolic activity of human placental trophoblast cells was not affected by O_2 tension. However, low O_2 (2% O_2) induced cell proliferation and reduced hormonal secretion, whereas 20% O_2 shifted the cell cycle from proliferation to differentiation (Ma et al., 2001).

Steroidogenesis

Progesterone production by GCs cultured under air increased significantly at 96 h of culture and further increases were observed at 144 h, whereas the production of P_4 at 5% O_2 increased at 96 h but remained unchanged at 144 h. Increase P_4 between 48 and 96 h of culture under low O_2 may be due to the luteinisation of some cells during collection and seeding. The effect of low O_2 on P_4 production has been examined in GCs and luteal cells in different species but the results were inconsistent.

Low O_2 at 1-2% inhibited P_4 production in rat GCs (Koos and Feiertag, 1989) but had no negative effect in pig GCs except at 1% (Basini et al., 2004). Low (5-10%) O_2 increased P_4 production in luteinizing but not non-luteinizing cultured bovine GCs (Fadhillah et al., 2014). In contrast 3% O_2 had no effect on cultured bovine early-luteal cells but reduced P_4 production in cultured mid-luteal cells (Nishimura et al., 2006, Nishimura and Okuda, 2010). The last group suggested that the contradictive outcome of O_2 concentration on the P_4 secretion depended on the cell differentiation status.

Upregulation of *HSD3B1* in GCs is indicative of luteinisation (McAllister et al., 1990, Wang et al., 2007). In the current study, transcript expression of *HSD3B1* was greater at 20 than 5% O_2. This is in contrast to studies with cultured luteal cells, where *HSD3B1* mRNA expression was unaffected by O_2 concentration (Nishimura et al., 2006). However, O_2 concentrations in luteal tissue have not been recorded and the possibility of higher O_2 concentration in the CL should be considered. Oxygen concentration during the mid-luteal stage in ovarian venous blood is around 15% (Wise et al., 1982). However, O_2 concentration in the follicle is much lower than this (de Castro e Paula et al., 2008). Consequently, GCs cultured under low O_2 may have different characteristics and roles depending on the extent of luteinisation.

In vivo, GCs could develop under low O_2 during follicular development and encounter further reductions in O_2 towards ovulation. After ovulation, ovarian follicular cells may be exposed to greater levels of O_2 in a more vascularized tissue bed. In this manner, ruptured follicular cells will be

exposed to higher O_2 levels compared with cells before ovulation. This may stimulate luteinisation.

Another factor that could explain contrasting results from the current study and other studies is duration of culture. Previous studies have tended to culture cells for just 24 h (Koos and Feiertag, 1989, Nishimura et al., 2006, Fadhillah et al., 2014).

In the current study, E_2 production by GCs cultured at 20% O_2 declined with time in culture. Cells under this condition still produced small amounts of E_2 at the end (144 h) of culture period. However, E_2 production by GCs cultured at 5% O_2 was increased at 144 h. Additionally, aromatase activity was reduced by culture time under high O_2 concentration but was unchanged under low O_2.

Roberts and Echternkamp (1994) reported that bovine GCs cultured under 5% compared to high 20% produced more E_2 after 2 to 4 days of culture. The production of E_2 (whilst higher under 5% than 20% O_2) nevertheless increased with time in culture for GCs from small follicles (2-8mm) but reduced with time in culture for GCs from large follicles (>8 mm). Whilst broadly supporting observations from the current study, temporal differences in oxygen-mediated E_2 production could be explained by the nature of GCs cultured (aspirated GCs recombined with scrapped cells from small and large follicles in the study of Roberts Echternkamp, 1994) and aspirated from 4-6 mm follicles in the current study. Another study cultured sheep follicles under varying O_2 and found that the highest concentrations of E_2 occurred only when follicles were cultured at 5% O_2 and stimulated by FSH (Cecconi et al., 1999).

Fluctuations in E_2 production by cultured GCs under air have been reported by (Gutierrez et al., 1997). The results in that study revealed that E_2 production by bovine GCs could be maintained throughout culture and variation in E_2 production depends on GC origin and applied treatments (Gutierrez et al., 1997). Similar pattern of E_2 secretion were observed in the current study but in GCs cultured at 5% O_2. However, E_2 production reduced over the culture period under atmospheric O_2. The current study reports for the first time that GCs maintain both E_2 production and

aromatase enzyme activity throughout 144 h of culture in bovine GCs cultured at physiological O_2 level.

The current study found that *CYP19A1* expression was low and virtually undetectable in cultured bovine GCs. However, the higher concentrations were detected in freshly collected GCs. It has been suggested that the low and/or undetectable *CYP19A1* due to the short half-life of transcript in cultured GCs (Silva and Price, 2000, Sahmi et al., 2006). Insignificant *CYP19A1* gene expression can contribute to the measurable changes in E_2 production (Luo et al., 2011, Castañon et al., 2012). Possibly, the lower expression of *CYP19A1* in *in vitro* cultured GCs would be the reason for the much lower E_2 concentration *in vitro* than *in vivo* (Nimz et al., 2009). In the current study CYP19A1 protein expression was detected in cultured bovine GCs cells, and aromatase expression was detected.

The mechanisms by which the E_2 production increases in 5% O_2 is not understood. It has been suggested that self-renewing division (cell proliferates without differentiation) of stem cells are conditioned by low O_2 (Ivanovic, 2009).

In summary, GCs cultured under high O_2 concentration (air) differentiate more into luteal cells than in low O_2. Inconsistencies between studies may be due to variations of culture model.

Haemoglobin and oxidative stress

The main role of haemoglobin (HB) is to transport gases such as oxygen (O_2), carbon dioxide (CO_2), carbon monoxide (CO) and nitric oxide (NO) (Saha et al., 2014). Haemoglobin is a tetramer structure containing two α and two β globin chains (Pieragostini et al., 2010). Genes encoding α and β chains are located on separate chromosomes in mammals and birds. The α globin gene is embedded in open chromatin in all cell types, whereas the β globin gene is packaged into inactive heterochromatin in non-erythrocyte cells. However, about eight other alleles from *HBB* have been identified in the bovine (Pieragostini et al., 2010). Despite these variations, both α and β globin is expressed equally in erythrocytes (Hardison, 1998). However, haemoglobin has been discovered not only in the erythrocytes but also in other tissues such as mouse avascular lens and cornea (Mansergh et al., 2008), the human endometrium (Dassen et al., 2008), rat and human

neural cells (Richter et al., 2009), human hepatocytes (Liu et al., 2011a), human matured cumulus cells *in vivo* (Kind et al., 2013), and recently in human GCs and cumulus cells (Brown et al., 2015). The results in the current study demonstrated the expression of both *HBA* and *HBB* mRNA in bovine GCs *in vivo* and *in vitro*. However, haemoglobin function in non-erythrocyte cells is still unclear. Haemoglobin alpha has been described as involved in nitric oxide regulation in endothelial cells (Straub et al., 2012) and has a cytoprotective role against oxidative insults in cervical cancer cells (Li et al., 2013b).

It's interesting to note that the expression of *HBA* mRNA in GCs was higher than the expression *HBB* mRNA. The same unequal expression has been shown in other alveolar epithelial cells and cervical cancer cells (Newton et al., 2006, Li et al., 2013b). However, the most important finding was that the expression of *HBA* mRNA increased in GCs cultured under 5% O_2 compared to those under atmospheric O_2. The reason why haemoglobin is expressed in GCs is uncertain. However, the higher concentration of *HB* under low O_2 could be related to the low O_2 level found in the ovarian follicle. It would be more applicable to say that HB protein may be used as O_2 transporter and the cells under low O_2 concentration need more O_2, or it could act as an O_2 reservoir. According to this mode of action *HB* mRNA expression will be stimulated by low O_2. This coincides with reduced O_2 concentration in follicles and further reductions were observed towards follicular ovulation (de Castro e Paula et al., 2008). It has been suggested that haemoglobin genes are regulated by O_2 concentration (Hardison, 1998). The result in the current study was supported by that shown in bacteria (Dikshit et al., 1990) that *HB* gene expression was induced by low O_2 concentrations. Another interpretation involves the potential role of HB in oxidative stress. It has been reported that HB has as antioxidant role (Liu et al., 2011a). Collectively, increased HB levels under low O_2 may regulate oxidative stress and may have an essential role in follicular development, ovulation and CL development.

To test the hypothesis that *HB* in GCs functions as an antioxidant or O_2 carrier, transcripts of reactive oxygen species inhibitory genes, superoxide dismutase (*SOD1* and *SOD2*), were analysed. Transcripts for these two

genes, however, were not affected by O_2 tension. Furthermore, no effects of O_2 tension were revealed on ROS. It had previously been reported in pigs that SOD and peroxidase activity are increased in cultured GCs under low O_2 and that ROS level were reduced by low O_2 level (Basini et al., 2004).

Further analysis of *HSPA1A* revealed that GCs cultured under 5% O_2 and 20% O_2 may not be under stress. It is well-known that HSP performs a role in cell protection against variety of stresses. In response to oxidative stress and heat stress, HSP70 acts as a chaperone and has the capability to contribute to cellular repair and cytoprotective function (Kregel, 2002). It prevents protein degradation and aggregation, folding of non-native protein, structural protein maintenance, translocation of protein, degradation of unstable protein and also reduces apoptosis reviewed by (Kregel, 2002).

Previous research reported the expression of *HSPA1A* in GCs (Velázquez et al., 2011). In that study, bovine GCs of large follicle showed lower expression of *HSPA1A* in large than in small and medium follicles. Perhaps mimicking the small to large follicle transition, *HSPA1A* expression in cultured GCs within the current study decreased towards the end of culture (96 and 144 h) compared with those cultured for 48 h under 5 and 20% O_2.

Effect of melatonin on cultured granulosa cells under low and high oxygen tension

The presence of melatonin in follicular fluid at high concentration (Nakamura et al., 2003, Tian et al., 2014) raises the question does hypoxia and melatonin in the follicle play a role in the ovarian follicular cell, ovulation and subsequent GCs luteinisation? To our knowledge this is the first study to evaluate melatonin action on GCs under physiological O_2 level. We found that melatonin did not have a consistent effect on GCs cultured under low and high O_2 concentration.

Cell number and viability

In the present chapter, melatonin at 20 pg/ml increased GC number. Melatonin helped to protect the integrity of GCs, it reduced DNA damage, lipid peroxidation, apoptosis and mitochondrial dysfunction (Takada et al.,

2012, Cruz et al., 2014, Tanabe et al., 2015). The apoptotic genes *BAX* and *P53* are other important factors in GC development under O_2 tension with melatonin treatment. The level of *BAX* mRNA decreased with culture time. Conceivably, replacing culture media during each time point could be the reason for reduction of *BAX* mRNA expression in bovine GCs.

Steroidogenesis

Regardless of other factors, melatonin did not have a consistent effect on steroidogenesis in the current chapter. Melatonin decreased E_2 production by GCs cultured under both conditions. As melatonin operates via different pathways to influence cells, different hypothesis can be considered to explain effects of melatonin on steroidogenesis. Endogenously produced melatonin can increase steroidogenesis through cyclic adenosine monophosphate (cAMP) and protein kinase (PKA). In contrast, exogenous melatonin working via its receptors reduces steroidogenesis by inhibiting cAMP (Tamura et al., 2009). However, it should be noted that melatonin operates via other pathways such as nuclear receptors which can freely pass through the cell membrane to affect cells (Luchetti et al., 2010). It has been reported that melatonin has inconsistent effects on follicular cells. It can increase (Woo et al., 2001, Wang et al., 2012), have no effect (Nakamura et al., 2003, El-Raey et al., 2011, Nakamura et al., 2014) or reduce (Chuffa et al., 2011) E_2 production. The inconsistent results could be due to the type of cell and culture system. Conceivably, endogenous melatonin production by GCs cells could reduce the main function of external melatonin treatments.

Melatonin biosynthesis and as antioxidant

It has been reported that melatonin is not only synthesized by the pineal gland but also by other tissues that have the ability to produce melatonin. On human (Kim et al., 2013) and bovine (Tian et al., 2014) melatonin in follicular fluid may originate directly from the follicle or from blood. Ovarian tissue has the capacity to synthesis melatonin (Kim et al., 2013). In the current study, transcripts of both *AANAT* (low levels) and high *ASMT*, which are the last two essential enzymes for melatonin biosynthesis, were expressed in bovine GCs. The enzymes responsible for melatonin biosynthesis were found in mammalian ovary; *AANAT* and *ASMT* in rat

ovary and human (Itoh et al., 1997, Itoh et al., 1999), *ASMT* in bovine (El-Raey et al., 2011).

The presence of melatonin in ovarian follicular fluid may reduce macromolecular oxidation and protect the follicle from oxidative stress or other environmental stress (Gao et al., 2012). Recently, increased oxidative stress had a direct negative outcome on the quality of oocytes and on female fertility (Prasad et al., 2016). Melatonin differs from other antioxidants because of its exceptional features of cascade reaction with free radicals and its ability to be modified under moderate oxidative stress (Tan et al., 2015). Melatonin as an antioxidant protects GC integrity through decreasing oxidative stress within the cellular compartment (Tanabe et al., 2015). Results in the present chapter show that ROS production was reduced by the inclusion of melatonin in culture media regardless of O_2 tension within GCs.

4.5 Conclusion

The present chapter assessed the physiological effects of different doses of melatonin on bovine GCs cultured during 144 h either at super physiological O_2 (20% O_2, 5% CO_2) or physiological O_2 conditions (5% O_2, 5% CO_2). The findings demonstrated that melatonin increased cell viability and reduced reactive oxygen species by cultured GCs under both conditions. Melatonin had no effect on steroid production, and expression of apoptotic (i.e. *BAX a*nd *P53*) and antioxidant-enzyme (i.e. *SOD1* and *SOD2*) genes. It is suggested that melatonin may be synthesised by GCs as genes encoding the last two enzymes (i.e. *AANAT* and *ASMT*, which are the rate limiting enzymes controlling melatonin production (Tan et al., 2015) were expressed in bovine GCs.

Low O_2 concentrations enhance GC proliferation and reduce GC differentiation. Bovine GCs differentiated more into luteal cells under atmospheric O_2, as evidenced by increased P_4 production and upregulation of *HSD3B1* transcripts. In contrast, under the same conditions both E_2 production and aromatase enzyme activity declined with culture time. On the other hand, physiological O_2 maintained E_2 production and aromatase enzyme activity for 144 h of culture and had no effect on *HSD3B1* transcript expression. These results suggest that O_2 concentration influences cell proliferation and differentiation.

The greater expression of haemoglobin transcripts in GCs cultured under 5% O_2 was another novel feature of this current research. Culture may offer a valuable system to determine GC function. The results reported in this chapter provide evidence that culturing GCs under low O_2 is a more physiological, providing a better model system to study the effects of heat stress (temperature) on granulosa cell function.

Chapter 5: Effect of temperature on bovine granulosa cells cultured under low oxygen in the presence and absence of melatonin

5.1 Introduction

Chapters 2 and 3 support the contention that season has an effect on cattle fertility in temperate climates such as the UK. During follicular development GCs provide a critical microenvironment for the oocyte (Tatone et al., 2008, Petro et al., 2012). Granulosa cells could be an early target of heat injury. Recent studies demonstrate that GCs can be undergo apoptosis under thermal stress (Li et al., 2016). This can be due to the oxidative stress and/or directly via molecular mechanisms of apoptosis. Evidence suggests that the deleterious effects of elevated temperature are due to oxidative stress (Ganaie et al., 2013). It has been reported that superoxide increased immediately after heat stress (Li et al., 2014).

Reactive oxygen species and apoptosis play a central role in reproductive physiology (Tamura et al., 2009). High concentrations of melatonin in follicular fluid, and expression of melatonin receptor are reported (Itoh et al., 1999, Taketani et al., 2011, El-Raey et al., 2011), with supporting evidence from Chapters 3 and 4. Melatonin as antioxidant exhibits unique features under moderate oxidative stress. Melatonin has a 10-fold greater capacity to scavenge ROS or to neutralize toxic oxygen derivatives than other classic antioxidants (Tan et al., 2015). It has been proposed that cattle fertility can be improved by chronic administration of melatonin (Garcia-Ispierto et al., 2013). In addition, melatonin can protect GC integrity in the preovulatory follicle and reduced apoptosis in GCs (Tanabe et al., 2015) and it could also interfere with luteinizing mechanisms of GCs (Taketani et al., 2011).

In the past GCs, were commonly cultured under atmospheric O_2 (20% O_2 with 5% CO_2). However, *in vivo* GCs develop under low O_2 concentrations (de Castro e Paula et al., 2008) and in the presence of the potent antioxidant melatonin. A culture model which mimics physiological

conditions was established in Chapter 4, and it was clear that atmospheric oxygen (20% O_2) enhanced luteinisation of GCs.

Hypothesis

1. Culturing bovine GCs at higher temperature under physiological O_2 tension reduces GC viability, steroidogenesis and stimulates ROS generation.
2. Melatonin can ameliorate the negative outcome of temperature under low O_2.

To separate the effect of temperature and melatonin on cultured GCs, cells were cultured under normal (37.5°C) and high (40.0°C) temperatures in the presence and absence of melatonin (0, 20, 200 and 2000 pg/ml) for up to 144 h. The main objectives are:

1. To explore the effect of high temperature on GC viability, apoptotic genes, steroidogenesis and transcripts in the presence and absence of melatonin.
2. To evaluate the effect of high temperature on the generation of ROS and the effectiveness of melatonin to reduce ROS production. In this respect, superoxide dismutase (*SOD1* and *SOD2*) expression was assessed as it is one of the first factors to be expressed during oxidative stress.
3. To explore the effect of temperature on *HBA* (which serves as an oxygen carrier), *ASMT* (the limiting melatonin biosynthesis enzyme) and *HSPA1A* (a stress biomarker) expression.

5.2 Materials and Methods

All the materials and methods were obtained from Sigma (Sigma-Aldrich, Company Ltd, Gillingham, Dorset), others were stated in context.

5.2.1 Experimental design

Bovine GCs were cultured in a 4 x 3 x 2 factorial arrangement (4 melatonin doses, 3 culture times and 2 temperatures; Table 5.1). Granulosa cells were cultured in M199 culture media (Media M199 supplemented with penicillin (50 IU/ml), streptomycin (50 µg/ml), bovine serum albumin (1 mg/ml) free fatty acid, testosterone (100 ng/ml), bovine FSH (1 ng/ml), insulin (10 ng/ml) from (ITS), transferrin (2.5 µg/ml), sodium selenite (4 ng/ml) and L-glutamine (3654 µg/ml)) under low oxygen (5% O_2 with 5% CO_2) for 48 h. After GCs attached to the bottom of the wells (48 h of pre-culture), the cells and supernatant were harvested from one plate at 48 h post seeding (duplicate wells) and the culture medium from remaining plate was replaced with fresh culture medium. At this time point, melatonin treatments (0, 20, 200 and 2000 pg/ml) were added to wells. To evaluate the effect of temperature on GCs in the presence and absence of melatonin at (5% O_2 with 5% CO_2), culture plates were separated into two incubators; one set at 37.5°C and the other at 40.0°C. The temperature were selected to mimic the physiological temperature in thermo-neutral and heat-stressed cow. It has been recorded that ovarian temperature is about 1°C cooler than rectal temperatures (38.5°C) and cooler temperatures were recorded in pre ovulatory follicles than ovarian stroma (Grøndahl et al., 1996). Commonly, GCs are cultured at 37.5°C which is recognised as normal (Woo et al., 2001, Basini et al., 2004, Li et al., 2014, Fu et al., 2014). Also it has been reported that body temperature of heat stressed cows increases to around 40 to 41°C (Roth et al., 2001b). Granulosa cells and supernatants from duplicate wells per melatonin treatment were harvested from two plates (i.e. one at 37.5°C and one at 40.0°C) for each time point (96 and 144 h) post seeding. At every 48 h of culture, cell supernatants were replaced with fresh media and melatonin.

Table 5.1 *Granulosa cells cultured in different plates and seeded at different cell densities. All experiments were incubated under high (40.0°C) or normal (37.5°C) temperatures at low O_2 (5%) for 144 h, with collections at 48, 96 and 144 h.*

Exp.	Plate	Cell density	Culture media	Replicated experiments	Within plate replicates	Growth area (cm^2)
1	12 wells	0.6 X 10^6	1 ml	4	2 wells	3.8
2	96 wells	6 X 10^4	250 µl	3	5 wells	0.143

5.2.2 Ovarian collection and granulosa cell culture

Ovarian collections from slaughter-house are described in Section 4.2.3 and GCs were cultured as described in Section 4.2.4. All plates were cultured at 5% O_2 under high (40.0°C) and normal (37.5°C) temperatures in the presence or absence of melatonin. Granulosa cells were harvested as described in Section 4.2.5.

5.2.3 Cell number and viability

Harvesting of GCs and counting cells by trypan blue were as described in Section 4.2.5.1. Granulosa cells number were measured by trypan blue. The viability of GCs by crystal violate were quantified by plotting optical density plotted against known cell numbers using linear standard curves. Cell numbers were adjusted by interpolating from a standard curve using prism software (R^2=0.98) as described in Section 4.2.5.2. Granulosa cells cultured in 96 well plates were cultured as described in Section 4.2.4; Exp **3**. Granulosa cells were cultured at a density of 60,000 cells per 250 µl of culture media in 96 wells plates.

5.2.4 Steroid hormonal analysis

Both P_4 and E_2 concentrations in spent media were analysed by ELISA as described in Section 4.2.6. The inter- and intra-assay CV were 14.4% and 6.6% for P_4 and 8.7 and 5.3% for E_2, respectively.

5.2.5 RNA extraction and reverse transcription

Total RNA was extracted from GCs using RNeasy Kit as described in Section 4.2.8. Details of mRNA quantification were described in Section 4.2.9 and the validation oh HKGs as described in Section 4.2.9.3 and detail in

appendix A.2. As in the previous chapter, to increase the specificity of the target gene, TaqMan technology was used, as TaqMan probes can detect only target product (Uyar et al., 2013).

5.2.6 Measurement of reactive oxygen species

The production of ROS by GCs were analysed by Nitroblue tetrazolium (NBT) assay as described in Section 4.2.10. Granulosa cells were cultured in pre-coated 96 well plates with fibronectin. The details were described in Section 5.2.1 with changing cell density according to the cellular growth area. Granulosa cells were seeded at 60,000 cells density in 250 µl of culture media. The ROS generation was adjusted by cell viability (Section 5.2.3). Nitroblue tetrazolium was used as a measure of the superoxide anion. It has been reported that superoxide increases after heat stress (Li et al., 2014).

5.2.7 Statistical analysis

Results were analysed by ANOVA using GenStat. Terms fitted to this 2 X 4 X 2 factorial model were temperature (Normal (37.5°C) *vs* High (40.0°C)), melatonin (0, 20, 200 and 2000 pg/ml) and time in culture (96 and 144 h). For transcript expression, terms fitted to this 2 X 2 X 2 factorial model were temperature (37.5°C and 40.0°C), melatonin (0 and 2000 pg/ml) and culture duration (96 and 144 h). The model was blocked by culture replicate, incubator and plate. Temperature level, melatonin and culture duration were considered as fixed effects. Oestradiol (pg/ml) and progesterone (ng/ml) production was expressed per x 10^5 cells. Natural log transformations of the data were used to correct for heteroscedasticity of the residuals. The data are shown as natural logs of the mean with SED. LSD were used to estimate significant differences between means.

5.3 Results

5.3.1 Effect of temperature on granulosa cell proliferation and steroid production at 5% oxygen tension in the presence or absence of melatonin

5.3.1.1 Number of viable cells

Granulosa cell number after 48 h of culture in basal media was 4.54 X 10^5 cells; SED=0.19. At this time, melatonin treatments were added to the culture media and plates placed in the incubator conditioned for the predetermined temperature treatment. Cell number increased with time of culture (Figure 5.1A, $P<0.001$) in a similar manner regardless of temperature (time x temperature interaction $P=0.85$). However, there was an interaction of temperature and melatonin on GCs number (Figure 5.1B; $P=0.004$). Melatonin treatment at 200 and 2000 pg/ml increased cell number when cultured at supra-physiological temperature (40.0°C). In contrast, when cells were cultured at physiological temperature (37.5°C), melatonin treatment (2000 pg/ml) reduced cell number.

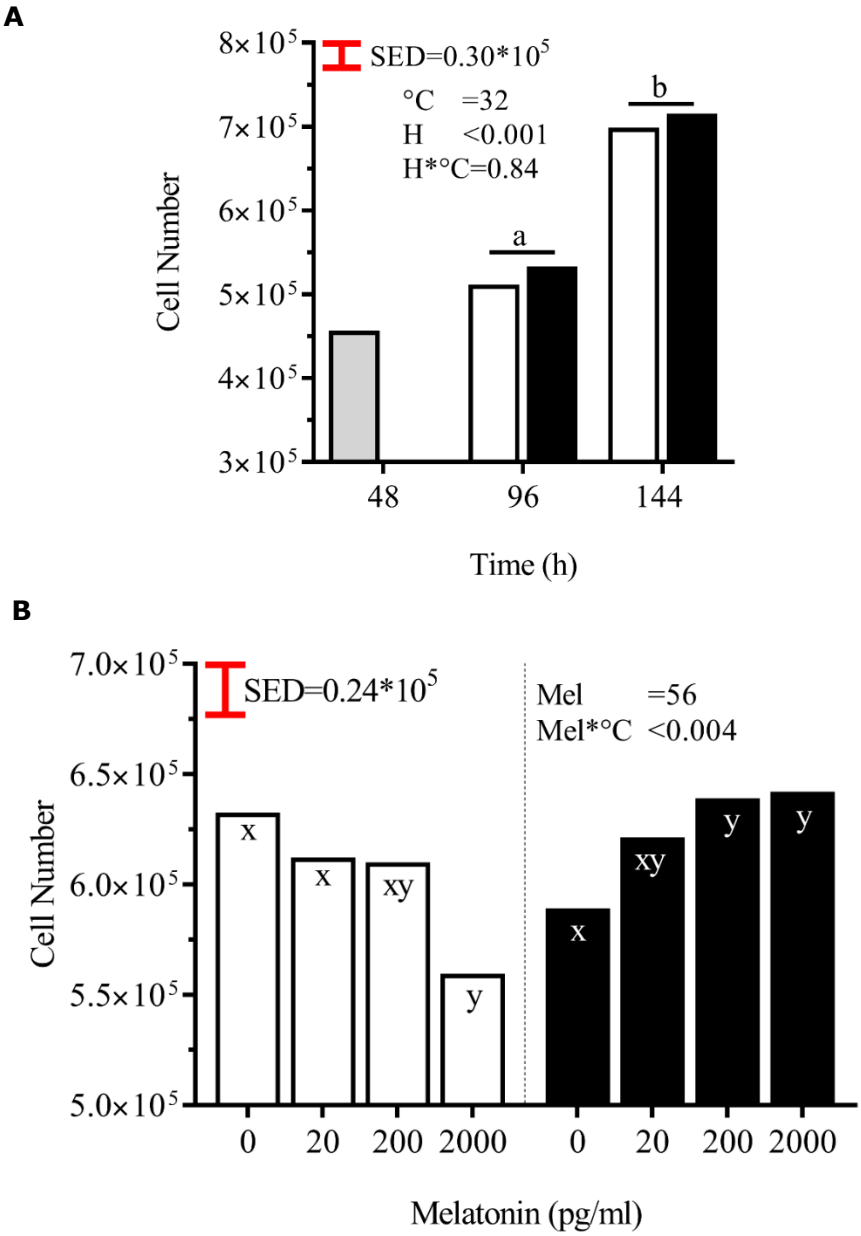


Figure 5.1 *Effect of temperature and melatonin on GCs number after 96 and 144 h of culture. Six hundred thousand cells were seeded in a 12 well plate at 37.5°C for the first 48 h at 5% O_2. At 48 h the plates were transferred to an incubator at 37.5°C (□) or 40.0°C (■) in the presence or absence of melatonin. Viable cells were quantified at 48, 96 and 144 h of culture after trypan blue exclusion. Cell number increased (P<0.001) with time in culture under both temperature levels (**A**). Melatonin increased cell number in super physiological temperature and reduced cell number in normothermia (P=0.004) (**B**). The data are presented as mean with SED. Significant differences between means was estimated with LSD. a, b highlight differences between time point (**A**) and X,y highlight differences within temperature (**B**). Data represented four replicate cultures with duplicates wells within plate replicate.*

5.3.1.2 Progesterone production

Progesterone production decline from 96 to 144 h of culture for GCs cultured at 40.0°C (P<0.05). However, cells cultured at 37.5°C sustained

their P_4 production at similar level between 96 and 144 h (time by temperature interaction, P<0.001; Figure 5.2A). In addition, increasing concentrations of melatonin caused an increased (P=0.007) in P_4 production by GCs cultured at 37.5°C. In contrast, for cells cultured at 40.0°C, P_4 production declined with increasing melatonin concentration (Figure 5.2B).

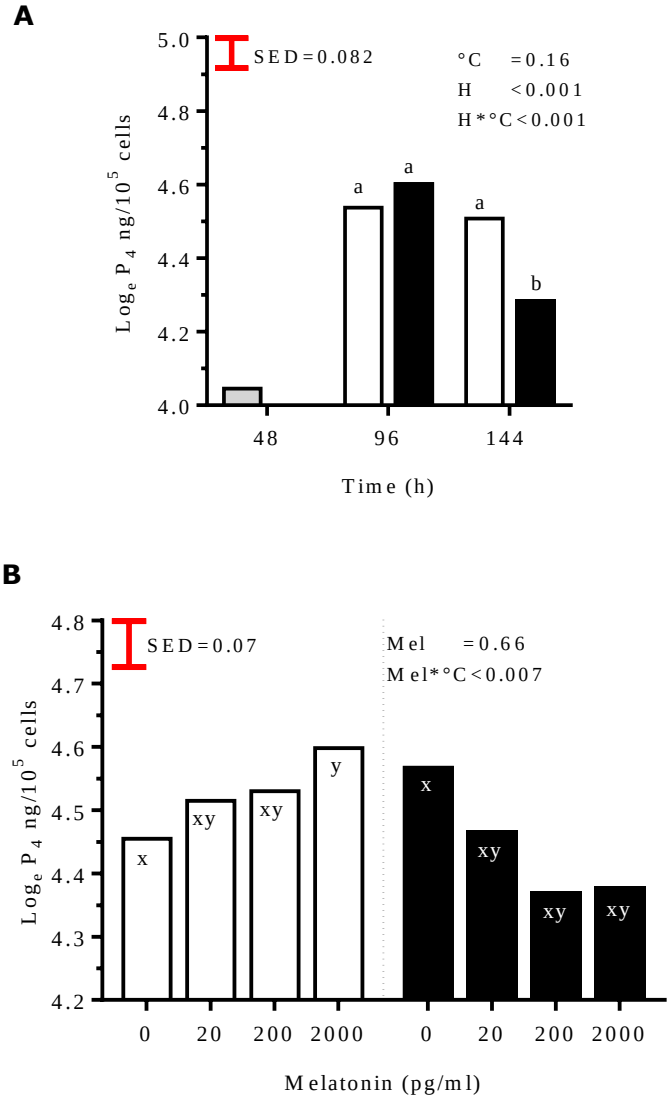


Figure 5.2 *Effect of temperature and melatonin on GCs P_4 production in vitro. Six hundred thousand cells were seeded in a 12 well plate at 37.5°C for the first 48 h at 5% O_2. At 48 h the plates were transferred to an incubator at 37.5°C (□) or 40.0°C (■) in the presence or absence of melatonin. Progesterone production decline at 144 h in cells cultured at 40.0°C but was unaffected at 37.5°C (**A**). Melatonin reduced P_4 production at 40.0°C and increased at 37.5°C (**B**). Data was analysed after transformation to the natural log of ng /ml media for 1 X 10^5 cells. Data are presented as mean and SED. Significant differences between means were estimated by LSD. a, b highlight differences between time point (**A**) and and X,y highlight differences within temperature (**B**). Data represented four replicate cultures with duplicates wells within plate replicate.*

5.3.1.3 Oestradiol production

Oestrogen production increased from 96 to 144 h of cells culture at both 37.5°C and 40.0°C (P<0.001; Figure 5.3A). Treatment with 20 and 200 pg/ml of melatonin caused a reduction of E_2 production (P<0.001) equally at both 37.5 and 40.0°C. Nonetheless, the effect of melatonin at the highest concentration used (2000 pg/ml) did not affect E2 production (Figure 5.3B).

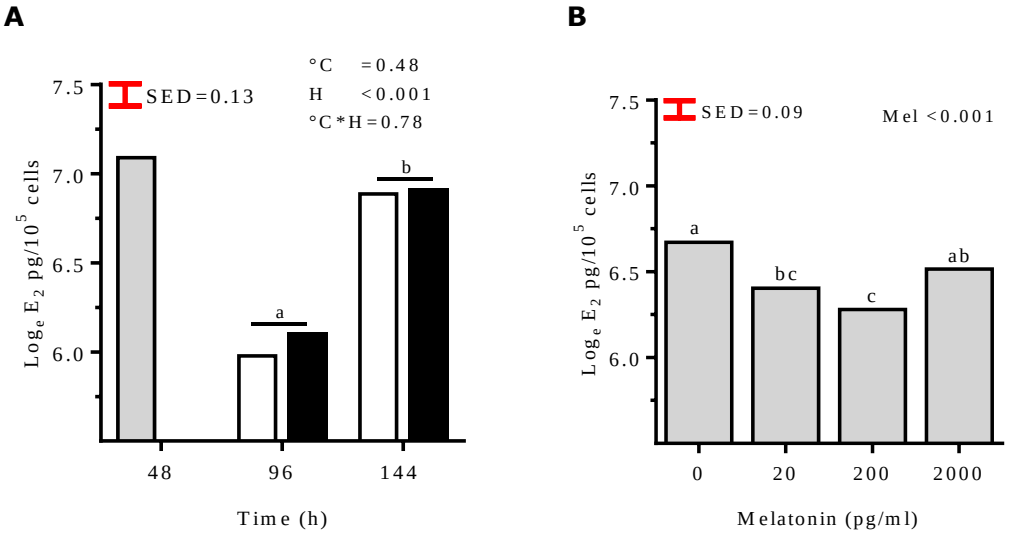


Figure 5.3 *Effects of temperature and melatonin on GCs E_2 production in vitro after 96 and 144 h of culture. Cells were incubated for 48 h at 37.5°C and then exposed to 37.5°C (□) or 40.0°C (■) in the presence and absence of melatonin. Oestrogen production increased between 96 and 144 h of culture (**A**). Melatonin reduced E_2 production under both temperature level (**B**). Data was analysed after transformation to the natural log of pg/ml media for 1 X 10^5 cells. The data are presented as mean and SED. Significant differences between means were estimated by LSD. a, b, c highlight differences between time point (**A**) and melatonin (**B**). Data represented four replicate cultures with duplicates wells within plate replicate.*

5.3.1.4 Oestrogen to progesterone ratio

The ratio of E_2:P_4 production increased (P<0.001) between 96 and 144 h of culture time and tended to be slightly higher at 144 h for cells under high temperature (temperature x time interaction, P=0.068; Figure 5.4A). There was no interaction of melatonin treatments with temperature (P=0.53). Treatment with melatonin at 20 and 200 pg/ml reduced E_2:P_4 production (main effect across all time frames and temperature

treatments; P<0.001), whereas melatonin at the highest concentration (2000 pg/ml) did not affect the E_2:P_4 ratio (Figure 5.4B).

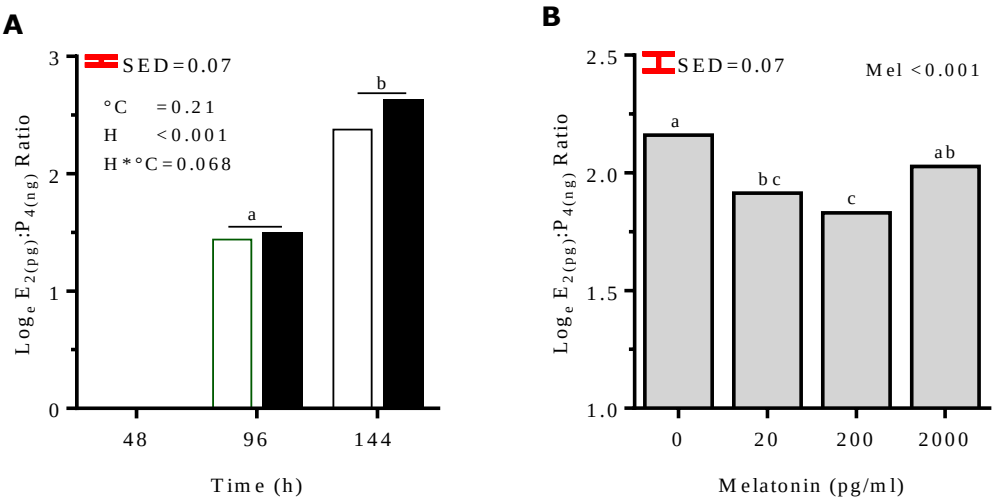


Figure 5.4 *Effects of temperature and melatonin on the E_2:P_4 production ratio in vitro after 96 and 144 h of culture. Cells were incubated for 48 h at 37.5°C and then exposed to 37.5°C (□) or 40.0°C (■) in the presence and absence of melatonin. Oestrogen to progesterone ratio increased between 96 and 144 h of culture (**A**). Melatonin reduced E_2:P_4 ratio under both temperature levels (**B**). Data was analysed after transformation to the natural log of pg/ng /ml media for 1 X 10^5 cells. The data are presented as mean with SED. Significant differences between means was estimated by LSD. a, b, c highlight differences between time point (**A**) and melatonin (**B**). Data represented four replicate cultures with duplicates wells within plate replicate.*

5.3.2 Effect of temperature on granulosa cell gene expression in the presence or absence of melatonin at 5% oxygen tension

5.3.2.1 Housekeeping genes

The expression of TATTA box binding protein (*TBP*) mRNA was the most stable reference gene in GCs cultured for up to 144 h under 5% O_2 at 37.5 and 40.0°C with the inclusion of melatonin (Table 5.2: for details see Appendix A.2). In consequence, all target genes in the present chapter were normalized against the expression of *TBP*.

Table 5.2 *The variable and interact (P values) of HKGs in GCs showed the effect of temperature, melatonin and culture time on the stability of HKG in GCs.*

HKG	°C	Mel	H	Mel*H	Mel*°C	H*°C	Mel*H*°C
RPLP0	0.60	0.96	**0.09**	0.47	0.33	0.39	0.45
TBP	0.63	0.82	0.26	0.76	0.25	0.67	0.38

°C= temperature, Mel=melatonin, H=culture duration hour, *= interaction. Using ANOVA test.

5.3.2.2 Apoptotic genes (*BAX* and *P53*)

Expression of *BAX* tended (P=0.068) to be higher at 40.0°C than at 37.5°C. Furthermore, the expression of *BAX* mRNA in GCs increased at 144 h of culture at 40.0°C. However, the mRNA expression of *BAX* in GCs cultured at 37.5°C declined at 144 h of culture (time x temperature interaction P=0.009; Figure 5.5A). Melatonin treatment tended (P=0.079) to increased *BAX* expression in bovine GCs cultured at 40.0°C, whereas reduced *BAX* expression was observed for those cells cultured at 37.5°C (Figure 5.5B).

Expression for *P53* mRNA was not affected by temperature (37.5°C vs 40.0°C; P=0.45). There was no effect of time and no significant interaction between temperature and time in culture (P=0.35) on *P53* mRNA expression (Figure 5.6A). However, melatonin treatment tended to reduce the expression of *P53* mRNA at 37.5°C, whilst at 40.0°C melatonin increased *P53* mRNA expression (temperature by melatonin interaction: P=0.091; Figure 5.6B).

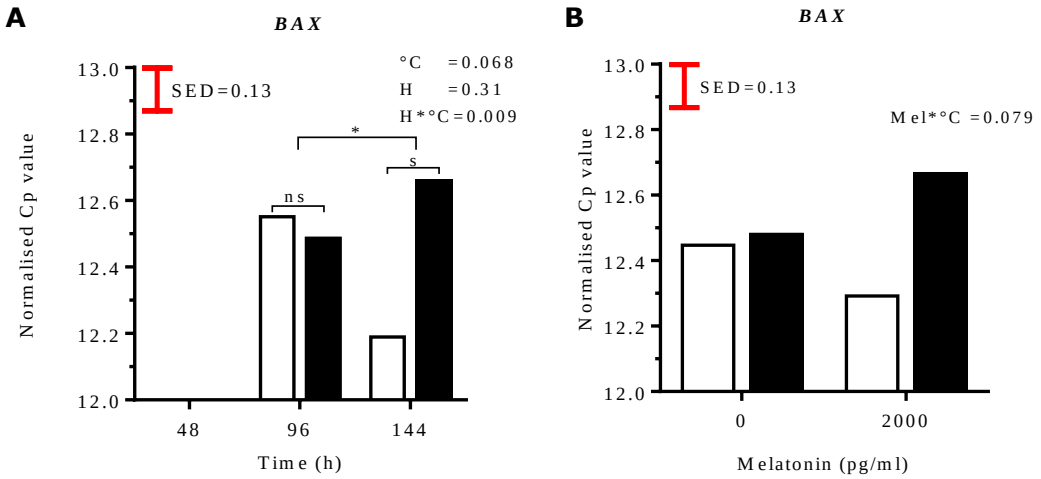


Figure 5.5 *Effects of temperature and melatonin on BAX mRNA expression in bovine GCs after 96 and 144 h of culture. Cells were incubated for the first 48 h at 37.5°C and then exposed to 37.5°C (□) or 40.0°C (■) in the presence and absence of melatonin. The expression of BAX mRNA increased at 144 h of cultured at 40.0°C, whereas reduced at 37.5°C (Temperature x time interaction; P=0.009;* ***A****). Melatonin tended to increase BAX expression in GCs cultured at 40.0°C, whereas it was reduced at 37.5°C (Temperature x melatonin interaction; P=0.079;* ***B****). The data are presented as mean with SED. Significant differences between means was estimated by LSD. S=significant, ns=nonsignificant and * interaction. Data represented four replicate cultures.*

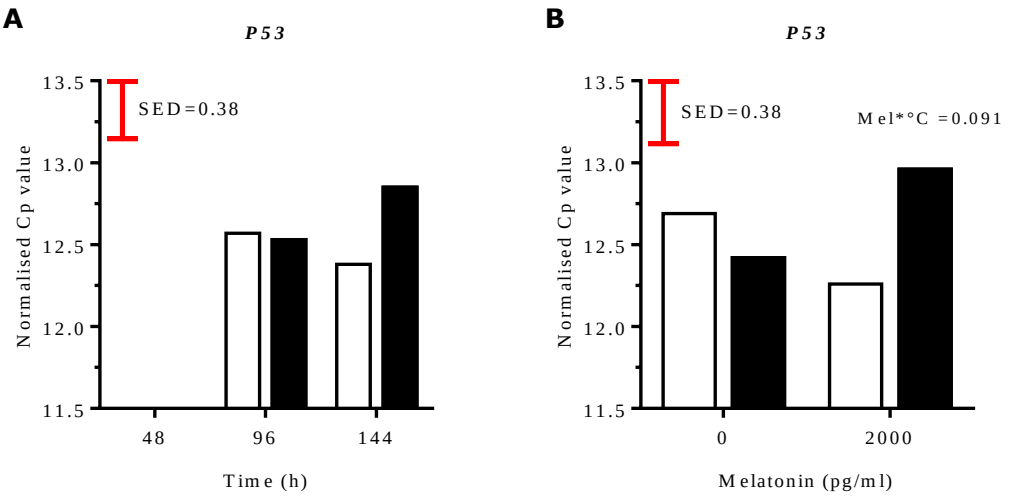


Figure 5.6 *Effects of temperature and melatonin on P53 mRNA expression in bovine GCs after 96 and 144 h of culture. Cells were incubated for the first 48 h at 37.5°C and then exposed to 37.5°C (□) or 40.0°C (■) in the presence and absence of melatonin. Melatonin treatment tended to increase the expression level of P53 mRNA in GCs cultured at 40.0°C, whereas cells cultured at 37.5°C reduced P53 expression by melatonin treated (Temperature x melatonin interaction; P=0.091;* ***B****). The data are presented as mean with SED. Data represented four replicate cultures.*

5.3.2.3 Steroidogenic enzyme (*HSD3B1*)

Progesterone producing enzyme *HSD3B1* mRNA expression was not affected by temperature or melatonin treatment (P>0.10; Figure 5.7).

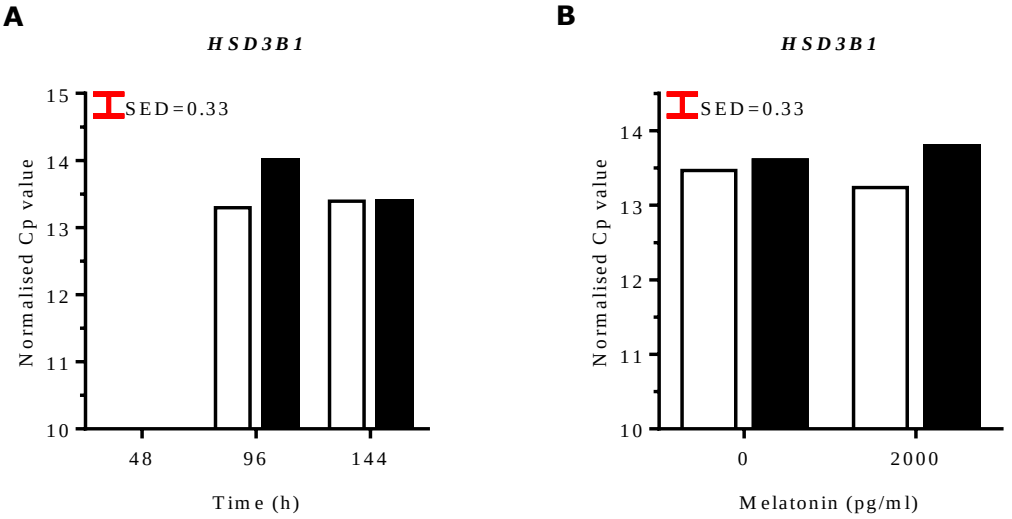


Figure 5.7 *Effects of temperature and melatonin on expression of HSD3B1 in bovine GCs after 96 and 144 h of culture. Cells were incubated for the first 48 h at 37.5°C and then exposed to 37.5°C (□) or 40.0°C (■) in the presence and absence of melatonin. The expression level of HSD3B1 mRNA in GCs cultured for 144 h at 37.5°C and 40.0°C (****A****) and treated with melatonin (****B****). The data are presented as mean with SED. Data represented four replicate cultures.*

5.3.2.4 Heat shock 70 KD protein 1A (*HSPA1A*)

The mRNA expression level of *HSPA1A* in cultured bovine GCs was not affected by temperature (P>0.10; Figure 5.8A). However, the inclusion of melatonin treatment to culture (main effect across culture time and temperature treatments) tended (P=0.087) to increase the mRNA expression of *HSPA1A* GCs (Figure 5.8B).

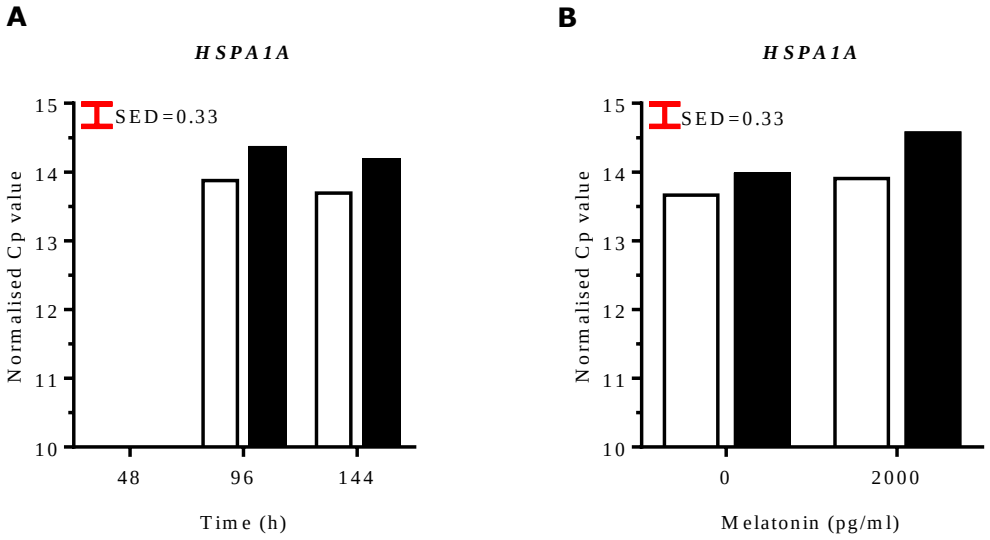


Figure 5.8 *Effects of temperature and melatonin on the expression level of HSPA1A mRNA in bovine GCs after 96 and 144 h of culture. Cells were incubated for the first 48 h at 37.5°C and then exposed to 37.5°C (□) or 40.0°C (■) in the presence and absence of melatonin. The expression level of HSPA1A mRNA in GCs cultured for 144 h at 37.5°C and 40.0°C (**A**). Melatonin tended (main effect; P=0.087) to increase HSPA1A mRNA level in cultured GCs (**B**). The data are presented as mean with SED. Data represented four replicate cultures.*

5.3.2.5 Melatonin biosynthesis

Expression of *ASMT* mRNA in cultured bovine GCs was higher (P=0.019) in cells cultured at 40.0°C than for cells cultured at 37.5°C **(**Figure 5.9A). Melatonin treatment (P=0.83) and culture time (P=0.74) did not affect *ASMT* mRNA expression. Additionally, no interaction was observed with the fixed effects (Figure 5.9B, C).

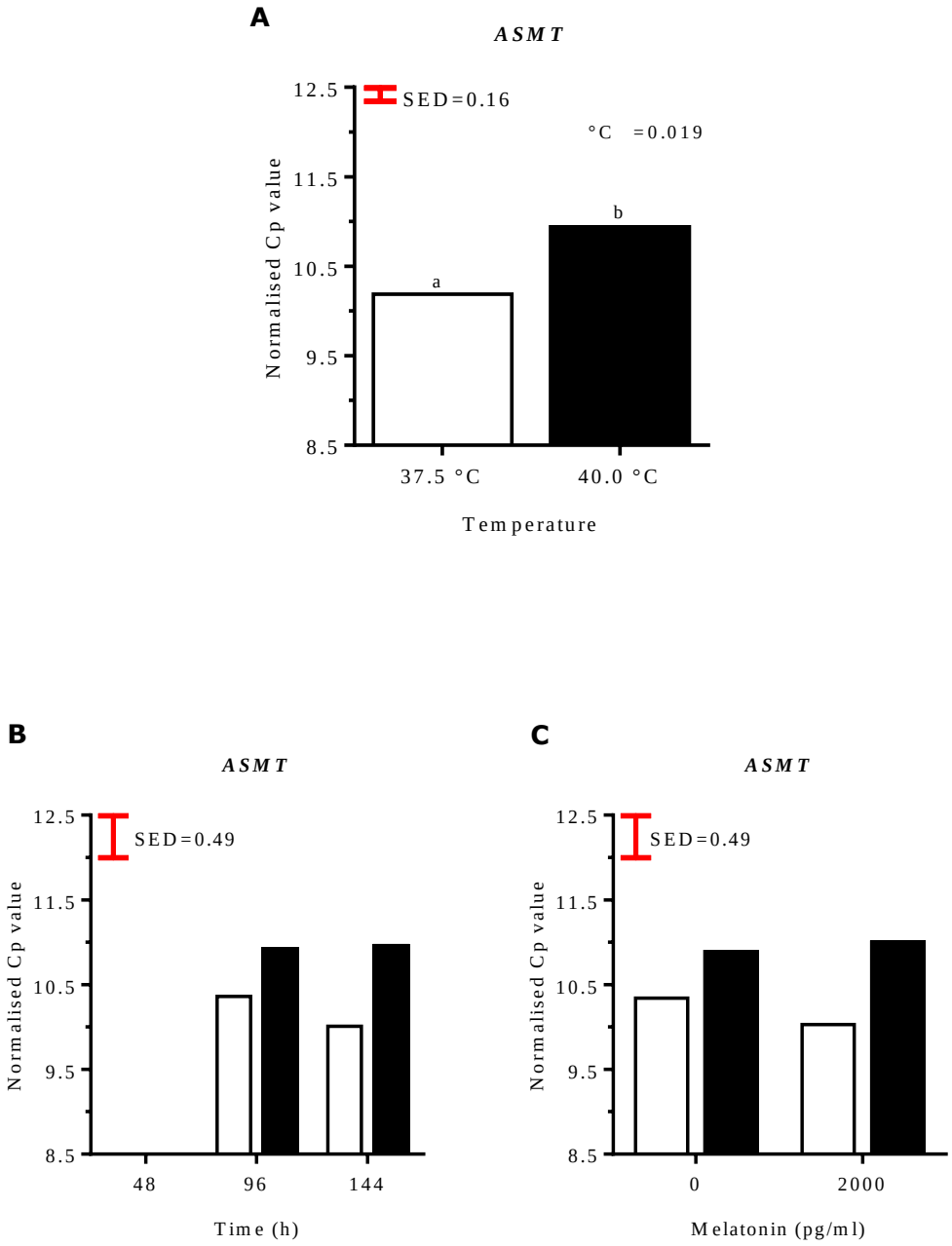


Figure 5.9 *Effects of temperature and melatonin on ASMT mRNA expression in bovine GCs after 96 and 144 h of culture. Cells were incubated for the first 48 h at 37.5°C and then exposed to 37.5°C or 40.0°C in the presence and absence of melatonin. The expression level of ASMT mRNA was higher in bovine GCs cultured at 40.0°C (■) than those cultured at 37.5°C (□) conditions (**A**) the higher expression was observed throughout the culture time 96 and 144 h (**B**). Transcript expression was not affected by melatonin treatment (**C**). The data are presented as mean with SED. Significant differences between means was estimated by LSD. a, b highlight differences between temperature (**A**). Data represented four replicate cultures.*

5.3.2.6 Haemoglobin (*HBA*, molecular oxygen carrier)

Haemoglobin mRNA was expressed in cultured bovine GCs at all times of culture. However, *HBA* mRNA levels were not affected (P=0.31) by temperature (37.5°C *vs* 40.0°C). Additionally, there was no effect of melatonin treatment (P=0.66) and time in culture (P=0.24) on the *HBA* mRNA expression on bovine GCs cultured under 37.5°C or 40.0°C (Figure 5.10).

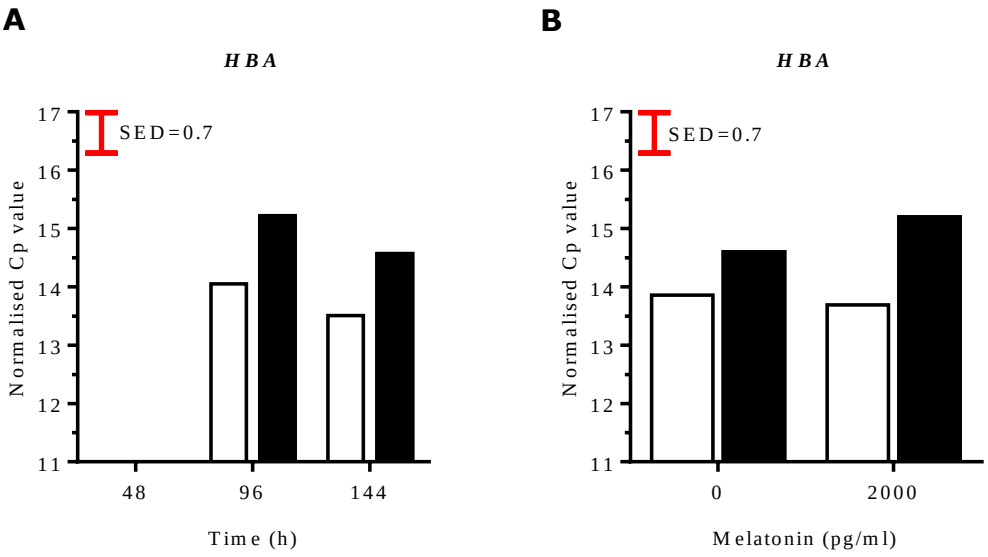


Figure 5.10 *Effects of temperature (**A**) and melatonin (**B**) on HBA expression in bovine GCs after 96 and 144 h of culture. Cells were incubated for the first 48 h at 37.5°C and then exposed to 37.5°C or 40.0°C in the presence and absence of melatonin. The expression level of HBA mRNA observed throughout the culture time 96 and 144 h of culture at 144 h at 37.5°C (□) and 40.0°C (■). The data are presented as mean with SED. Data represented four replicate cultures.*

5.3.2.7 Superoxide dismutase (*SOD1* and *SOD2*)

The expression of superoxide dismutase mRNA (*SOD1* mRNA, Figure 5.11A; and *SOD2* mRNA, Figure 5.11B) were not affected (P>0.10) by temperature (37.5°C *vs* 40.0°C), melatonin treatment (0 *vs* 2000pg/ml) or time in culture (96 to 144 h of culture).

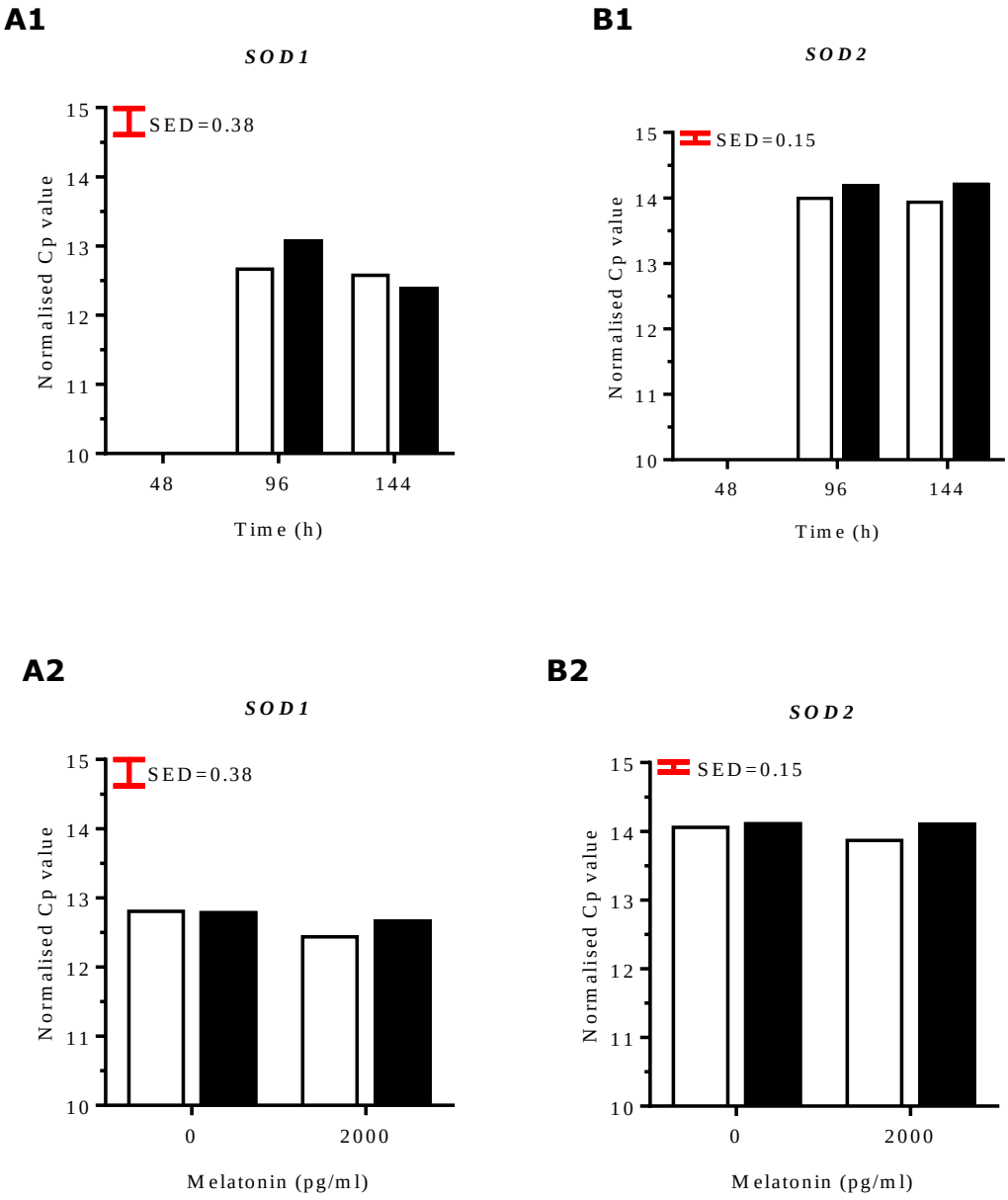


Figure 5.11 *Effects of temperature (**1**) and melatonin (**2**) on the level of superoxide dismutase (SOD1 **A** and SOD2 **B**) in bovine GCs after 96 and 144 h of culture. Cells were incubated for the first 48 h at 37.5°C and then exposed to 37.5°C or 40.0°C in the presence and absence of melatonin. The expression level of SOD mRNA expression observed throughout the culture time 96 and 144 h of culture at 37.5°C (□) and 40.0°C (■) (**A1** and **B1**). The data are presented as mean with SED. Data represented four replicate cultures.*

5.3.3 Effect of temperature on reactive oxygen species generated by cultured granulosa cells at 5% oxygen in the presence or absence of melatonin

Production of ROS by bovine GCs was not affected (P=0.37) by temperature (37.5°C *vs* 40.0°C). Generation of ROS was lower at 96 h (P<0.001) of culture compared with those observed at 144 h of culture (Figure 5.12A). However, the presence of melatonin significantly reduced (P<0.001) ROS generation at both temperatures (37.5°C and 40.0°C) (Figure 5.12B). There were no interactions between fixed factors.

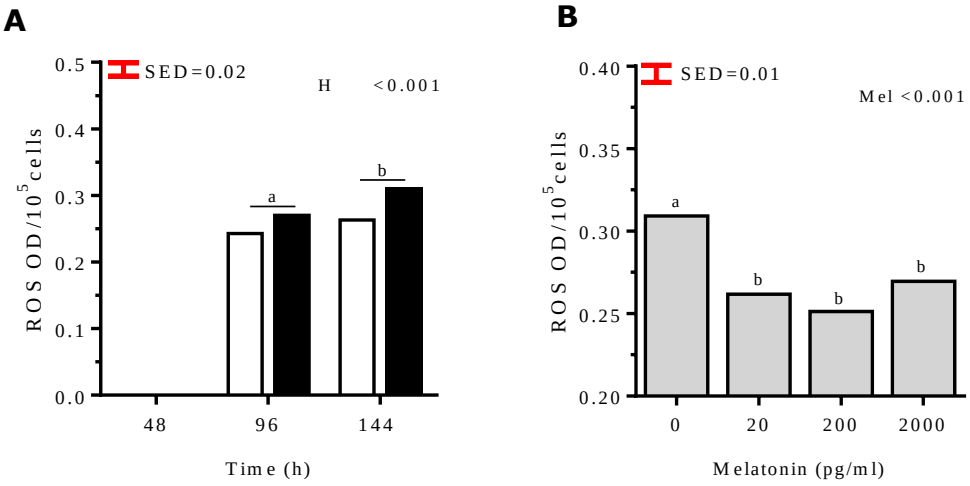


Figure 5.12 *Effect of temperature and melatonin on ROS production by cultured bovine GCs after 96 and 144 h of culture. Cells were incubated for the first 48 h at 37.5°C and then exposed to 37.5°C (□) or 40.0°C (■) in the presence and absence of melatonin. Cells were cultured at 37.5°C and 40.0°C at low O_2 environment (5% CO_2, 5% O_2 and 90% N_2). Reactive oxygen species affected by culture time (**A**) and melatonin treatments (**B**). The data are presented as mean with SED. Significant differences between means was estimated by LSD. a, b highlight differences between time point (**A**) and melatonin (**B**). Data represented three replicate cultures with five wells within replicate.*

5.4 Discussion

This is the first study to evaluate the effects of temperature on GCs cultured under physiological O_2 in the presence of melatonin. For GCs cultured under 5% O_2 high temperature did not reduce GCs viability. However, the expression of apoptotic *BAX* transcripts increased with culture time under high temperature, whereas its expression reduced at normal temperatures. Progesterone production by cells cultured at 40.0°C for 144 h was reduced. Oestradiol production increased between 96 h and 144 h of culture.

Melatonin treatments increased cell number under high temperature. Melatonin tended ($P<0.10$) to reduced *BAX* and *P53* mRNA expression level at 37.5°C, whereas it was increased at 40.0°C. Melatonin inclusion to culture media reduced P_4 production at 40.0°C and increased P_4 production at 37.5°C. Melatonin treatment reduced E_2 production and at 20 and 200 pg/ml reduced the E_2:P_4 ratio. Melatonin reduced ROS production by GCs cultured either under normal and high temperatures. This chapter report, for the first time, that mRNA expression level of *ASMT* in cultured GCs increased at 40.0°C compare with 37.5°C. Together, these results indicate that high temperature may increase GCs dysfunction (increased apoptotic expression and reduced P_4 production) progressing the understanding of ovarian function and may suggesting a possible the molecular mechanisms for summer infertility.

Several studies reported the effect of temperature or heat stress on GC culture. However, no published reports considered the effect of temperature on GCs cultured under low O_2 tension (5% O_2 with 5% CO_2). Also the inclusion of melatonin in culture media over an extended period of culture is also a novel feature of this study.

Cultured granulosa cells under 37.5°C and 40.0°C

Cell number and viability

High temperature (40.0°C) in the current study did not affect cell number. However, the expression of apoptotic genes such as *BAX* increased at 144 h for cells cultured at 40.0°C relative to 37.5°C. Shimizu et al. (2005) demonstrated that apoptosis in rat GCs increased under heat stress in a

time dependent manner and *BAX* mRNA level was increased. In general, apoptosis has two main pathways. The 'internal pathway' relies on mitochondrial followed by cytochrome C release induced activation of *BCL2* family members such as *BAX* that lead to apoptosis. The 'external pathway' relies on death receptors such as *TNF* family members on the cell membrane (Rodriguez et al., 2013). It has been reported that heat stress can increase cytochrome C release and induce apoptosis possibly through *P53* activation which activates *BAX* directly. The process of heat stress induced apoptosis in GCs points to the involvement of mitochondrial pathways (Gu et al., 2015).

Steroidogenesis

Several published studies showed that temperature altered steroidogenesis in GCs. However, this is the first study to test the effect of heat stress on steroid production and gene expression in GCs cultured for 144 h. Additionally, cells were cultured at physiological O_2 (5% O_2). The findings indicate that temperature interacts with culture time to effect P_4 production. Progesterone production by GCs cultured at 40.0°C was reduced significantly after 96 h of culture. Thus, results of the present study could mimic previous studies performed *in vivo*, which showed that temperature interacts with stage of the oestrous cycle to affect plasma P_4 (Trout et al., 1998, Wolfenson et al., 2002). These authors suggested that chronic heat stress reduced P_4 production. Luteal cell collected in winter and cultured under 38.0°C produced more P_4 than cells incubated at 40.0°C (Wolfenson et al., 1993a). Temperature induced P_4 production could result from the effect of temperature on apoptosis. In the current study high physiological temperatures induced *BAX* transcript expression in GCs collected at 144 h of culture. At this time point P_4 production was reduced, however, no effect of temperature was observed on E_2 production.

With respect to the cattle fertility, the above findings of reduced P_4 production at 40.0°C indicate that this could be a key factor for reduced fertility during hot seasons. Mann et al. (1999) demonstrated that reduced P_4 secretion after ovulation and during CL development compromised

reproductive performance and lead to low interferon-tau secretion. Low P_4 secretion can affect uterine function through $PGF_{2\alpha}$ secretion (Shaham-Albalancy et al., 2001) and reduced P_4 concentration with lengthening luteal phase promote dominant follicle persistence and altered follicle dynamics (Sirois and Fortune, 1990). Progesterone production by GCs exposed to 40.5°C for 2 h was not affected by heat stress. However, the expression level of *CYP11A1* and *STAR* decreased under heat exposure (Li et al., 2016). It would be better to suggest that GCs cultured at 5% O_2 for 96 h of culture at 40.0°C could enhance luteinisation. However, after 96 h of culture time, P_4 production was reduced under 40.0°C and unchanged under 37.5°C. Bridges et al. (2005) suggested that the premature luteinisation of ovarian follicular cells can be related with the cattle infertility.

It has been shown that high temperature can inhibit E_2 production by GCs and reduced the expression of nuclear steroidogenic factor 1 (*SF-1*) and *CYP19A1* mRNA (Li et al., 2016). Consistent with the current results no effect of heat stress on steroid secretion was observed by de Castro e Paula et al. (2008). Granulosa cells cultured under physiological O_2 levels confirmed previous results in Chapter 4 that E_2 production decreases between 48 and 96 h and increases at 144 h of culture at both temperatures.

However, E_2:P_4 ratio increased between 96 and 144 h of culture time. The ratio tended to be higher under high than normal temperature. This is because the production of P_4 was reduced in the high-temperature group. There is no apparent explanation for increased E_2:P_4 ratio. Roth et al. (2001b) domenstrated slightly higher E_2:P_4 ratio in follicular fluid in heat stressed cows compared to non-heat stressed cows. Also E_2:P_4 ratio at day 5 of the pregnant rat under heat stress were higher in the evening and heated group had lower E_2:P_4 in afternoons than the control group (Hamid et al., 2012).

Haemoglobin and oxidative stress

Previous studies reported that the O_2 level in follicular fluid was not affected by heat stress. They suggested by that the low O_2 concentration in the

follicular fluid during acute reduction of blood supply to follicle in heat stressed conditions did not lead to the reduction of O_2 level (de Castro e Paula et al., 2008). However, the authors suggested that prolonged exposure to heat stress may compromise O_2 levels in bovine ovarian follicles. Two proposals have been offered to explain these observations: (i) oxygen delivery system could reduce their capacity and decrease O_2 delivery under high temperature, and (ii) extra O_2 required under thermal stress could lead to increased haemoglobin gene expression (Liu et al., 2013). In the current findings, genes involved in O_2 transport and stress were expressed in GCs. As discussed in Chapter 4, *HBA* was increased at 5% O_2. However, in current chapter, the expression level of *HBA* mRNA was not significantly affected by temperature.

In the current chapter there was no effect of temperature on *SOD1* and *SOD2* transcripts expression. Additionally, ROS generation by GCs did not differ under both conditions. However, elevated temperature increased the expression level of *ASMT* transcripts, the last enzyme responsible for melatonin biosynthesis. Previous studies indicate that melatonin is produced by GCs (Kim et al., 2013). However, mechanisms regulating melatonin enzyme expression in GCs are not known; i.e. the mechanism of higher mRNA level for *ASMT* at 40.0°C is not clear. It appears that regulation of melatonin synthesis in extra-pineal gland tissues is non-responsive to light cycle. It could be regulated by different mechanisms. The proposed mechanism is that melatonin is regulated by the demands of tissues for melatonin (Tan et al., 2015). However, earlier studies in the frog showed that melatonin level in short and long photoperiod elevated by high temperature (Delgado and Vivien-Roels, 1989). Several studies demonstrated melatonin as being thermoregulatory (Cagnacci et al., 1995b, Kräuchi et al., 2006). Regrettably, in the current study we were unable to find a reliable technique to measure melatonin concentration in spent culture media. This could be due to the high background and/or the low stability of melatonin in culture media. The expression of melatonin biosynthesis enzyme transcripts could indicate that increased endogenous melatonin plays a role in protecting cells from environmental stress.

Effect of melatonin on cultured GCs under 37.5°C and 40.0°C

Cell number and viability

In the present chapter, melatonin inclusion increased GC number under high temperatures. Melatonin helps to protect the integrity of GCs (Takada et al., 2012, Cruz et al., 2014, Tanabe et al., 2015). Sheep GCs cultured at 43.0°C revealed that colony forming of GCs under heat stress was lower and melatonin increased colony formation (Fu et al., 2014). The proportion of apoptotic GCs and *P53* expression were higher under heat stress, and melatonin treatment reduced *P53* transcript during heat exposure (Fu et al., 2014). However, herein bovine GCs cultured at 5% O_2 revealed that melatonin tended ($P<0.09$) to interact with temperature to increase apoptotic gene *BAX* and *P53* expression at 40.0°C and reduced both transcripts at 37.5°C.

Melatonin appear to have activated both intrinsic and extrinsic apoptotic pathways, but the mechanisms of melatonin involvement in apoptotic pathways are still unclear (Hardeland et al., 2011, Rodriguez et al., 2013). These results are consistent with other findings, that melatonin induced apoptotic genes *BAX*, *FAS*, *BCL2* and *P53* (Gholami et al., 2013) and heat increased the apoptotic rate and increased *BAX* mRNA expression (Li et al., 2016). However, melatonin tends to interact with temperature to affect *BAX* and *P53* in GCs still need more investigation. It seems both temperature and melatonin promote apoptosis via the internal pathway and could synergistically influence apoptotic gene expression.

Steroidogenesis

The interaction between temperature and melatonin treatments showed that melatonin increased P_4 production under physiological temperature and reduced P_4 under high temperatures in dose dependent manner. Increased P_4 may be due to the effect of melatonin on O_2 transporting mechanisms and increased tissue oxygenation (Hlutkin and Zinchuk, 2008). As discussed in Chapter 4, increased O_2 enhances cell differentiation (Ivanovic, 2009) and the results in Chapter 4 support the contention that GCs cultured under air produce more P_4 than under low O_2. In this respect the fact that GCs produced more P_4 under low O_2 with added melatonin could be due to the effect of melatonin in stimulating oxygenation. It has

been reported that the concentration of P_4 in follicular fluid had a positive correlation with melatonin (Taketani et al., 2011).

The explanation for the lower P_4 production by GCs treated with melatonin under 40.0°C could be related to temperature stress. Melatonin receptor expression in spleen tissue increases under heat stress (Acharjee and Singh, 2015) and melatonin signalling via its receptors reduced steroidogenesis through inhibiting the second key messenger for steroid production, cAMP (Tamura et al., 2009).

Melatonin had no direct effect on E_2 and P_4 production by rat GCs cultured in serum free media for 48 h (Nakamura et al., 2014). In the current study E_2 production by GCs cultured at 5% O_2 under both temperature conditions (37.5 and 40.0°C) was reduced by melatonin treatments. However, E_2 concentration in follicular fluid did not have any correlation with melatonin (Taketani et al., 2011). A recent study demonstrated that melatonin and/or temperature treated mice increased melatonin receptor and *HSP70* proteins in spleen (Acharjee and Singh, 2015). The above findings are consistent with the current results that melatonin reduces E_2 production and tended ($P<0.10$) to increase *HSPA1A* mRNA expression in bovine GCs. It has been reported that the expression of melatonin receptor increased under high temperature (Acharjee and Singh, 2015). Melatonin signalling through receptors can reduce steroidogenic hormone secretion (Tamura et al., 2009). We could suggest that the reduction of steroidogenesis by melatonin in the current result could due to the melatonin signalling through its receptors.

Haemoglobin and oxidative stress

Melatonin reduced ROS level under both temperature conditions (37.5 and 40.0°C). However, following its cascade reaction with ROS, melatonin retains the capability to scavenge free radicals. Following degradation of melatonin to N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK) by interaction with ROS, and its enzymatic breakdown such as cytochrome P450, melatonin metabolite AFMK has an ability to donate electrons to function as a reductive molecule and subsequently neutralized radicals (Tan et al., 2001, Tan et al., 2015). Furthermore, this AFMK inhibited

prostaglandin functions and has a potent anti-inflammatory action (Kelly et al., 1984, Mayo et al., 2005). There is strong evidence suggesting that melatonin reduces synthesizing enzyme for $PGF_{2}\alpha$ cyclooxygenase-2 gene expression (Tamura et al., 2009). It has been reported that prostaglandin produced by GCs (Fowkes et al., 2001, Dozier et al., 2008). This cascade could support the present results that P_4 concentration increased by melatonin under normal physiological temperature.

Increased melatonin (Nakamura et al., 2003, Tamura et al., 2013) and decreased O_2 and temperature in the preovulatory follicles could provide novel mechanisms of ovarian development, ovulation and subsequent CL formation. Cells from ruptured follicles exposed to higher O_2 levels either from blood and/or surrounding environment, are also exposed to more melatonin either from follicular fluid and/or blood. These two factors probably interact with the mechanisms of GC luteinisation and CL development. As a potent antioxidant and enhancer of tissue oxygenation, melatonin would be important in order to differentiate GCs to luteal cells. As temperature increases and melatonin decreases in summer blood supply to ovulated follicles could be reduced and subsequently reduce O_2 concentration to the newly formed CL, thus compromising cattle fertility during summer.

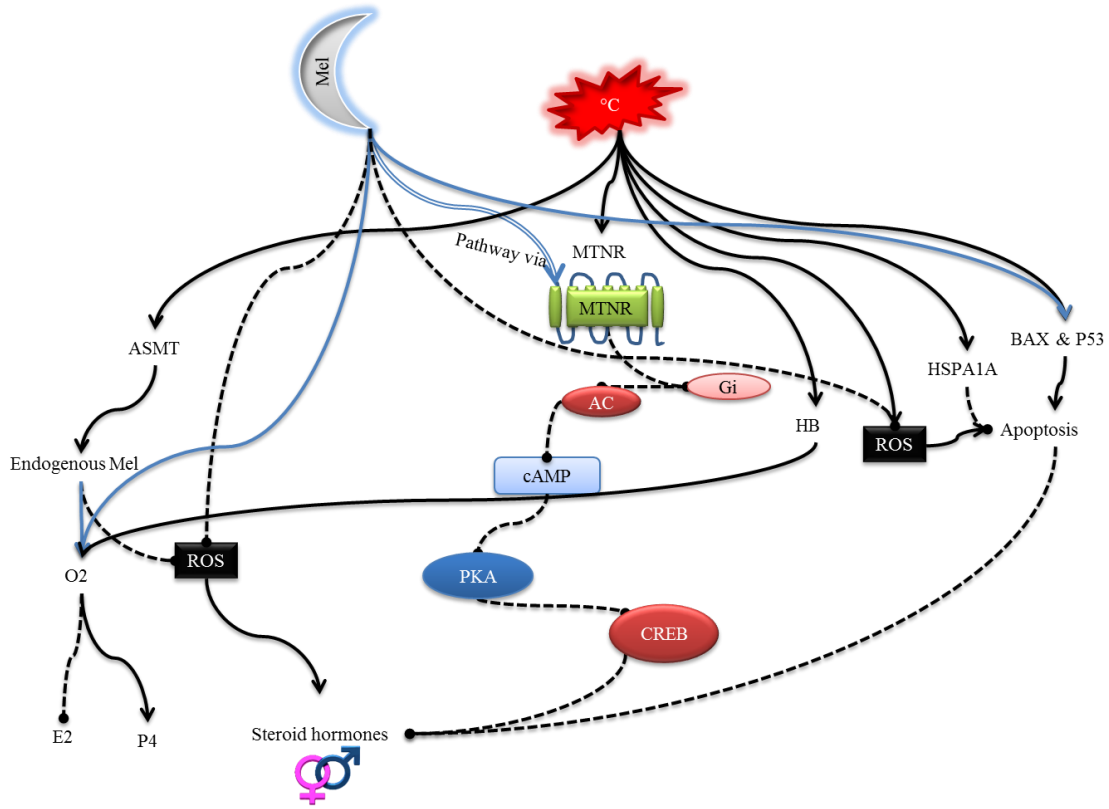


Figure 5. 13 *Mechanisms of melatonin interactions with temperature to affect granulosa cells function. Melatonin could increase progesterone (P_4) through reduction of reactive oxygen species (ROS), tissue oxygenation and it actions on Prostaglandin F2alpha ($PGF_2\alpha$). However, melatonin could also reduce steroidogenesis via it receptor signalling pathways. By enhancing apoptosis and it may also decrease oestradiol (E_2) as it stimulates cell differentiation. Solid lines indicate stimulatory effects and dotted lines indicate inhibitory effects. ASMT=acetyl serotonin O-methyltransferase, O_2=oxygen, MTNR=melatonin receptor, Adenylyl cyclase (AC), cyclic adenosine monophosphate (cAMP), Protein kinase A (PKA), cAMP response element-binding protein (CREB), HB=haemoglobin, HSPA1A=heat shock protein family A (Hsp70) member 1A, BAX= BCL2 associated X, apoptosis regulator and P53= tumor protein p53.*

5.5 Conclusion

In the present study, we have developed a model to evaluate the effect of environment and other factors on GCs *in vitro*. These models maintain bovine GCs to produced E_2 and P_4 for at least 144 h. The effects of temperature on GCs at different culture durations in the presence of melatonin could open a new window for researchers to address the effects of season on cattle fertility. The findings revealed that temperature increased apoptotic gene expression and melatonin could successfully protect bovine GCs from negative effect caused by high temperature.

Cells exposed to long-term high temperatures increase apoptotic gene expression. This effect is also indicated by inhibited P_4 production by GCs under high temperatures at 144 h of culture. Melatonin interacts with temperature to effect P_4 secretion. It increases P_4 at 37.5°C and reduces P_4 at 40.0°C. *ASMT* gene expression was increased in GCs collected under high temperature. This study confirmed that melatonin reduced ROS generated by GCs cultured at 5% O_2 and incubated at 37.5 and 40.0°C.

The current results suggest that there are interactions between melatonin, temperature and O_2 concentration in follicular fluid. These may affect follicle development, ovulation and subsequently CL formation. Important biological purposes may be served by the consequences of these modifications via providing a link of O_2, temperature and melatonin effects on follicular cells development and steroidogenesis. This study described GC viability, steroidogenesis and expression gene analysis which helps to advance our understanding of molecular mechanisms responsible for seasonal effects on cattle fertility. Subsequent research should consider melatonin and physiological O_2 concentrations during follicular development. However, the melatonin cascade reaction and its interaction with oxidative stress and follicular development could improve our understanding of the follicle development and it relationship with stress.

Chapter 6: General discussion and general conclusion

6.1 General discussion

One of the major factors causing economic losses in the global dairy industry contributing to reduce dairy cow fertility is the hot season. Even with modern cooling systems, fertility still remains low. Tolerance of dairy cows to heat stress decreases with increasing milk yield (Hansen, 2000, West et al., 2003). Photoperiod is another factor effecting animal fertility. It has been reported that moderate hot summers have an effect on oxidative stress in dairy cows (Bernabucci et al., 2002). Possibly seasonal variation in oxidative stress and antioxidant defence mechanisms lead to various physiological activities (Chainy et al., 2016). Therefore, the overall goals of this thesis were to determine the mechanisms of seasonal effects in dairy cattle under temperate UK climatic conditions.

Effect of season of birth on first breeding

Studies within this thesis demonstrate that cows born during the autumn were inseminated, conceived and calved earlier than cows born in spring and summer. It is suggested that animals exposed to short daylight during the first 6 months post birth could stimulate heifer maturity, whereas heifer reared under long daylight during second 6 month post-natal hastened puberty, the results support that season of birth influenced puberty and maturity (Schillo et al., 1983, Rius et al., 2005). Schillo et al. (1992) reported that autumn born cows attained sexual maturity at an earlier age, suggesting that photoperiod may enhance the onset of puberty. Buffalo heifers exposed to light during winter enhanced the onset of puberty (Roy et al., 2016).

Effect of season on cattle fertility

In the current study, the highest conception rates within 90 days of calving was recorded in autumn, and the highest conception rate according to the insemination season was recorded in winter (Chapter 2). Furthermore, total CL weight was heavier in autumn (Chapter 3). Silva et al. (2006) demonstrated that the oocyte quality increased during autumn.

Cattle are homoeothermic animals, and maintain constant body temperature by the balance between the amounts of metabolic heat produced and heat dissipated. Heat dissipation depends on the surrounding temperature and humidity (Hansen, 2004). The imbalance between heat gain and loss leads to molecular, cellular, metabolic and physiologic changes. Furthermore, females of numerous species experiencing hyperthermia near breeding time are susceptible to heat (Cavestany et al., 1985, Putney et al., 1988b). High environmental factors (temperature and THI) around insemination date had a negative outcome on conception rate and 10 days before insemination and this was more sensitive to temperature, and temperature humidity index (chapter 2). Conception rate in moderate temperate climates was reduced by high THI around the time of insemination (Schüller, 2015). Conceivably, insemination during the autumn and winter after removal of hot and long daylight could result in a better conception rate; this could be due to the nutritional factors and/or seasonal variation of hormonal production such as melatonin.

It has been reported that late rise of P_4 after ovulation and low P_4 production during luteal phase lead to poor embryo development (Mann et al., 1999). In our *in vitro* studies high temperature increased apoptotic gene expression and reduced P_4 production by cultured bovine GCs (Chapter 5). The reduction of P_4 concentration with lengthening luteal phase alters follicular dynamics and promotes dominant follicle persistence (Sirois and Fortune, 1990). The persistence of dominant follicles could support the results in Chapter 3, that the incidence of multiple ovulations and follicular cysts in dairy cattle is influenced by season with the higher frequency during summer and autumn. Several studies reported that high occurrence of multiple ovulations and multiple births is associated with season (Karlsen et al., 2000, Fitzgerald et al., 2014). Multiple ovulations in dairy cattle increased in summer, whereas multiple ovulation rates in lactating cows were similar to those in dry cows in winter (Sartori et al., 2002). This could support the hypothesis that season could affect follicular development, ovulation and CL development.

Effect of melatonin on cultured granulosa cells

Other factors such as oxidative stress could be the reason for lower fertility in hot and long daylight seasons. It has been reported that during the summer cows had lower antioxidant enzyme activity and lower total antioxidant status in the summer (Turk et al., 2015). Melatonin serves as a potent antioxidant to reduce ROS production and enhance tissue oxygenation (Korkmaz et al., 2009, Hlutkin and Zinchuk, 2008). This could protect luteinisation of GCs from oxidative stress. In an *in vitro* study, P_4 production by follicles increased with melatonin treatments, after the ovulation processes has been stimulated (Adriaens et al., 2006). However, the dark light cycle is often overlooked as a main factor that compromises reproductive activity. Using artificial light could lead to false signalling to the hypothalamus via melatonin. This defective signal or faulty information would have altered circadian gene expression, disrupted the signal of the master clock and subsequently perturbed normal physiological function of tissue (Reiter et al., 2014). Melatonin interaction with temperature to affect GC viability and alter steroidogeneses (Chapter 5) could indicated that season has direct and indirect effects on reproduction function.

The expression of melatonin biosynthesis enzyme transcripts in GCs and increased *ASMT mRNA* in GCs cultured under high temperature suggest that endogenous melatonin could reduce the exogenous melatonin role. The proposed mechanisms of melatonin and temperature in the control of steroid production by GCs are shown in (Figure 6.1).

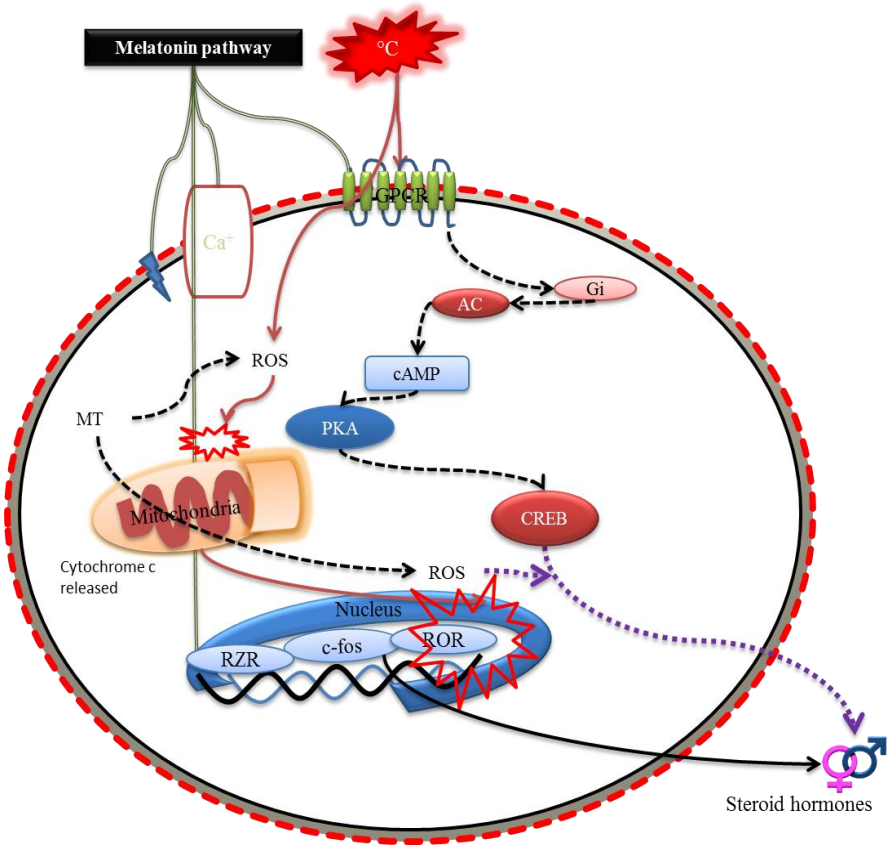


Figure 6.1 *Proposed mechanisms of melatonin and temperature on steroid production by GCs. Melatonin may increase P_4 as antioxidant and enhance tissue oxygenation. Whereas melatonin acting through its receptor could reduce steroidogenesis. Temperature may reduce P_4 production via its effect on apoptosis and enhance melatonin receptor expression. Solid line mean increase and dashed line mean decrease.*

Effect of oxygen tension on granulosa cells culture

The majority of data available in the literature has been generated from GCs cultured under atmospheric O_2. The physiological O_2 level for many tissue/cells is much lower than that employed in cell culture *in vitro.* It has been shown that physiological O_2 is essential to recreate micro environmental niches such as local cytokines level (Redshaw and Loughna, 2012). In an effort to identify a new culture system to more closely mimic physiological cell function, cells were cultured under physiological O_2 concentration (5%). Physiological O_2 increased GC proliferation, reduced GC differentiation and maintained E_2 production and aromatase activity while reducing progesterone production (Chapter 4). Thus, low oxygen tension reduced GC luteinisation and maintained the follicular phenotype. These results shed light on how O_2 tensions, which is often not considered

in experimental settings, can impact on the function and differentiation of GCs.

To this end, the findings described herein provide novel insight related to the mechanism of steroidogenesis, molecular and cellular biology. Insights extended from these findings may serve as the foundation for the future therapies of fertility and cell study.

To further confirm that GCs are under stress, heat shock proteins act as chaperones within the cells (Ellis, 1993). *HSP70* family are the most temperature sensitive and are induced by hypoxia (Kregel, 2002). In the present results *HSPA1A,* superoxide dismutase gene expression and ROS production was not affected either by O_2 tension (Chapter 4) or temperature conditions (Chapter 5). However, the novel gene expression on GCs *HBA* was influenced by O_2 concentration and the higher expression was revealed at physiological O_2 compared with atmospheric O_2 (Chapter 4).

6.2 General conclusion

In summary the results of this thesis demonstrate that season has clear effects on fertility parameters such as age at first insemination and first calving and conception rate. In addition, environmental stress around the insemination period can affect conception rate in dairy cattle. Additionally, the incidence of multiple ovulations and follicular cysts was another factor significantly affected by season in the temperate UK climate.

In term of *in vitro* study, the results in this thesis support and promote the culturing of GCs at physiological O_2 concentration. As the results indicated that atmospheric O_2 induced cells differentiation and possibly causes abnormal cellular function, proliferation, hormonal secretion and gene expression, these results are not only of benefit for the culturing of GCs, but are also aimed at optimizing *in vitro* studies in general to improve reproductive health in both human and animals. The results for the first time reported that in response to O_2 tension, the transcript for *HB* was active under low O_2. To quantify the effect of environmental factors (temperature and melatonin) on GCs, results herein shown that high

temperature reduced P_4 production and enhance apoptotic gene expression. Additionally, high temperature increased melatonin biosynthesis enzyme transcripts *ASMT*.

Future Directions

The results in the present thesis revealed that season does influence cattle fertility in the UK temperate zone. However, one of the limitations in the current studies is the lack of information on nutrition and management factors that contribute to seasonal effects on cattle fertility. It would be intriguing to investigate the seasonal interaction with management factors. Further investigation will need to evaluate the mechanisms of multiple ovulations and follicular cysts related to the seasonal effect. It will be beneficial to address the seasonal effect on oocyte quality and embryonic development both *in vivo* and *in vitro*.

The cultures here have provided enough evidence to support the fact that GC differentiation is controlled by O_2 tension. These novel results generated important questions for the future study. We did not look at the effect of O_2 on theca cells. Also it will be important to consider the ideal physiological level of O_2 in follicle cells. More clearly, the cells within the follicle grow under low O_2 until ovulation and, after ovulation, cells will be exposed to higher O_2 levels during differentiation into luteal cells. It seems that shifting follicular cells from proliferation to differentiation is a critical period for cattle fertility that is controlled by O_2.

In the present study melatonin biosynthesis enzyme transcripts were expressed in GCs. More investigation will need to address the role of haemoglobin in follicular development and it correlation with melatonin. It is important to investigate melatonin and O_2 concentration in the follicular fluid and its correlation with the follicular development. Both O_2 and the melatonin cascade in follicular cells still need more investigation. This study investigated the transcript expression of *HB* in GCs and the expression was upregulated by low O_2 concentration in culture system. Therefore, molecular marker could indicate that cells will adapt to the variation of O_2 level. It will be important to investigate more about the role of haemoglobin in ovarian follicles and culture systems.

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A. Appendix

A.1 Validation of housekeeping genes under low and high O_2 tension in the presence or absence of melatonin for long term cultured bovine GCs

Internal control selection of reference gene is essential for normalization of target gene expression. To study transcripts and obtained more accurate results in mRNA analysis, most stable HKG should be addressed under present GCs culture system. Relative quantification of mRNA for gene of interest was quantified by qRT-PCR technique to compare the Cp value. Perhaps this technique challenges to insufficient reverse transcription, unequal amounts of RNA and cDNA, poor RNA integrity (Ståhlberg et al., 2004, Cecconi et al., 1999). Recently, common housekeeping genes (HKG) expression is modulated by hypoxia and cell growing (Khan et al., 2014, Baddela et al., 2014).

In this study GCs were cultured in different conditions to investigate reference gene control which did not affected by working conditions. GCs were cultured under atmospheric (20% O_2) and low O_2 (5% O_2) with 5% CO_2 in the presence or absence of melatonin, total RNA from four biological replicates during different time points, namely 48, 96 and 144 h of culture were collected from GCs.

There was no significant effects and interactions of O_2 (atmospheric and 5% O_2), melatonin (0 and 2000 pg/ml) and culture duration (48, 96 and 144h) on TBP (Table 4.1). The mRNA level of *B2M* was increased (P<0.004) by culture time. Oxygen tension had a significant effect (P0.02) on *RPL19* transcription and it up regulated (p<0.019) under air conditions. Oxygen tension tended to interact with time to affect *RPLP0* transcript (p<0.06). The supplementary (Table A.1 and Figures A.1, A.2 and A.3) show the level of HKG in GCs throughout culture duration under different conditions.

To summarise the validation of HKG in this study, the analysis revealed that the *TBP* was the most stable reference gene according to general ANOVA, Cp value, CV, and available computational programs.

Table A. 1 *The %CV value of HKGs, the most stable gene had lowest CV.*

HKG	*M2B*	*RPL19*	*RPLP0*	*TBP*
%CV	3.91	3.468	4.245	1.496

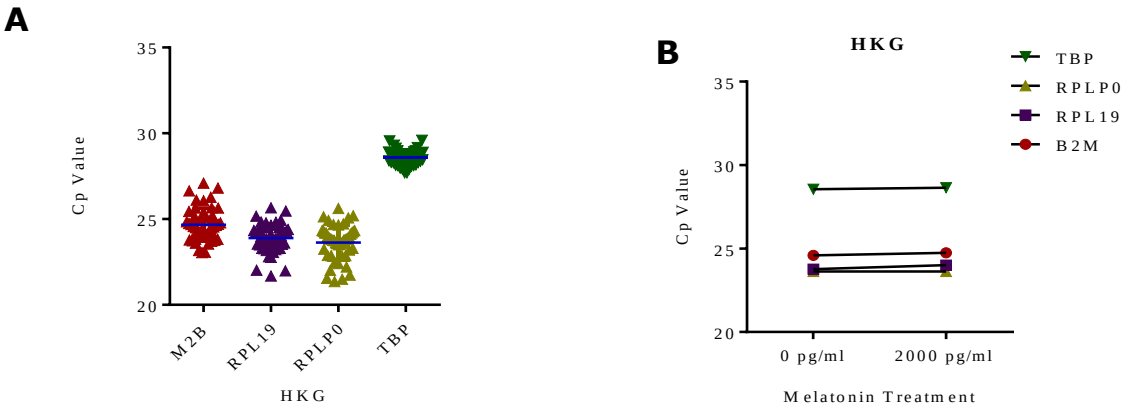

Figure A. 1 *Evaluation of HKG expression level. Cp value for individual duplicated sample mean (**A**) and Cp value of GCs cultured under melatonin treatment and compared by general ANOVA (**B**).*

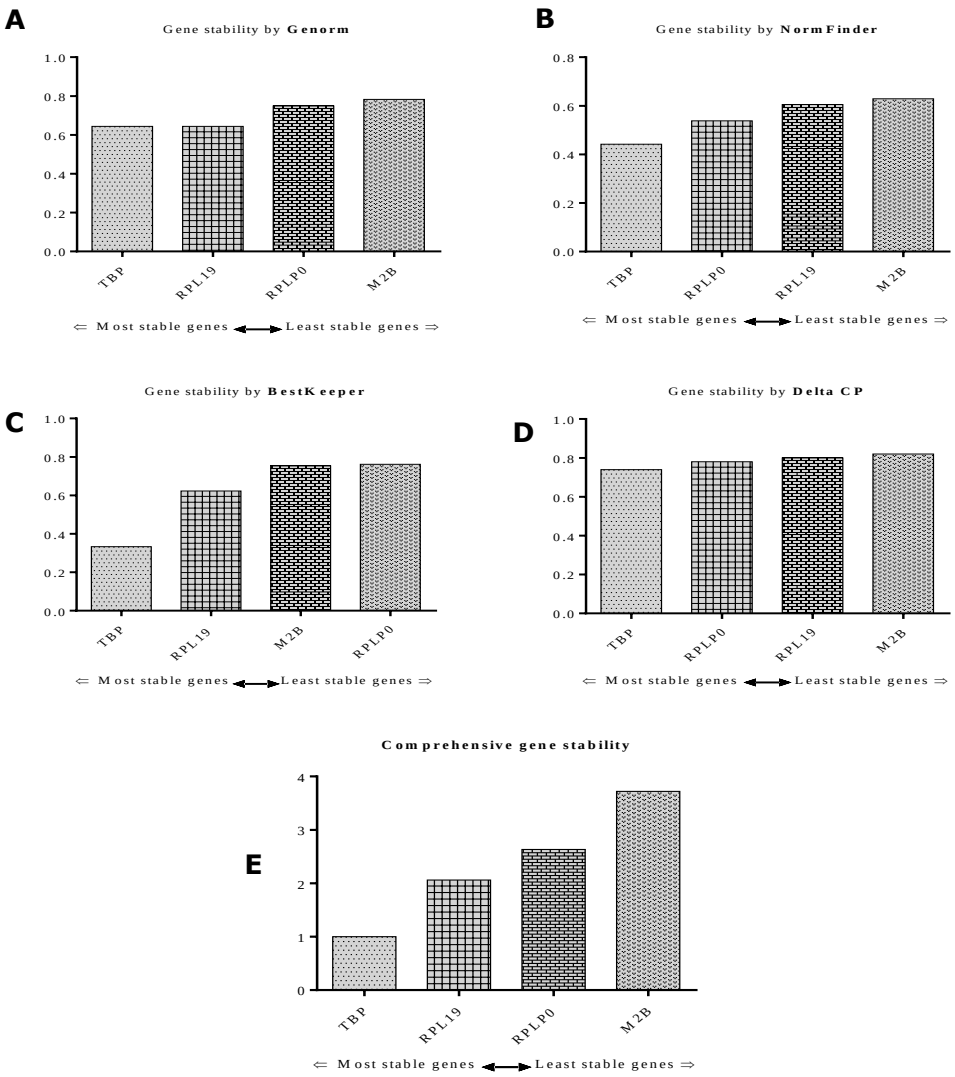

Figure A. 3 *To explore whether the expression of the more stable four (TBP, RPLP0, RPLP19 and M2B) HKGs are modulating by O_2, melatonin and cell growth. We evaluating and compare the genes expression level in GCs cultured by currently available computational programs **RefFinder**. It reveals that the TBP had the highest stability rank among HKGs. The data were analysed by Genorm (**A**), NormFinder (**B**), Bestkeeper (**C**), Delta Cp (**D**) and comprehensive stability (**E**). Genes to the left of the chart or the most stable HKG have lower value.*

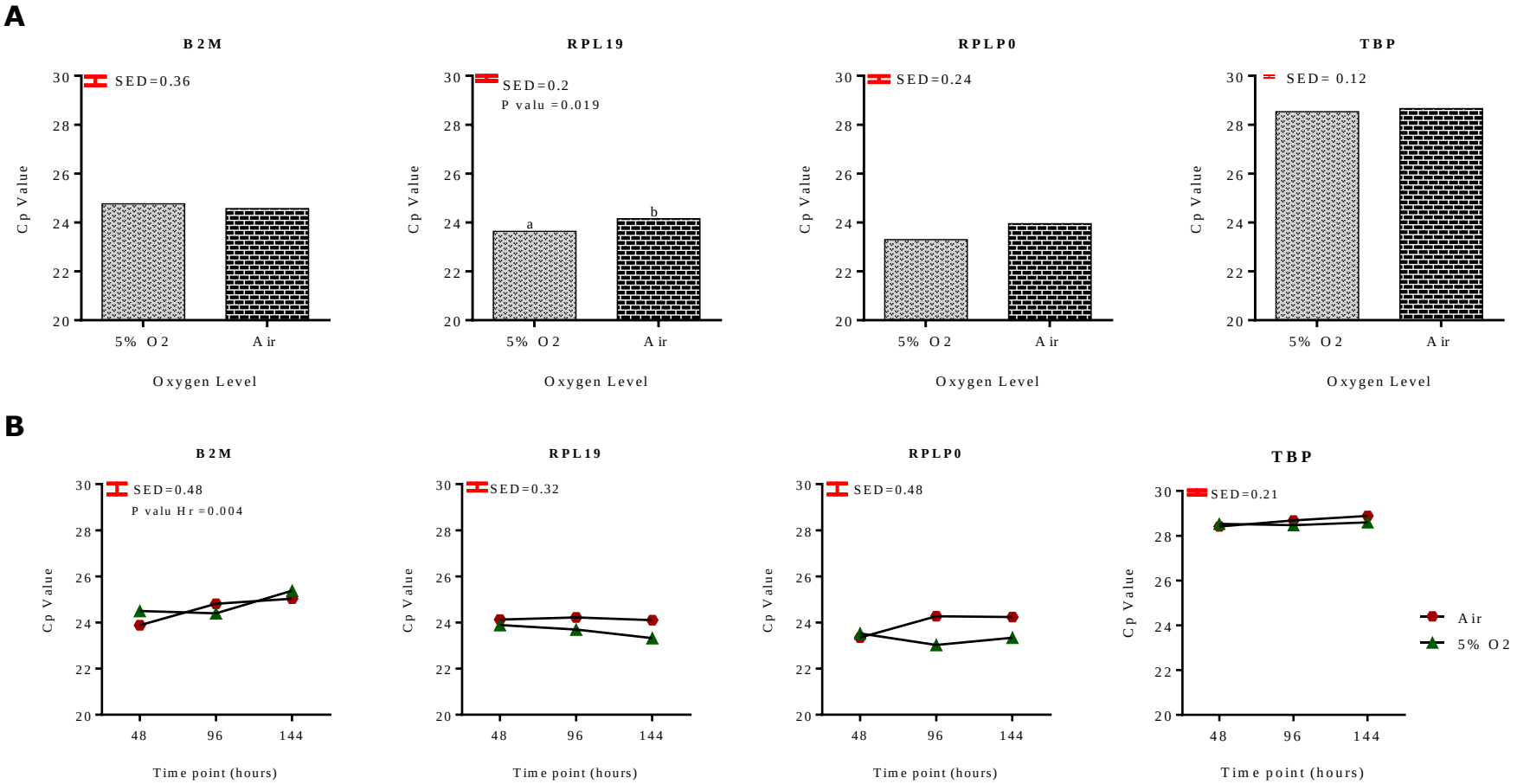

Figure A. 4 *Expression level of HKG in GCs culture investigated under air (21% and 5% CO_2) and physiological O_2 (5% O_2 and CO_2) (**A**). Oxygen tension (5% O_2 and Air) interacts with culture period to affects HKGs (**B**). General ANOVA model were used to reveal the significant effect of culture conditions on gene expression. The data presented as mean of HKG Cp value with SED.*

A.2 Validation of housekeeping genes in bovine GCs cultured under 5% O_2 at 37.5°C and 40.0°C

In the present validation of HKG, most stable HKGs (*TBP and RPLP0*) were selected from the previous section. Total RNA from GCs of four biological replicates cultured in humidified 5% O_2 with 5% CO_2 collected at 48, 96 and 144 h described in section 5.2.1**.** GCs cultured at 37.5°C and 40.0°C group in the presence or absence of melatonin (0 and 2000 pg/ml) were collected for gene expression.

The result analysed by general ANOVA and RefFinder (Figure A.5) demonstrated that both of transcript were stable. However, *TBP* addressed again ranked to be more stable reference gene by BestKeeper and ANOVA. The Cp value of *RPLP0* transcript was not affected by culture condition. However, *RPLP0* mRNA tended to increase (P=0.09) by culture time (the Cp 21.26 for 48 h, 20.9 for 96 h and 21.40 for 144 h), whereas *TBP* Cp value was not changed under all conditions. Therefore, the target genes in current culture system will be analysed by the stable reference gene *TBP*.

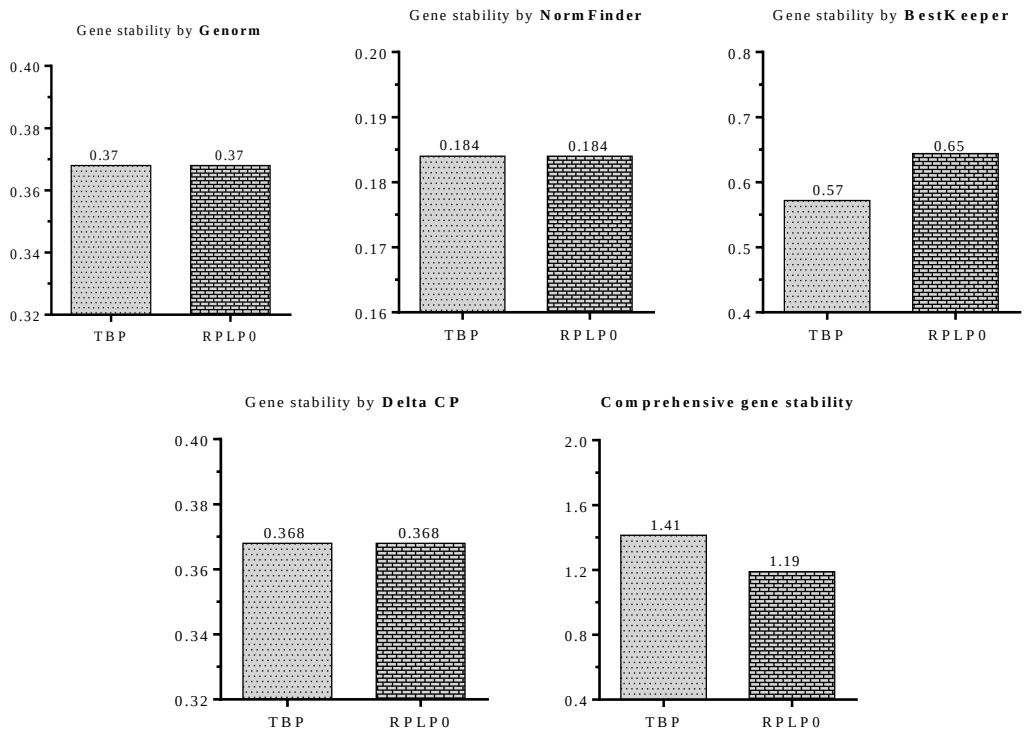

Figure A.5 *To explore whether the expression of the more stable HKGs (TBP and RPLP0) are modulating by temperature, melatonin and cell growth. The genes expression level were analysed by available computational programs RefFinder. It reveals that the both TBP and RPLP0 had the similar stability rank. The data were analysed by Genorm (**A**), NormFinder (**B**), Bestkeeper (**C**), Delta Cp (**D**) and comprehensive stability (**E**). The most stable HKG have lower value.*

Table A.3 *Detail of the primer and probe of bovine used in the current study.*

Genes	Primer and Taqman probe Sequence (5" -> 3")		Positions		length	product length	Accession number	Stander efficiency	Exon		coding
		Probe 5"- FAM, 3"-TAM	Start	End					location	Total	CDS
RPL19	Forward	AGCGGATCCTCATGGAACAT	290	309	20						
	Revers	CCTGGTCCGCCAGAAGCT	344	361	18	71	NM_001040516	1.959	(3-4)	4	35-625
	Probe	CCACAAGCTGAAGGCAGACAAGGCTC	312	337	26						
RPLP0	Forward	TGGCTTTGTCTGTGGAGACTGA	746	767	22						
	Revers	CCACAAAGGCAGATGGATCA	810	829	20	83	NM_001012682	1.837	(7-8)	9	61-1017
	Probe	CCCACTTGCTGAAAAGGTCAAGGCCTT	777	803	27						
TBP	Forward	ACAGCCTCCCACCCTATGC	14	32	19						
	Revers	ATCATTGGACTAAAGATAGGGATTCC	70	95	26	81	NP_001069210	1.987	(1-2)	2	1-319
	Probe	CCTCCCCTCAGGGTGCCATGACTC	44	67	24						
B2M	Forward	GCCAAATTACCTGAACTGCTATGTG	117	141	25						
	Revers	CCCATTCTTCAGCAAATCG	170	189	20	72	NM_173893	1.85	1	4	34-394
	Probe	TGGGTTCCATCCACCCCA GATTGAA	144	168	25						
SOD1	Forward	GCCGTCTGCGTGCTGAA	92	108	17						
	Revers	ACTGTATTTCCCTTTGCCTCGA	141	162	22	70	NM_174615	1.883	1	5	80-538
	Probe	CCCGGTGCAAGGCACCATCC	118	137	20						
SOD2	Forward	CGCTGGAGAAGGGTGATGTT	283	302	20						
	Revers	GCCCCCACCGTTGAACTT	333	350	18	67	NM_201527	1.84	(2-3)	3	70-738
	Probe	CAGCTCAGATAGCTCTGCAGCCTGCG	304	329	26						
CYP19A	Forward	GGACACCTCTAACATGCTCTTCCT	770	793	24						
	Revers	TGCATCAAAATACCCCTGGATT	830	851	22	81	NM_174305	1.98	(5-6)	10	183-1694
	Probe	TCCCCTTGGATGAAAGTGCCATCG	799	822	24						
CYP19A	Forward	TGGGTTGCCATTGCCTTC	2082	2099	18						
	Revers	GGACAGTAAGGAGCTGGGGTGAG	2130	2152	23	70	NM_174305	1.98	non coding	10	183-1694
	Probe	CAGTTGGAAAAGACAAGCCACCAGCAA	2101	2127	27						
HSD3B1	Forward	CCAGGCCAGCGTACCGGTCTTT	339	360	22						
	Revers	CTCCCTGTAGGCGTTGGGCC	389	408	20	69	NM_174343	1.83	4	4	105-1226
	Probe	TCCACACCAGCACCATAGAAGTGGCT	362	387	26						
HSPA1A	Forward	TGATGACCGCCCTGATCAA	1226	1244	19						
	Revers	CGCCCGGCTGGTTGT	1298	1312	15	86	NM_203322	1.947	1	1	173-2098
	Probe	CGCAACTCCACCATCCCCACGA	1246	1267	22						

Gene	Primer and Taqman probe Sequence (5" -> 3")		Positions		length	product length	Accession number	Stander efficiency	Exon		coding
		Probe 5"- FAM, 3"-TAM	Start	End					location	Total	CDS
P53	Forward	AACACCAGCTCCTCTCCACAG	1157	1177	21						
	Revers	CCAAGGCATCATTCAGCTCTC	1251	1271	21	114	XM_010815982	1.802	(9-10)	11	434-1408
	Probe	AAGAAGAAACCACTGGATGGA	1181	1201	21						
CASP3	Forward	CAACGGACCCGTCAATTTGAAA	390	411	22						
	Revers	GGTTTTCCAGTCAGACTTCTGCAAT	440	464	25	74	NM_001077840		4	7	99-926
	Probe	CCCCTCTGAAGAACT	424	436	13						
BAX	Forward	GTTGTCGCCCTTTTCTACTTTGC	328	350	23						
	Revers	CAACTCGGGCACCTTGGT	379	396	18	68	NM_173894	1.99	(4-5)	6	1-579
	Probe	ACAGGGCCTTGAGCACC	360	376	17						
BCL2	Forward	GGCTGGGACGCCTTTGT	550	566	17						
	Revers	CCTTCAGAGACAGCCAGGAGAA	604	625	22	75	NM_001166486	2.32	(1-2)	2	1-690
	Probe	CCCTAGCATGCGGCCCCTGTTT	579	600	22						
HBA	Forward	TGACCAAAGCGGTGGAACA	200	218	19						
	Revers	GTGCAGGTCACTCAGTTCAGACA	242	264	23	64	NM_001077422.3	1.95	3	3	40-468
	Probe	CTGGACGACCTGCCCGGTGC	220	239	20						
HBB	Forward	GGCATGAAGCATCTCGATGA	217	236	20						
	Revers	CAGCTTATCACAGTGCAGCTCACT	262	285	24	68	NM_173917		(2-3)	3	53-490
	Probe	CTCAAGGGCACCTTTGCTGCGC	238	259	22						
MTNR1A	Forward	CATCAACCGCTATTGCTGCAT	414	434	21						
	Revers	ACACGTAGCAGAGGGAATTCGT	472	493	22	79	XM_010820272		2	2	180-1136
	Probe	TGCCACAGCCTCAGATACGACAAGCTG	436	462	27						
MTNR1B	Forward	GCGGAGCTTTCTGAGCATGT	741	760	20						
	Revers	GGCGAGGCCGATGCA	805	819	15	78	NM_001206907.1		2	2	1-1131
	Probe	CCATCTGCTGGGCGCCGC	782	799	18						
ASMT	Forward	GATCCCATTGGTTTGTTTTGATC	949	971	23						
	Revers	AACAATTTGAGATGTGTCCACGAT	1006	1029	24	80	NM_177493.2	2	(3-4)	4	124-1176
	Probe	ACCCAGGACACTCACACGCAAGGAA	973	997	25						
ANNAT	Forward	TTGAGCGAGAGGCCTTCATC	152	171	20						
	Revers	CGGACCTCGTCCAGATTCA	194	212	19	60	NM_177509.2		2	4	(111-734)
	Probe	CTGTCTCCGGCAACTGCCCCC	173	193	21						

Table A.4 *Cell culture media solution*

Reagents	Concentration
M199 (M7528)	
FSH	1 ng/ml
Testosterone	100 ng/ml
Penicillin-Streptomycin (P4333)	50 µg/ml
Insulin from ITA	10 ng/ml
Apo-transferrin bovine (T1428)	2.5 µg/ml
Sodium selenite	4 ng/ml
L-glutamine	365 µg/ml
BSA (free fatty acid)	1 mg/ml
Cell were cultured in pre-coated wells with Fibronectin from bovine (F1141)	6 µg/ml

Table A.5 *Concentration for preparation of Aromatase working Buffer*

Reagent	Concentration		Desired Con. g/l
TES	20	mM	4.585
EDTA	10	mM	3.3621
KCL	150	mM	11.182
PH	7.3 - 7.4		
protein inhibitor cocktail tablet	Two tablet per 10 ml		

TES (T1375) = N-TRIS (Hydroxymethyl) methyl-2 aminoethan sulfuric acid).

Aromatase Working solution:

- Prepare stock testosterone 100 nM
- Prepare desired concentration of sample 60 µg/tube
- Freshly add 10mM NADPH to each reaction

NADPH prepration.

- Dilute 100 mg in 2ml of NaOH 0.01M STOCK =6X
- Add 6x to each sample 1:5 (protein, testosterone and Buffer) = 200 µl final volume (PH 7.4).

NADPH= Nicotinamide adenine dinucleotide phosphate (Cofactor, Cytochrome P450 reductase).

A. 6 Tissue on paraffin embedded sections

70% IMS	(2 hour)
80% IMS	(2 hour)
95% IMS	(2 hour)
100% IMS	(2 hour)
100% IMS	(2 hour)
10% + 90% Histo-clear II	(4 hour)
100% Histo-clear II	(4 hour)
100% Histo-clear II	(4 hour)
Paraffin	(3 hour)
Paraffin	(3 hour)

Immunohistochemistry

Histo-clear II	(2 x 10 min)
100% ethanol	(5 min)
95% ethanol	(5 min)
70% ethanol	(5 min)
PBS	(2 x 5 min)
Immunostaining	(section 3.2.5)
Counterstaining	
PBS	(2 x 5 min)
Haematoxylin	(20s)
Tap water	(5 min)
Ammoniated water	(8dip)
Tap water	(5 min)
70% IMS	(5 min)
95% IMS	(2min)
100% IMS	(2min)
Histo-clear II	(2 x 5 min)