The Effect of Age and Gonadotropin Stimulation on the Embryo Development of Holstein Heifer Calf Oocytes

Luke Currin

Department of Animal Science
Macdonald Campus, McGill University,
Montreal, Quebec, Canada

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“*An understanding of the natural world and what’s in it, is a source of not only great curiosity, but great fulfilment”*

- Sir David Attenborough

Abstract

Increased availability of marker-assisted genomic selection is promoting the reproduction of elite domestic livestock at prepubertal ages using *in vitro* embryo production (IVEP) technologies, since genetically valuable animals can be identified at birth. However, past literature has reported that although prepubertal donors produce high numbers of oocytes, many of these oocytes exhibit poor development rates to the blastocyst stage *in vitro*. Therefore, the objective of this study was to examine the effect of age and gonadotropin stimulation prior to oocyte collection on follicular and embryo development dynamics. In order to assess this, 14 Holstein heifer calves were subjected to laparoscopic ovum pickup (LOPU), every two weeks between 2 and 6 months of age. Recovered oocytes were then matured, fertilised and embryos cultured *in vitro* to assess cleavage and development to the blastocyst stage.

In order to assess the effect of gonadotropin stimulation, three different gonadotropin regimes were tested: A long treatment, i.e. ≥72 hours prior to LOPU; a short treatment, i.e. 36 to 48 hours prior to LOPU; and a control group receiving no gonadotropin stimulation. Among treatments, no significant difference in the number of follicles available for aspiration (16.9 vs. 20.7 vs. 19.8, $P>0.05$, respectively) was found. However, the longer treatment resulted in a higher proportion of larger follicles (>5mm in diameter), which also resulted in a higher proportion of good-quality usable oocytes (95.3% vs. 85.4% vs. 82.2%, $P<0.05$). Furthermore, the long treatment also resulted in a significantly higher cleavage rate (72.7% vs. 59.0% vs. 66.0%, $P<0.05$), along with a significantly better blastocyst rate (36.7% vs. 18.3% vs. 16.7%, $P<0.05$).

To evaluate the effect of age, animals were grouped into three categories based on their age at the time of LOPU: 98 days of age or less, 99-130 days, greater than 130 days. Results indicate that as animals got older, although the number oocytes steadily declined (17.5 vs. 14.7 vs. 11.9, $P<0.05$), the proportion of usable oocytes steadily increased with age (84.2% vs. 87.5% vs. 93.6% $P>0.05$). Moreover, although cleavage rates did not differ among age groups (66.3% vs. 69.7% vs. 65.0%, $P>0.05$), the blastocyst rate steadily increased as animals got older (12.8% vs. 17.1% vs. 21.8%, $P<0.05$). Finally, the transfer of blastocysts (n=21) to adult recipient cows

resulted in 61.9% pregnancy, which is similar to rates when embryos derived from adult cows are used. Pregnancy was monitored to term in nine cows, seven of which delivered normal live calves, which confirms full embryo developmental capacity. At the end of the experiment, no adverse health or fertility effects were observed.

In conclusion, prepubertal heifers hold great potential in that animals as young as two months, can respond to gonadotropin stimulation and produce viable embryos when coupled with *in vitro* embryo production techniques. LOPU/IVEP technology can be safely applied in Holstein calves to produce more offspring and reduce the inter-generation interval. However, more research is required to better understand the reasons for reduced blastocyst rates in oocytes derived from prepubertal animals.

Résumé

L'augmentation de la disponibilité de marqueurs pour la sélection génomique assistée promouvoit la reproduction de betails élites à des âges prépubères en utilisant des technologies de production d'embryons *in vitro*. Ceci est possible puisque les animaux de grande valeur génétique peuvent être identifiés à la naissance. Par contre, la literature précédente a signalé que meme si les donneuses prépubères produisent des nombres élevés d'ovocytes, la plupart de ces ovocytes démontrent des taux faibles de development au stage de blastocyte *in vitro*. Donc, l'objectif de cette etude était d'examiner l'effet de l'âge et de la stimulation par gonadotrophine précédant la collecte d'ovocytes sur les dynamiques de développement folliculaire et embryonnaire. Afin d'évaluer ceci, 14 génisses Holstein ont été soumis à des prélèvements d'ovocytes par laparoscopie (LOPU) aux deux semaines de 2 à 6 mois d'âge. Les ovocytes récupérées ont ensuite été mis en maturation, fécondés, et les embryons ont été culturés *in vitro* (IVEP) afin de determiner leur potentiel de clivage et de développement au stage de blastocyte.

Afin d'évaluer l'effet de la stimulation par gonadotrophine, trois régimes différents ont été testé: un traitement long (≥72 heures précédent LOPU), un traitement court (36-48 heures preécédent LOPU), et un groupe controle ne recevant aucune stimulation de gonadotrophine. Selon les traitements, aucune différence significative a été observé pour le nombre de follicules disponible pour aspiration (16.9 vs. 20.7 vs. 19.8, $P>0.05$, respectivement). Par contre, le traitement long a entraîné une proportion plus élevée de follicules larges (>5mm en diameter), ce qui a aussi entraîné une proportion plus élevée d'ovocytes utilisables et de haute qualité (95.3% vs. 85.4% vs. 82.2%, $P<0.05$). En plus, le traitement long a entraîné un taux de clivage significativement plus élevé (72.7% vs. 59.0% vs. 66.0%, $P<0.05$), et un taux de blastocyte significativement plus élevé (36.7% vs. 18.3% vs. 16.7%, $P<0.05$).

Pour évaluer l'effet de l'âge, les animaux ont été groupé en trois categories basé sur leur âge au temps de LOPU: 98 jours ou moins, 99-130 jours, et plus que 130 jours. Les résultats indiquent que lorsque les animaux vieillissent, malgré que le nombre d'ovocytes a décliné régulièrement (17.5 vs. 14.7 vs. 11.9, $P<0.05$), la proportion d'ovocytes utilisables a augmenté régulièrement avec l'âge (84.2% vs. 87.5% vs. 93.6% $P>0.05$). En plus, malgré que les taux de clivage n'ont pas changé parmis les groups d'âge (66.3% vs. 69.7% vs. 65.0%, $P>0.05$), les taux

de blastocyste ont augmenté régulièrement avec l'âge des animaux (12.8% vs. 17.1% vs. 21.8%, $P<0.05$). Finallement, le transfert de blastocystes (n=21) aux vaches receveuses adultes a entraîné 61.9% de grossesse, ce que est similaire aux embryons dérivés de vaches adultes. Les gestations ont été suivi à terme dans neuf vaches, sept desquelles ont accouché des veaux normaux et vivants. Ceci confirme la capacité de développement embryonnaire complet. À la fin des experiments, aucun effet adverse de santé ou de fertilité a été observé.

En conclusion, des génisses prépubères démontrent beaucoup de potential que des animaux aussi jeunes que 2 mois peuvent répondre à des stimulations par gonadotrophine et produire des embryons viables lorsque couplé avec des techniques de production d'embryons *in vitro*. Les technologies LOPU/IVEP peuvent être appliqués sans encombre dans les veaux Holstein afin de produire plus de progéniture et réduire l'interval entre generation. Par contre, plus de recherche doit être menée afin de mieux comprendre les raisons pour la reduction des taux de blastocyste d'ovocytes derivées d'animaux prépubères.

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Contribution of authors

Authors:

Luke Currin, Laura Michalovic, Karina Gutierrez, Werner Glanzner, Rodrigo Bohrer, Naomi Dicks, Rosalba Lopez, Anne-Marie Bellefleur, Hernan Baldassarre and Vilceu Bordignon.

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List of Abbreviations

AI .....Artificial Insemination

AMH .....Anti-Mullerian Hormone

ARC .....Arcuate Nucleus

ARTs .....Assisted Reproductive Technologies

AVPV .....Anteroventral Periventricular Nucleus

BC .....Before Christ

BMP-4 .....Bone Morphogenic Protein 4

BSA .....Bovine Serum Albumin

Ca^{2+} .....Calcium

CDIC .....Canadian Dairy Information Centre

CDK1 .....Cyclin-Dependent Kinase 1

CG .....Cortical Granules

CIDR .....Controlled Internal Drug Release

CA .....Corpus Albicans

CH .....Corpus Hemorrhagicum

CL .....Corpus Luteum

cm .....Centimetre

CO_2 .....Carbon Dioxide

COC .....Cumulus-Oocyte Complexe

DAG .....diacylglycerol

DAPI .....4',6-diamidino-2-phenylindole

E1 .....Estrone

E2 .....Estradiol

E3 .....Estriol

eCG .....Equine Chorionic Gonadotropin

EDTA .....Ethylenediaminetetraacetic acid

ERα .....Oestrogen Receptor α

ET .....Embryo Transfer

FAO.....Food and Agriculture Organisation

Folr4 .....Folate Receptor 4

FSH .....Follicle Stimulating Hormone

G.....Gauge

g.....Gram

GH .....Growth Hormone

GnRH .....Gonadotropin Releasing Hormone

GNX .....Gonadectomized

GPCR.....G-Protein Coupled Receptor

Gpr54 .....G-protein Coupled Receptor 54 (Kisspeptin Receptor)

GSH .....Glutathione

GVBD.....Germinal Vesicle Breakdown

h^2 .....Heritability

hCG .....Human Chorionic Gonadotropin

HEPES .....4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HPO .....Hypothalamic-Pituitary Ovary

I .....Inhibin

I.M. .....Intramuscular

I.V. .....Intravenous

ICM .....Inner Cell Mass

ICSI .....Intracytoplasmic Sperm Injection

IFN-τ.....Interferon Tau

IP_3 .....Inositol 1,4,5-trisphosphate

IVC .....*In Vitro* Culture

IVEP.....*In Vitro* Embryo Production

IVF .....*In Vitro* Fertilisation

IVM .....*In Vitro* Maturation

KBW .....Kilogram Body Weight

kg.....Kilogram

Kp.....Kisspeptin

LH.....Luteinizing Hormone

LOPU.....Laparoscopic Ovum Pickup

M I.....Metaphase 1

M II.....Metaphase 2

mg.....Milligram

mL.....Millilitre

mm.....Millimetre

mM.....Millimolar

mmHG.....Millimetre of Mercury

MOET.....Multiple Ovulation Embryo Transfer

MPF.....Maturation-Promoting Factor

mRNA.....Messenger Ribosomal Nucleic Acid

mSOF.....Modified Synthetic Oviductal Fluid

N_2.....Nitrogen gas

ng.....Nanogram

NGF.....Non-Growing Follicle

O_2.....Oxygen gas

OPU.....Ovum Pickup

OS.....Ovarian Stimulation

P4.....Progesterone

PA.....Parthenogenetic Oocyte Activation

PBS.....Phosphate-buffered Saline

PGF-2α.....Prostaglandin F2α

PIP_2.....Phosphatidylinositol 4,5-bisphosphate

PKC.....Protein Kinase C

PLCζ.....Phospholipase C Zeta

PMSG.....Pregnant Mare Serum Gonadotropin

POA.....Pre-Optic Area

RyR.....Ryanodine Receptor

SCNT.....Somatic Cell Nuclear Transfer

SNP.....Single Nucleotide Polymorphism

SOAF.....Sperm-Oocyte Activating Factor

SUZI.....Sub-Zona Insemination

T II.....Telophase 2

TCM.....Tissue Culture Medium

TE.....Trophectoderm

TLH.....HEPES-buffered Tyrode's Medium

tvOPU.....Trans-vaginal Ovum Pickup

w:v.....Weight/volume

ZP.....Zona Pellucida Sperm Binding Protein

ΔG/t.....Genetic Gain

ΔS.....Selection Differential

µg.....Microgram

µL.....Microlitre

µm.....Micrometre

[GI].....Generation Interval

°C.....Degrees Celsius

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I. Introduction

The purpose of this research project goes beyond understanding reproduction and fertility. The advancement of knowledge concerning the female reproduction system could have impacts in many scientific fields, and may lay the ground work for many discoveries in future research, not only in reproduction, but in other areas as well.

A better understanding of the reproductive capacity of prepubertal animals could have implications in many sectors including: agriculture, medicine, business and conservation. First and foremost, with the world's population continually increasing, there is increased demand and pressure put on the agricultural sector to produce more and more food. In fact, it is estimated that by the year 2050, agricultural production will need to increase by 70% over 2005 levels [1]. Considering this, a better understanding of bovine reproductive physiology could aid in the production of more dairy and beef products, in important source of food and nutrients. In terms of medicine, prepubertal models are an excellent negative model for fertility [2]. Therefore, knowledge about the reproductive system at prepubertal ages could further expand our scientific understanding of human infertility. In fact, human infertility estimated to affect 16% of Canadian couples, a number which has doubled since the 1980s, and is most often attributed to problems in the female [3]. For business, the Canadian dairy industry is a cornerstone of trade dealings, an industry which contributes over $6 billion annually to the Canadian economy [4]. Concerning conservation, there are currently over 16 000 species of plants and animals at imminent risk of extinction [5]. With increasing numbers of invasive species, ongoing habitat loss and global warming, the number of conservation-reliant endangered species is expected to increase in the coming years [6-8]. Therefore, the continued development of assisted reproductive technologies (ARTs) and a better understanding of fertility to aid in captive breeding programs could have critical impacts on conservation efforts worldwide [9, 10].

With the advent of molecular markers such as single nucleotide polymorphisms (SNPs), genetically superior animals can be identified at birth. In other words, a cow's production potential can be assessed long before her first lactation. Therefore, in order to maximise genetic gain, it is preferable to reproduce these animals as young as possible, at a prepubertal age, to

reduce the interval between generations. However, it has been documented that oocytes collected from these animals are less developmentally competent, with lower cleavage and blastocyst rates, when compared to their adult counterparts [11-13]. It has also been well documented that age has a significant influence on oocyte competence and the future efficiency of embryo development [14, 15]. Therefore, the purpose of this research is to optimise the priming of calf oocytes for *in vitro* embryo production (IVEP). Armstrong defined oocyte competence as "the ability of an oocyte to undergo fertilisation and develop to an embryo of a particular stage, while full competence is the ability to produce an embryo capable of establishing pregnancy that leads to the birth of normal offspring" [15]. However, oocyte competence is still not fully understood since there is a complex molecular basis governing oocyte growth and development including nuclear and cytoplasmic factors such as mRNA and protein synthesis [16].

In vivo, oocyte competence is acquired sequentially during follicular growth and development, with full competence attained just prior to ovulation in the final stages of pre-ovulatory follicle growth. This development is driven under the influence of pituitary gonadotrophin hormones, namely follicle-stimulating hormone (FSH) and luteinising hormone (LH) [15]. In mature cattle, ovulated oocytes that have matured *in vivo* have high developmental competence [15]. However, oocyte growth and maturation leading to the acquisition of competence is the result of a complex interplay, where endocrine and paracrine factors acting on follicular cells play a key role. In prepubertal animals, however, these factors may be missing or inadequate given the immaturity of the hypothalamic-pituitary ovary (HPO) axis. The core purpose of this study is to expand our understanding of the potential for the development of calf oocytes, as well as the impact of gonadotropin regimes and the *in vitro* embryo production conditions required to improve their developmental capacity.

This thesis is presented and organised in various sections, outlining the current state of knowledge surrounding various topics such as oogenesis, the hypothalamic-pituitary ovary axis and *in vitro* embryo production. This is followed by experimental design, results as well as discussion and conclusion of the findings.

II. Review of the Literature

1.0 - Canadian Dairy Industry

Dairy farming is a large industry in Canada and plays an important role in the Canadian economy, especially in the province of Quebec (**See Table 2.1**). There are almost 1 million dairy cows in Canada, with 37% of those in Quebec, making Quebec, Canada's largest dairy farming province [4]. The dairy industry is a 17 billion dollar industry and employs thousands of people [4].

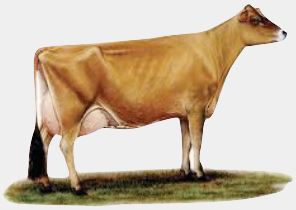
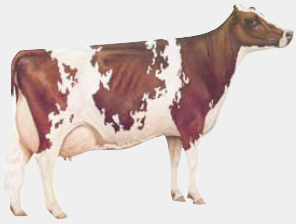
Table 2.1: Overview of the Canadian Dairy Industry at the Federal and Provincial Level.

Canadian Dairy Industry Overview		
	Canada	**Quebec**
Location		
Dairy Cattle	953 000 animals	354 000 animals
Dairy Heifers	444 600 animals	151 000 animals
Dairy Farms	11 683 farms Tie Stalls: 70.8% Free Stalls: 22.4% Robotic: 6.8%	5 766 farms Tie Stalls: 88.3% Free Stalls: 6.1% Robotic: 5.5%
Annual Milk Production	8.176 billion L	3.001 billion L
Dairy Farm Employment	22 055 people	10 955 people
Dairy Product Processing Jobs	22 429 people	9 183 people

Quebec is Canada's largest and most important dairy-producing province, home to over 350 000 cows, the province produces over 37% of Canada's milk annually. Data courtesy of the Canadian Dairy Information Centre (CDIC) [4].

There are over 800 breeds of cattle worldwide, each specialised for certain roles and climates. For example, Holsteins are recognised for producing large volumes of milk, while Jersey cattle are known for their creamy milk with a high fat content. In Canada, there are three principal breeds of dairy cattle, Holstein, Jersey and Ayrshire (**See Table 2.2**). According to the FAO, Holsteins are the most widespread breed of cattle worldwide, and are present in over 150 different countries [17]. Therefore, since they are the dominant breed in the Canada, we decided to focus our study on Holsteins (also known as Friesians, or Hostein-Friesians, in other parts of the world). Holsteins, Jerseys and Ayrshires all belong to the *Bos taurus* species. Some breeds such as Nelore and Brahman, popular in other parts of the world, belong to the *Bos indicus* species, sometimes known as Zebu. It is important to differentiate these species, since Zebu cattle are known to have distinct reproductive properties compared to taurine cattle [18]. For example, *indicus* cattle have been shown reach puberty at a much older age [19], have more visible follicles present on the ovary [20], and metabolise exogenous hormones differently when compared to taurine cattle [20]. Therefore, this review will focus specifically on Holstein cattle.

Table 2.2: Comparison of the Three Popular Breeds of Dairy Cattle in Canada.

Canadian Dairy Cattle			
Breed	**Holstein**	**Jersey**	**Ayrshire**
			
Herd Share	93%	4%	3%
Average Milk Production per Lactation	10 102 kg	6 610 kg	7 781 kg
Average Fat Content	3.87%	5%	4.11%
Average Protein Content	3.19%	3.8%	3.37%

The Holstein is the most popular dairy breed in Canada and makes up over 90% of national herd,

it is also the most widespread breed worldwide. Data courtesy of the Canadian Dairy Information Centre (CDIC) [4].

2.0 - Folliculogenesis & Oogenesis

The ovary is a complex and dynamic organ which is not only the part of the reproductive system, but also the endocrine system. It is composed of two distinct layers of tissue, the inner medulla and the outer cortex. The medulla houses blood vessels, and branches of both the nervous and lymphatic system. Conversely, the cortex is mostly avascular in nature and houses all of the follicles, with many different cell types which play important roles in steriodogenesis. Early on during embryonic development, primordial germ cells (PGCs) migrate from the genital ridge and establish a finite population of oocytes in the ovary [21]. Folliculogenesis takes place later, a dynamic process which occurs in conjunction with cellular events ultimately leading to ovulation [22]. Both oogenesis and folliculogenesis are outlined in the following section, starting with a brief introduction of the bovine oestrous cycle.

2.1 The Oestrous Cycle

The bovine oestrous cycle is a series of changes that occur in the female reproductive system over a 21 day period in response to changes in various hormone levels. Animals can be referred to as ‘cycling’, meaning that normal oestrus cycles are occurring, or ‘anestrus’ meaning the animal is not cycling. Therefore, in cattle, the principal times an animal is referred to as in anestrus is before puberty, during gestation and the post-partum period following birth.

All ruminants, including domestic cattle, are spontaneously ovulating, polyestrous animals [23]. This is opposed to monoestrous species which only go into heat once a year (bears, foxes and wolves). Furthermore, some species are seasonally polyestrous. These species have more than one oestrous cycle during specific times of the year. They can be divided into short day breeders and long day breeders. Short day breeders are sexually active in the fall and winter (sheep, goat, deer). Long day breeders are sexually active in the spring and summer (horses, hamsters, ferrets).

During the oestrous cycle, many small primordial follicles grow and are recruited from the finite pool present in the ovary at birth, under the influence of follicle stimulating hormone. Many of these follicles die and under-go atresia. However, some will develop and grow in size under the influence of luteinising hormone. This continues until a single follicle becomes dominant and ultimately ovulates. Cows are a mono-ovulatory species meaning that for each oestrous cycle, a single dominant follicle will ovulate [24]. This is opposed to some other domestic livestock species, such as pigs and some breeds of sheep, which are polyovulatory and ovulate multiple follicle each cycle [25]. Following ovulation, the ovulatory follicle will undergo luteinisation and a corpus hemorrhagicium (CH) is formed out of a blood clot. This CH, will eventually develop into a corpus luteum (CL), which produces large quantities of progesterone (P4). Finally, under the influence of prostaglandins from the uterus, the CL will degrade into a corpus albicans (CA), and a new wave of follicles will start to develop [26].

2.1.1 The Hormones of the Oestrous Cycle

The bovine oestrous cycle, is a highly complex system, which is orchestrated by a series of endocrine feedback mechanisms. It is managed in principle by eight hormones, outlined in **Table 2.3.**

Table 2.3: The Principle Hormones Managing the Female Reproductive System in Bovine.

The Hormones of the Bovine Oestrous Cycle		
Hormone	**Abbrev-iation**	**Main Function**
Follicle Stimulating Hormone	FSH	Secreted from the anterior pituitary under the influence of GnRH • Recruits a cohort of small follicles from the pool of growing follicles on each ovary during the follicular waves
Luteinizing Hormone	LH	Secreted by the anterior pituitary under the influence of GnRH • Stimulates the ovary and the continued growth of the follicles following selection • A pre-ovulatory surge of LH results in the ovulation of the ovulatory follicle • Believed to trigger resumption of meiosis in maturing oocytes • Responsible for the luteinization of follicular cells following ovulation

The Hormones of the Bovine Oestrous Cycle		
Hormone	**Abbrev-iation**	**Main Function**
Progesterone	P4	Produced by the Corpus Luteum • Inhibits ovulation • Maintains pregnancy • Prepares uterus (endometrium) for pregnancy • Stops the animal from showing signs of estrus
Prostaglandin F2α	PGF-2α	Secreted by the endometrium • Induces luteolysis
Estradiol	E2	Produced by developing follicles • During the luteal phase, responsible for negative-feedback on the hypothalamus • Following luteolysis, during the follicular phase, responsible for positive-feedback on the hypothalamus, and subsequent LH surge • Sexual behaviour in the animal • Various intra-follicular events, affecting both granulosa cells and the oocyte
Gonadotropin Releasing Hormone	GnRH	Produced by the hypothalamus • Acts of the pituitary gland • Results in the production of FSH and LH
Interferon Tau	IFN-τ	Produced by the developing embryo • The maternal recognition signal in cattle which is recognised by the endometrium • Blocks PGF-2α release and subsequent luteolysis
Inhibin	I	Produced by the granulosa cells of developing follicles • At low concentrations during the selection process • At high concentrations during the dominance phase, FSH stimulates the secretion of inhibin, Which in turn, acts on the anterior pituitary and suppresses FSH secretion

GnRH is produced by the hypothalamus, FSH & LH are produced by the pituitary gland, finally E2 & P4 are produced by the ovary.

2.1.2 Phases of the Oestrous Cycle

The oestrous cycle varies in length among species. In cattle, it ranges between 17-24 days, with a typical length of 21 days. Each cycle gives the cow the opportunity to become pregnant. It is composed of two phases: the luteal and follicular phase. During the luteal phase, which accounts for around 80% of the oestrous cycle, the corpus luteum is the dominant ovarian structure. Hence, progesterone levels increase, and oestrogen levels decrease. During the

follicular phase, which accounts for about 20% of the oestrous cycle, the growing follicle is the dominant ovarian structure, and oestrogen levels increase, but progesterone levels decrease.

The oestrous cycle is composed of four stages:

- **Estrus (day 0-1):** In cattle, estrus lasts between 4 and 24 hours, with an average length of around 15 hours. During this time, the dominant follicle reaches its maximum growth, matures and ovulation occurs between 24 and 32 after the onset of estrus. High levels of E2 and LH are present during stage.
- **Metestrus (Day 2-4):** The period from ovulation to the formation of the corpus luteum, where progesterone levels begin to rise
- **Diestrus (Day 5-17):** Longest stage of the oestrus cycle. Time during which the corpus luteum is secreting sustained levels of P4.
- **Proestrus (day 17-20):** The time between when progesterone levels begin to diminish (luteolysis) and the following estrus of the next cycle (**See Figure 2.1**).

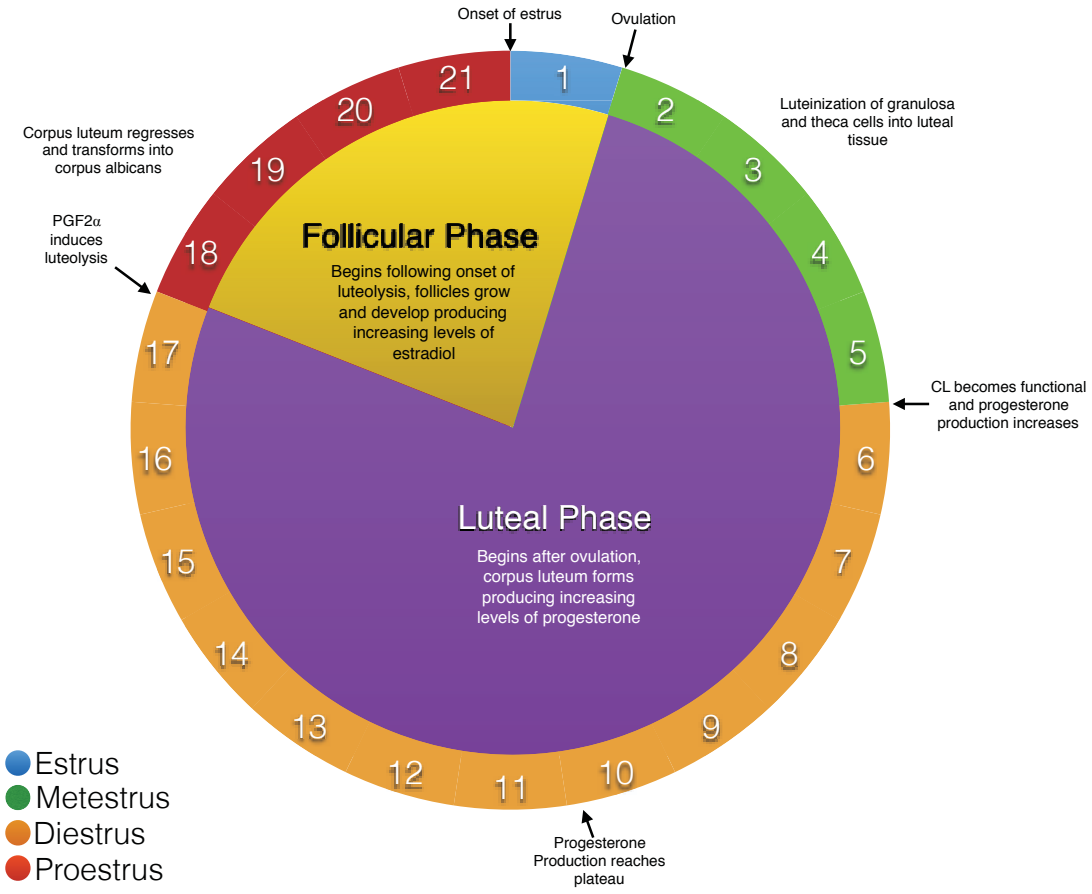


Figure 2.1: The Phases of the Oestrous Cycle in Bovine. Schematic diagram of the oestrus cycle, showing the stages of oestrus cycle on the outer ring: estrus (blue), metestrus (green), diestrus (orange) and proestrus (red). The inner graphs shows the phases of the oestrus cycle: luteal phase (purple) and follicular phase (yellow).

2.2 Follicular Waves

The bovine oestrous cycle is characterised by two or three follicular waves and three stages: recruitment, selection and dominance. The growth and development of the follicles are predominantly an effect somatic cell proliferation and differentiation [27]. Each of which are stimulated under the influence of FSH and LH, which are secreted from the anterior pituitary gland.

- **Recruitment:** During recruitment, antral follicles develop under the influence FSH which increases in serum concentrations prior to each follicular wave [28]. FSH recruits and stimulates a new pool of follicles to grow [28]. Following this, FSH concentrations decrease and LH allows the follicles to continue developing until around the time of selection.
- **Selection:** As the follicles enter the selection phase, inhibin and estradiol, which are produced by the follicle, repress FSH secretion from the pituitary. Thus, the relative roles of FSH and LH begin to shift. FSH secretion decreases during selection, while LH secretion increases and is responsible for regulating the continued growth and development of selected and dominant follicles. Developing follicles produce estradiol; hence, circulating levels of estradiol increase and decrease as the follicular waves grow and regress.
- **Dominance:** The largest follicles produce more and more estradiol. This has a positive feedback loop, causing a surge of GnRH from the hypothalamus. However, inhibin suppresses FSH secretion, which leads to the LH surge. The low levels of FSH are believed to cause other antral follicles to undergo atresia and prevents a new wave of follicles to be recruited. However, the dominant follicle will remain eminent for a short period of time, typically no longer than 3-6 days, until it is either ovulated or undergoes atresia.

Should the dominant follicle be present when a CL is present, progesterone prevents follicles from producing more oestrogen and the follicle will undergo atresia. It is only when the CL begins to regress and P4 levels recede that ovulation will occur (**See Figure 2.2**). It is also important to note that oestrogen is a general term used to describe three hormones: estrone (E1), estradiol (E2), and estriol (E3). E2 is the most important modulator of the oestrous cycle and is primarily produced in by granulosa cells in the ovary, it is the most potent of the three.

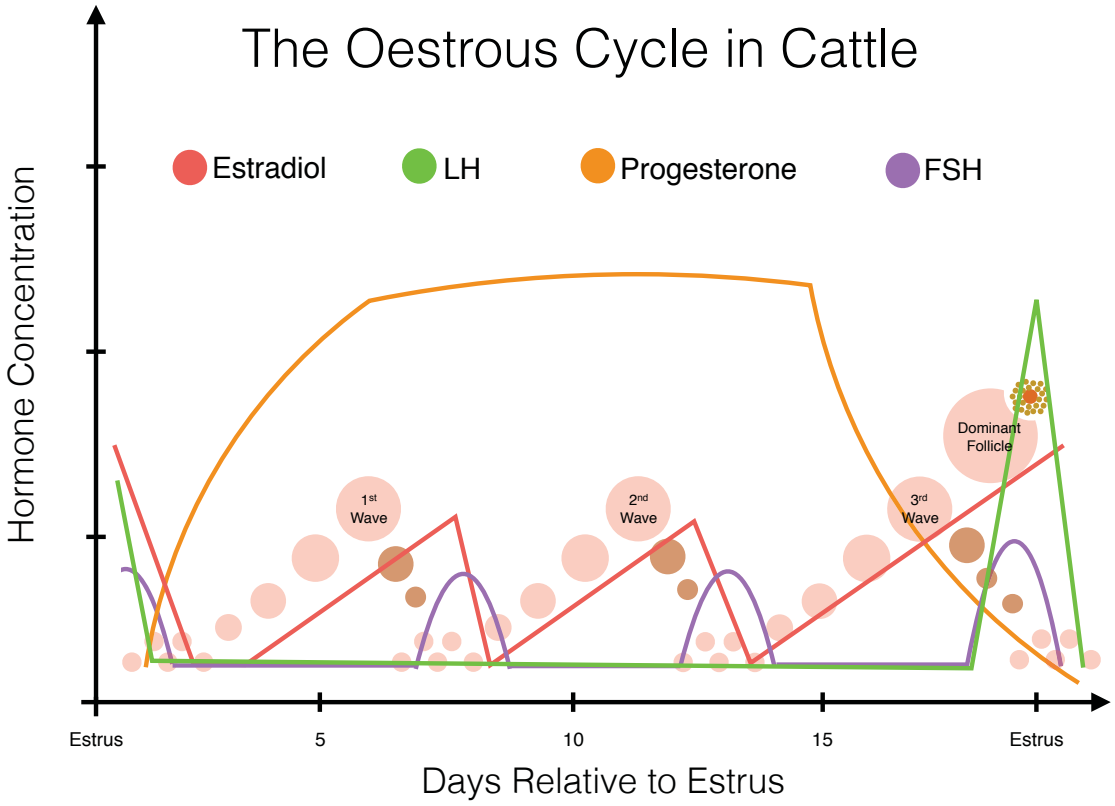


Figure 2.2: Follicular Waves of the Oestrous Cycle. Schematic of the relative hormone concentrations and how they relate to the follicular waves inside the ovary.

2.3 Oogenesis

In mammals, germ cells and somatic cells share a common origin. PGCs first develop in a specific region in the posterior portion of the embryo known as the primitive streak. Bone morphogenic protein 4 (BMP-4) and WNT signalling drive this development. These germ cells then migrate to the developing genital ridge where sexual differentiation takes place under the influence or absence of the SRY gene on the male Y chromosome. In the absence of the SRY transcription factor, in females, somatic cells in the genital ridge undergo differentiation into granulosa and future thecal cells. In females, PGCs develop into oogonia, a type of totipotent stem cell, which stay with the animal their entire life. Next, oogonia divide into primary oocytes. After several rounds of proliferation, primary oocytes enter meiosis after expression of *Stra8* is induced from retinoic acid produced by the mesonephros. During meiosis, these cells become arrested during prophase 1, which is maintained until the oocyte is ovulated. This period of development is important since chromosome decondensation and transcription occurs during this time allowing the oocyte to grow and mature [29]. Various hormonal cues affect this development, with the LH surge which occurs just prior to ovulation, believed to cause oocytes to resume meiosis at the time of ovulation [30]. Consequently, secondary oocytes develop in the infundibulum/oviduct with the extrusion of the first polar body. Following this, these cells undergo meiosis II, ultimately arresting at metaphase II. It is only after fertilisation occurs, that the oocyte will resume and complete meiosis II, with the extrusion of the second polar body [29].

2.3.1 Oocyte and Follicular Growth

Autocrine, paracrine, juxtacrine and endocrine factors are all essential and work together to drive ovarian folliculogenesis [22]. Throughout an animal's reproductive lifetime, follicles are repeatedly recruited and grow. However, a large proportion of these follicles will never reach the pre-ovulatory stage and undergo atresia before the oocyte within can be ovulated [31]. Before formation of the follicle, clusters of germ cells known as germ cell nests break down and primordial follicles form when oocytes are surrounded by squamous pre-granulosa cells. Primordial follicles are characterised by having one layer of flat granulosa cells. Next, primordial follicles develop into primary follicles which are characterised as having cuboidal pre-granulosa cells. Following this, primary follicles develop into secondary, antral and finally pre-ovulatory

follicles, sometimes referred to as Graafian follicles. This folliculogenic timeline is typically divided into two parts the gonadotropin-independant pre-antral follicle phase and the gonadotropin-dependant antral follicle phase (**See Figure 2.3**). Preantral folliculogenesis is characterised by the acquisition of theca cells, granulosa cell proliferation and oocyte growth [22]. This phase of growth is known to be dependant on various autocrine and paracrine regulatory factors, but is believed to be gonadotropin-independant as shown by various knock-out mice models lacking certain gonadotropin factors [22]. The final phase of folliculogenesis is antral follicle growth, marking a passage from mainly intra-ovarian regulation to extra-ovarian regulation, with the HPO axis playing an important role. During this time, a fluid-filled antrum develops inside the follicle and two distinct populations of granulosa cells develop: cumulus and mural granulosa cells. Cumulus cells surround the oocyte and support growth and developmental competence, while mural granulosa cells which line the wall of the follicle, are important in steriodogenesis and ovulation [22]. This phase is coordinated by FSH and LH. Finally, during ovulation, together with the oocyte, the cumulus cells which surround it are expelled, a structure known as the culumus-oocyte-complex (COC).

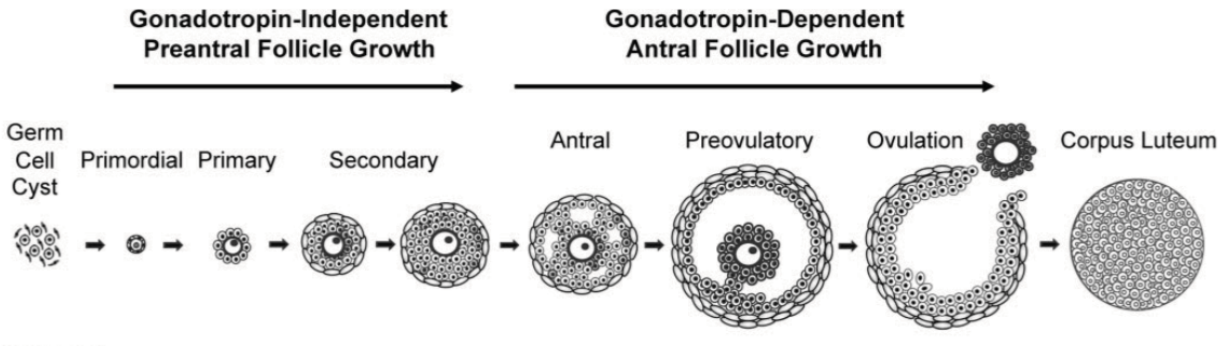


Figure 2.3: Folliculogenesis in the Bovine Ovary outlining Gonadotropin-Independant and -Dependant Growth. Figure reproduced from Edson and colleagues (2009) [22].

As in most mammals, folliculogenesis begins during foetal development in cattle [32]. Between 220 and 240 days of gestation, the foetal bovine ovary begins to form vesicular follicles in-utero [33]. At birth, fully grown oocytes are present in antral follicles in cows, this is in

contrast to some other species such as mice [34]. Furthermore, neonates as early as 5 days old have follicles up to 5 mm in diameter, and may contain between 75 000 and 300 000 oocytes [35]. Follicular recruitment occurs early on during gestation and the largest numbers of follicles 3-5mm in diameter are present at two months of age [35, 36].

After birth, the ovary grows quickly, increasing from 0.5 g at two weeks of age to 4 g at 4 months, and reaches 10 g by 12 months [33]. The increase in weight also corresponds with an increase in the number of vesicular follicles present, and, by 7 weeks of age, a constant pool of approximately 50 follicles can be present [33]. However, it has been shown that many of these follicles are atretic in nature, and the majority of oocytes collected from these follicles are non-viable [37]. Ovarian weight increases nearly 4 times faster than the total body weight during the first 5 months of life (**See Table 2.4**) [38]. This is in contrast to some other species, such as rats, which have been shown to follow the same growth to body weight curve [39].

Table 2.4: Ovarian Characteristics Compared to Body Weight from Birth to One Year of Age in Cattle.

Age (Months)	Body Weight (kg)	Paired Ovarian Weight (±SE) (g)	Number of follicles	
			< 5 mm	> 5 mm
Birth	36.7	0.5 ± 0.1	0	0
1	48.3	1.2 ± 0.1	1	1
2	69.9	3.2 ± 0.4	8.8	0
3	91.6	4.2 ± 0.6	11.4	1
4	101.9	3.5 ± 0.6	22.4	5.4
5	143.3	7.1 ± 1.5	13.0	2.0
6	178.5	6.8 ± 1.8	8.8	2.4
7	186.8	6.9 ± 0.8	14.8	1.4
8	207.3	7.1 ± 1.0	5	1.2
9	228.5	9.0 ± 1.5	5	2
10	282.6	9.4 ± 0.8	6	0.4
11	286.7	12.6 ± 3.0	5.2	1.0
12	329.7	12.4 ± 2.2	4.6	0.4

Adapted from Desjardins and Hafs 1968 [38].

2.3.2 - Loss of Ovarian Reserve over Time

It is widely believed that female mammals are born with a fixed number of oocytes at birth which decline over their lifetime, as they do not generate any new germ cells (**See Figure 2.4**) [40]. Therefore, younger animals have larger ovarian reserves present in the ovary. In fact, it has been postulated that mammals have the greatest number of non-growing follicles (NGFs) present in the ovary during in-utero development, and that populations decline throughout life until death or menopause [40]. It is interesting to note, that in humans, only 50-60% of NGFs are present in the ovary after puberty. Although data specific to bovine is scarce, it is believed most mammals follow a similar pattern of decline. In cattle, the foetal reserve is estimated to be around 130 000 oocytes (however estimates as high as 300 000 exist), with variations between individuals and breeds [35, 41]. Throughout an animal's lifetime, many of these oocytes will either be ovulated or die and undergo atresia, and by adulthood only a fraction of these oocytes remain, until eventual reproductive senescence [22].

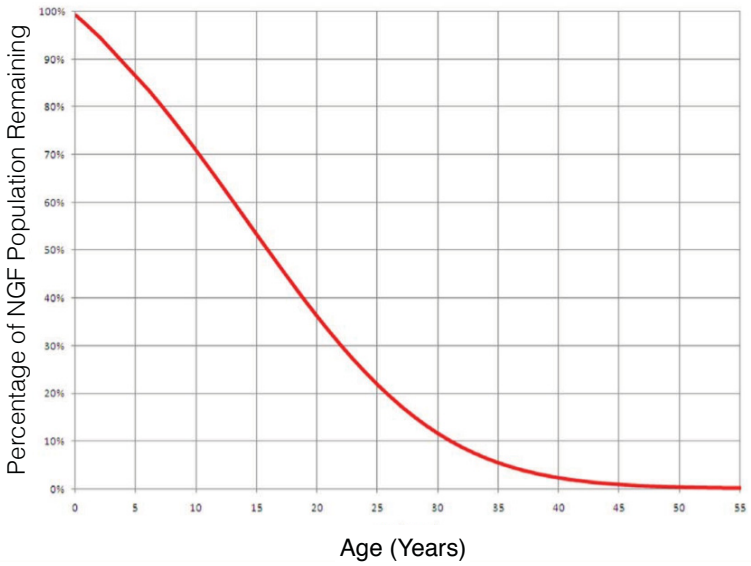


Figure 2.4: Theoretical Ovarian Reserve in Humans from Birth to Menopause. Note that by puberty, humans only have around 50-60% of

their ovarian reserve remaining. Figure reproduced from Wallace and Kelsey 2010 [40].

3.0 - HPO Axis Before and After Puberty

In female reproductive physiology, the hypothalamic-pituitary-ovarian axis is the conductor of the orchestra. Although, often referred to a single entity, the HPO axis is composed of three distinct components (**See Figure 2.5**):

1. **Hypothalamus:** Part of the limbic system in the brain and is the gateway between the nervous and the endocrine system. The hypothalamus is responsive to estradiol and progesterone, and produces GnRH. It is composed of two centres.
    - **Surge Centre:** The surge centre is sensitive to to positive feedback and releases high amplitude, high frequency pulses of GnRH in a relatively short period of time (hours) after E2 reaches a threshold concentration.
    - **Tonic Centre:** The tonic centre releases small quantities of GnRH in a pulsatile fashion. This episodic release is continuous throughout reproductive life.
2. **Pituitary Gland:** Part of the endocrine system and is composed of two lobes: the anterior and posterior. It has an intimate vascular connection with the hypothalamus and in response to GnRH, the anterior lobe releases FSH and LH.
3. **Ovary:** Part of the reproductive system. In response to FSH and LH, developing follicles produce E2 and I, while the corpus luteum produces P4. Estradiol can have both a positive and negative feedback loop on the hypothalamus. While, inhibin has a negative feedback loop on FSH production in the pituitary.

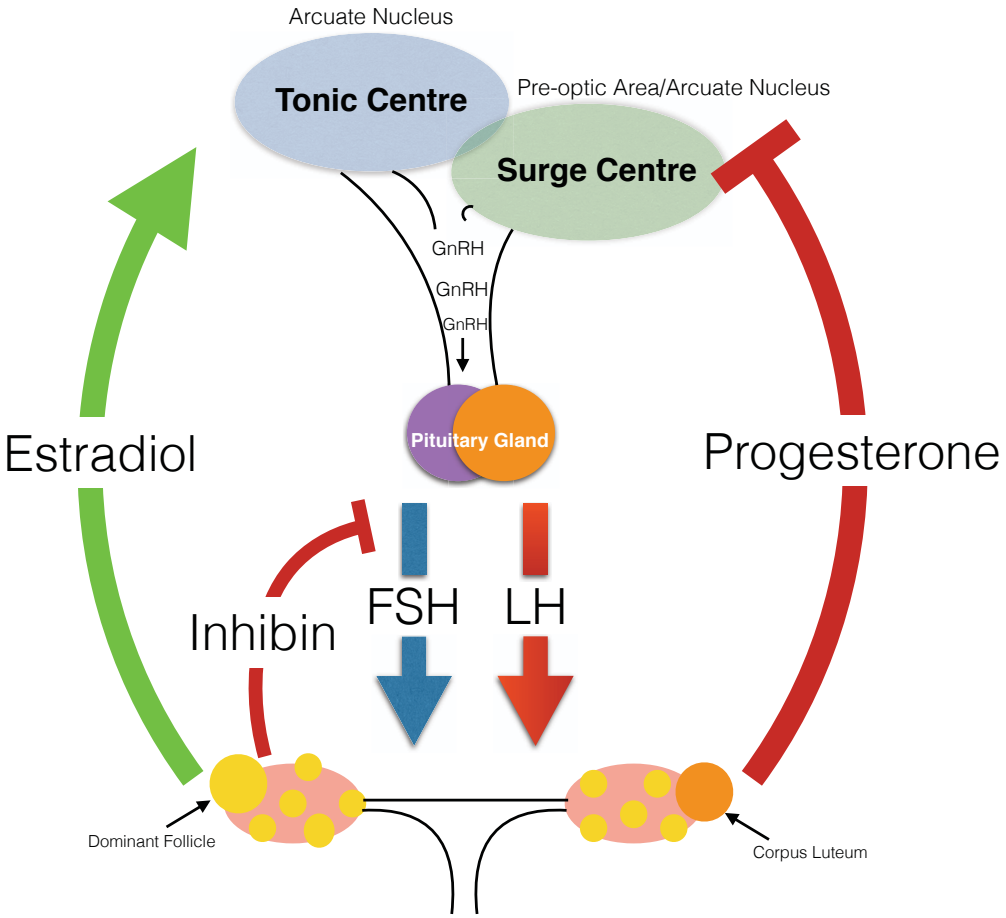


Figure 2.5: The Hypothalamic Pituitary Ovary Axis in Cattle. With the presence of progesterone from the corpus luteum, the surge centre is repressed and estradiol acts on the tonic centre to release small quantities of GnRH, to the pituitary gland which releases FSH and LH. In the absence of Progesterone, there is a positive feedback loop where estradiol causes the hypothalamus to release a flood of GnRH. Inhibin from the dominant follicle acts on the pituitary gland to suppress the release of FSH, inducing an LH surge. This LH surge in turn causes ovulation.

3.1 - Kisspeptin

Kisspeptins (Kp) are a family of neuropeptides synthesised by the *Kiss1* gene in the hypothalamus of the brain. They are produced in two neuronal cell populations and were discovered in 2003 to operate upstream of the GnRH system [42]. Much research has focused on the function of the *Kiss1* system recently, and it has now been shown to play seminal roles in many areas, including:

1. Sexual differentiation of the brain
2. Timing the onset of puberty
3. Secretion of gonadotropins
4. Transmission of the negative and positive feedback loops of sex steroids
5. Generation of the pre-ovulatory LH surge
6. Metabolic regulation of fertility
7. Modulation of reproductive function by environmental cues (including photoperiod and stress) (Reviewed in [43] & [44]).

Upon synthesis, the precursor protein is proteolytically cleaved into multiple peptides of various lengths, known as kisspeptin-10, -13, -14, and -54 [43]. These peptides share a common binding site and all act as a ligand to the G-protein coupled receptor, *GPR54*, also known as *Kiss1R,* which is expressed by GnRH neurons [43, 44].

3.2 - Kisspeptin and Puberty

Kp signalling was first discovered to play a pivotal role in timing the onset of puberty when individuals expressing mutated inactive genes of *Kiss1* and/or *Gpr54* failed to reach puberty normally [42]. Following this, later studies found that the converse is also true and that repeated administration of Kp-10 to prepubertal rats accelerated the onset of puberty [45]. The exact mechanism of function remains to be fully understood, however it has been found that *Kiss1* expression increases in the hypothalamus during puberty, and that the number of Kp-positive cells with close appositions with GnRH neurons increases during puberty [46-48]. However, later evidence suggests *Kiss1/Kp* expression in the hypothalamus is dependent on an estrogenic input from the ovary, suggesting some sort of feedback-loop plays a role [49].

In the simplest, broadest terms, puberty is defined as the process and period by which animals become capable of reproduction themselves [50]. Ovarian function is at the centre of puberty; however, the first ovulation is not synonymous with puberty in most heifers, as is the first standing estrus [50]. Ovulation has been shown to occur spontaneously in some calves less than one month of age, and can occur at any age before puberty, however, individuals do not go into estrus [51]. Therefore, a more precise definition is "the first opportunity for a heifer to conceive and should be defined as the first ovulatory estrus followed by a luteal phase of normal duration" and is characterised by the establishment of LH secretions in a pulsatile manner [18, 52]. In cattle, the age of puberty can depend on a variety of factors, including genetics, nutrition and weight, and can vary drastically from 6 to 24 months [50]. However, genetics is believed to the be the principle factor driving the age of onset [18]. For example, although Holsteins generally reach puberty around 12 months of age, Zebu cattle do not reach puberty until at least 22-36 months of age [19].

3.3 - Kisspeptin and GnRH

Numerous studies have documented that the *Kiss1* system is an essential regulator of gonadotropin release and injecting animals with Kp has been shown to induce LH and FSH release in many species including various ruminants [53-55]. Further research has pointed to Kp mediating a stimulatory effect on GnRH release from the hypothalamus, rather than a downstream effect on the pituitary, however, this remains controversial [53, 56-58]. As such, Kp has been suggested as a useful tool in order to stimulate the quiescent HPO axis in anestrous animals [59]. A single dose of Kp has been shown to elevate FSH levels for several hours following injection in ewes (**See Figure 2.6**) [60]. Furthermore, in prepubertal ewe lambs, Kp administration over a 24 hour period resulted in enhanced gonadotropin production and ovarian steroidogenesis, as well as stimulation of the LH surge and ovulation [61]. However, Kp-10 injection in prepubertal Holstein heifers, also resulted in a growth hormone (GH) surge in addition to LH, suggesting Kp also has some effect on the somatotrophic axis [54]. This remains to be studied further since work with Japanese Black beef calves (5-6 months of age), resulted in both LH and FSH secretion, but had no significant effect on GH [62].

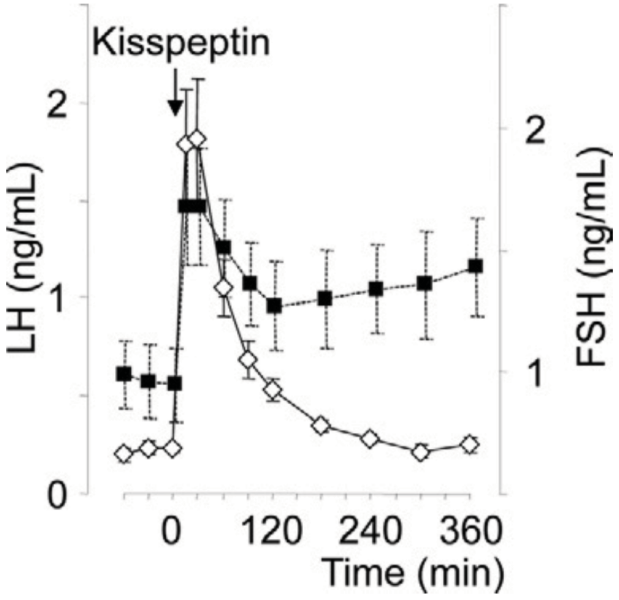


Figure 2.6: LH and FSH concentrations following Administration of Exogenous Kisspeptin-10 in a Representative Ovariectomized Ewe. Serum concentrations of LH (open diamonds) and FSH (filled squares). Figure reproduced from [59]**,** which itself was adapted from [60]**.**

3.4 - Feedback Loops and Development of the Surge Centre

The HPO axis contains both negative (inhibitory) and positive (stimulatory) feedback loops. During the luteal phase and most of the follicular phase, there is a negative feedback loop in place. However, at the end of the follicular phase, when E2 levels are high and P4 levels are low, a positive feedback loop is initiated in mature animals. In prepubertal animals, there is no positive feedback loop. Both the positive- and negative- feedback loops are believed to be mediated through neuronal oestrogen receptor alpha (ERα) signalling [63]. However, GnRH

neurons do not express these receptors and therefore kisspeptin neurons were proposed to play a intermediary role [64, 65].

Different subpopulations of kisspeptin neurons in the hypothalamus are thought to be responsible for the different feedback loops they feed [44, 66]. Neurons in the arcuate nucleus (ARC) are believed to be responsible for the negative feedback loop, in a variety of species including ruminants [44, 67]. Evidence for this is based on gonadectomized (GNX) models, where *Kiss1* mRNA levels rise following gonadectomy and the loss of negative-feedback-mediating gonadal hormones [46]. In turn, sexual steroid supplementation in GNX-models not only prevented the post-GNX rise in gonadotropins, but also the increase in *Kiss1* mRNA levels [46]. In rodents, the best studied model, the anteroventral periventricular nucleus (AVPV) is believed to be responsible for the positive feedback loop [66]. This hypothesis is supported by the fact that E2 appears to up-regulate *Kiss1* mRNA expression specifically at this nucleus and that these neurons are activated during the pre-ovulatory LH surge [68, 69]. In ruminants however, the location of these neurons is believed to be in the pre-optic area (POA) and/or specific regions of the ARC [70].

Although, prepubertal cattle do not cycle like mature cows, there is still HPO axis feedback and ovarian wave activity. Young calves, as young as two weeks old have been shown to have follicular growth patterns similar to the follicular waves found in mature cattle [71]. Furthermore, it was shown that a transient increase in serum FSH levels occur just prior to the emergence of a new follicular wave, suggesting the recruitment stage of follicular development is functional at all ages, and not just sexually mature animals [71]. However, young animals have been found to have a smaller ratio between the diameter of dominant follicle, and the next largest subordinate follicle [71]. Suggesting that there is some sort of imbalance in the selection and dominance phase of the follicular cycle, with the largest follicle asserting less dominance over other follicles in the cohort [71].

GnRH receptors on the pituitary have been shown not to change with age, and indeed, the pituitary secretes gonadotropins in response to GnRH at a very young age [52, 72]. Hence, it is believed that the pituitary gland is fully capable of synthesising the releasing gonadotropins in adequate concentrations to promote follicular growth and ovulation given the correct GnRH

stimulus [18]. However, it should be noted that Fraser and associates found evidence to the contrary in monkeys [73]. However, the upstream hypothalamus is believed to be the last component of the HPO axis to mature in prepubertal heifers, and is the limiting factor determining the HPO functionality before puberty [18]. Specifically, the number of *Kp* positive cells in the ARC and POA have been shown to increase during prepubertal development in the ewe, however, these numbers do not always correlate wth increases in LH pulse frequency [74]. Redmond and associates found that the number of *Kiss1* positive cells in the POA increased in number from 25 to 35 weeks of age in lambs, but these cells did not influence LH pulses. However, increased numbers of cells in the middle ARC, did have an influence [74]. Hence, although it is known Kp plays a definite role in the development of the surge centre and onset of puberty in ruminants, the exact details remain unclear [70].

4.0 - Ovum Pickup Technologies

Over the years, multiple ovum pickup (OPU) technologies have been developed. Many variations exist depending on the circumstances and the application at-hand. Since this project is based on cattle, special attention will be paid to laparoscopic ovum pickup (LOPU), and compared to what is typically used in adult animals ultrasound-guided trans-vaginal ovum pickup (tvOPU).

4.1 - Laparoscopic Ovum Pickup

LOPU is a reliable technique where follicles can be aspirated by using a laparoscope to visualise the ovary. This is typically done under general anaesthesia, and depending on the number of follicles to be aspirated, an experienced surgeon can usually perform the procedure in between 10-20 minutes [75]. Briefly, animals are restrained and placed in a dorsal recumbency position. Three small incisions are made on the ventral surface of the abdomen and trocars are inserted. Next, using a laparoscope, and a pair of atraumatic grasping forceps, the ovary is visualised. Finally, using a puncture pipette attached to a vacuum line, follicles are punctured and the follicular contents aspirated [76, 77].

4.1.1 - History of LOPU

Laparoscopic surgery is increasingly being used to replace traditional open surgery since it is less invasive and results in faster recovery times. It has also led to significant advances in animal production, facilitating easier and more precise diagnosis and management of fertility issues [78]. Despite rudimentary attempts to look inside a human body dating back to around 400 BC, it wasn't until the 20th century that the modern connotation of the word laparoscopy was developed [79]. It was first introduced in 1901 by George Kelling and Dimitri Ott, working independently of each other. Kelling was a German surgeon, who visualised the peritoneal cavity and examined the viscera of a living anaesthetised dog via laparoscope, he went on to name this procedure "Kölioskopie" [79]. In the same year, Ott, a Russian gynaecologist, used a similar approach in humans to examine the abdominal cavity of a pregnant woman [79, 80].

73 years later, in 1974, Snyder and Dukelow, two researchers from Michigan State University, first described LOPU using the ovine model [81]. They built off past research by Roberts, who was the first to use a mid-ventral incision and laparoscope using a remote light source in sheep [82]. As well as, Boyd and Ducker, who used a sectional table to elevate the abdominal region [83, 84]. In their work, Snyder and Dukelow used a laparoscope to diagnose pregnancy, detect ovulation and aspirate ovarian follicles *in vivo*. In order to develop their technique, they used 19 ewes and performed 170 laparoscopic examinations over an 11 day period, performing the procedure every other day with no adverse effects noted [84].

The first researchers to work with cattle and perform repeated in vivo oocyte collection were Canadian, who in 1983, accessed the ovary via the right paralumbar fossa [85]. In their work, using 50 mature Holstein heifers, they performed a total of 129 laparoscopies, testing various technical aspects such as needle gauge and suction device. A total of eight animals underwent more than four interventions, and noted that although adhesions were occasionally observed, they never interfered with follicular aspiration. They found that using a 19G needle, with a 45º bevel and a vacuum pressure of 250 mmHg yielded the best results, with a recovery rate of 72% and a mean of 7.7 oocytes per animal per laparoscopy [85].

Several years after this, in 1987, follicular aspiration in cattle using an ultrasonic guide was used by a Danish team of researchers [86]. In their work, they used seven gonadotropin-

treated mature heifers and positioned the ovary by rectal palpation against the sacrosciatic ligament in order to visualise the follicles by ultrasound which was placed on the sacra-ischiadic region. Follicles were punctured trans-cutaneously, resulting in a 42% recovery rate. The following year, in 1988, this method was elaborated upon by using trans-vaginal ultrasound-guided follicle aspiration (tvOPU) in cattle for the first time by a dutch team of researchers [87]. Using 10 Holstein cows, they performed a total of 36 trans-vaginal aspirations and recovered a total of 54 oocytes from 197 follicles. Together, they demonstrated that repeated aspirations could be performed, with little adverse health effects [87]. In the coming years, tvOPU became an industry standard and is widely used in cattle around the world today [88].

The use of laparoscopic technology became increasingly prominent for use in small ruminants such as sheep and goat, where it is difficult to manipulate the reproductive tract via the rectum due to their small size [78]. Hence, effective embryo transfer and retrieval techniques via laparoscope have been fully developed in various species including sheep and deer [89]. Puncturing follicle in order to retrieve oocyte complexes via laparoscope was developed after this however, since its inception, the LOPU technique has been refined and adapted for use in a variety of species, by adapting the needle type, gauge and vacuum pressure used in order to maximise oocyte recovery and blastocyst production rates [76, 78]. In the coming years, the technology was developed for use in both domestic livestock such as sheep and goat, as well as wild species including pumas, and African game [76-78, 90, 91].

4.1.2 - Technical Aspects and Recovery Rates

Various technical aspects such as the needle size and vacuum pressure are known to have a large influence on the quantity and quality of COCs that are collected [88]. In the very first LOPU, Snyder and Dukelow used a 23 G, 11 cm needle in an 18 G cannula. They managed to aspirate a total of 21 follicles, and recovered 6 COCs, for a recovery rate of 28% [84]. From the very beginning, Snyder and Dukelow acknowledged the large potential for the technology, stating “this appears to be a rapid and relatively efficient method of obtaining follicular oocytes for in vivo studies” [84]. Since then, however, many researchers have tested various aspects of the procedure, and modern recovery rates are often in the 80-90% range.

In the very first study using laparoscopy via the right paralumbar fossa in mature heifers, Lambert and colleagues tested various needle sizes and compared a syringe to vacuum pump. In their results, they found that using a vacuum pump dramatically improved recovery rates and that using a vacuum with a pressure of 250 mmHg attached to a 19 G needle yielded the best results [85]. Bols and associates tested various needle sizes and vacuum pressures with needles designed for tvOPU. In their work, they found that an 18 G with a long-beveled tip offered the best results [92, 93]. Regarding pressure, they found that as pressure increased, recovery rate also increased, however, there was a trade-off with the blastocyst production rate, as rates decreased [92, 93]. Therefore, a delicate balance must be achieved in order to maximise the recovery rate while minimising the stress placed on the COCs during collection [88]. Blastocyst production rates decrease significantly with pressure increasing beyond 50 mmHg [94].

4.1.3 - Potential applications

Most research is done using abbatoir-derived oocytes. However, using ovaries from the slaughterhouse has certain limitations:

1) It only allows for the recovery of a limited number of gametes with respect to the potential population present in the ovary.
2) Only oocytes present in follicles involved in the follicular wave have proper competence.
3) Prevents the same donor from being used in multiple replicates.
4) The life history (age, housing, diet, medications etc.) or reproductive health of the animal is usually unknown [88, 95].
5) If collecting at a commercial slaughterhouse, the animal cannot be treated (with FSH or any other desired drug) prior to collection.

Therefore, LOPU has great potential for the expansion of scientific knowledge when work with a specific animal or group of animals is required. For example, a single highly valuable animal (monetary or genetic), can be subjected to LOPU using some sort of ovarian stimulation protocol multiple times. This would have been impossible before LOPU technology, and not only can multiple offspring be produced, but valuable scientific data can also be collected, with no harm to the animal.

4.2 - Ultrasound-guided Ovum Pick-up

Ultrasound-guided trans-vaginal techniques have proven effective and popular in cattle, and has been used for a variety of purposes including ovum pick-up (OPU) [96, 97], follicular fluid collection [98, 99], dominant follicle ablation [100], foetal fluid collection [101, 102] and intra-follicular injection [103, 104]. It was first used in cattle by a team of dutch investigators in 1988 [87]. In large animals, this is usually performed, as it is more practical as it does not require general anaesthesia. However, this technique can only be performed on certain animals, with size being the limiting factor. The size is important since the ultrasound probe must fit into the vagina, and the technician's arm must fit inside the rectum to be able to palpate the ovary. tvOPU can be performed on animals as young as 5-6 months of age, with the size of the vagina and rectum being the limiting factor [96, 105]. However, ultrasound-guided OPU can also be performed as young as 14 weeks of age, without supporting the ovary by palpation [106]. Therefore, LOPU is essential for the study of young calves, and other small ruminants such as sheep and goat.

LOPU has several advantages over tvOPU primarily because the ovary can be viewed with a depth of field:

1) Primarily superficial follicles can be aspirated.
2) The ovary can be viewed directly to ensure proper aspiration technique.
3) Recovery problems can be mitigated.
4) Has high recovery rates.
5) A reduced risk of injury to the ovary and surrounding structures [78, 88].
6) Requires less expensive equipment than the ultrasonography equipment required for tvOPU [78, 107].

However, it also has some disadvantages:

7) It is more invasive than tvOPU on the animal (although much less traumatic on the ovary).
8) The requirement for anaesthesia, and the risks associated with it.

5.0 - *In vitro* Embryo Production Technologies

In vitro embryo production (IVEP) is a blanket term used to describe the combination of *in vitro* maturation (IVM), *in vitro* fertilisation (IVF) and finally *in vitro* culture (IVC). In recent years, various reproductive biotechnologies have become increasingly popular for use with bovine, for example, embryo transfer (ET). Hence, with an increase in demand, also comes an increase in the demand for IVEP procedures. IVEP, holds several advantages over traditional methods, namely:

1. It can be applied to animals which do not respond well to alternate breeding efforts.
2. Can be used to salvage the genetic potential of a terminally ill or dead animal.
3. Oocytes from a single donor can be inseminated with semen from different bulls at the same time.
4. Oocytes from various sources can be used, *in vivo* and *in vitro* [108].
5. Can be used to produce embryos from prepubertal donors.

Since early attempts at IVEP, improved methodology and protocols has significantly enhanced the development rate of embryos *in vitro*. This along with a reduced cost, has allowed IVEP to be used in commercial practices across the world. Most recent research has centred around the improvement of media and optimising conditions for IVEP, and successful blastocyst development rates are steadily increasing.

5.1 - In Vitro Maturation

Prior to IVF, oocytes must undergo a period of maturation which can either be done *in vitro* or *in vivo*. This is an important event, since it allows the oocyte to accumulate mRNA and proteins in preparation for activation, and could contribute to the reduced competence of calf oocytes [2]. Hence, the experimental design is an important consideration when determining the hormone treatment to be used. *In vivo* maturation is most commonly used in human IVF medicine and the first calf born from IVF was a product of *in vivo* maturation [109, 110]. In cattle, oocytes are believed to mature *in vivo* as a result of the pre-ovulatory LH surge, this can be induced by administering animals an intravenous (i.v.) injection of LH prior to LOPU [107,

111]. In recent years, more modern work has deemed *in vitro* maturation to present more consistent results than attempts at maturation *in vivo*. In cattle, maturation media is typically supplemented with LH, FSH and E2, in order to improve the fertilisation and blastocyst rate *in vitro* [112]. Oocyte maturation is a process which has two goals:

1. Nuclear maturation: Facilitate the resumption of meiosis and allow the oocytes to transition from germinal vesicle and progress to metaphase II [2].
2. Cytoplasmic maturation: Allow for morphological, functional and biochemical changes to take place in the cytoplasm of the oocyte, a process which is necessary for fertilisation and early embryonic development [2].

5.2 - *In Vitro* Fertilisation

In vitro fertilisation has been a source of interest for almost 80 years. Early (largely unsuccessful) attempts with rabbits were reported in the 1930s, however, it wasn't until the 1970s that the technology took off, when, in 1978, the first human IVF baby was born in the United Kingdom. Following this, the first successful calf born from IVF in bovine was reported in 1982 by Benjamin Brackett and colleagues, a research team based in Pennsylvania [109]. Since then, the technology has been applied in various domestic livestock including lambs (1986) [113], pigs (1986) [113], goats (1985) [114] and foals (1990) [115]. Most recently, in 2015, the first puppies born as a product of IVF was reported by a team of scientists at Cornell University [116].

5.3 - *In Vitro* Culture

A major source of economic loss in cattle farming is early embryonic death. In fact, it has been shown that that highest prevalence of embryonic death occurs prior to day 16, especially during blastocyst development between days 1 and 8 in lactating dairy cows [117]. Hence, proper IVC protocols are imperative since embryos are extremely sensitive and vulnerable during this time. Moreover, some evidence suggests that calf oocytes are less robust and tolerant to suboptimal handling and culture conditions than mature animals, underlining the requirement for strict provisions [15]. However, *in vitro* culture does not appear to be the limiting factor inhibiting development, since studies culturing presumptive zygotes (produced from IVM and

IVF) *in vivo* also found heifers to produce significantly lower blastocyst rates compared to mature cows (25.2% vs. 53.1% respectively) [118]. In cattle, IVC usually lasts for seven days, with cleavage rates checked after 48 hours, and blastocyst development rates checked on day seven. Following this, embryos can either be frozen for later use, analysed in a laboratory for scientific purposes, or transferred to a recipient using embryo transfer (ET).

6.0 - From Oocyte to Embryo: Fertilisation, Activation and Block to Polyspermy

Fertilisation is the union of the secondary oocyte and spermatozoon, which, *in vivo*, typically occurs in the ampulla of the oviduct in cattle and involves several steps. It is the culminating event of mammalian reproduction in which two haploid cells, the oocyte and sperm, fuse to give rise to a new genetically distinct, diploid organism [119]. The result of fertilisation is the production of a zygote, or single-cell fertilised egg which will eventually give rise to the embryo. The first step in fertilisation is recognition, in that the sperm must recognise the oocyte of being from the same species in order for fusion to occur. Following fusion, and binding of the spermatozoon to the zona pellucida, the acrosome reaction in the sperm head will occur which will allow the sperm to penetrate the zona and fuse the oolemma.

6.1 - Recognition

Recognition is the first step of fertilisation, which, with adhesion, is believed to be mediated by species-specific complementary molecules. However, the exact molecular basis for this remains unknown and controversial [120]. After insemination, molecules on the sperm head are modified and sperm acquire the ability to fertilise the egg within the female reproductive tract in a process known as capacitation [120]. Once capacitated, sperm will swim to the oocyte and initially bind with glycoproteins, which make up the zona pellucida. These glycoproteins are known as zona pellucida sperm-binding proteins 1 to 4 (ZP1- ZP4). The expression profiles of these proteins are species-specific, for example: mice possess ZP1-ZP3, humans possess ZP1-ZP4, while bovine and porcine express ZP2, ZP3 and ZP4 [121]. In mice, ZP3 is known to be one of the main sperm-receptors, however other factors are known to play a role since replacing ZP3 on mouse oocytes with human ZP3 fail to bind human sperm [122]. An alternative theory is

that for sperm-binding to occur the ZPs collectively present a three-dimensional binding site on the zona. ZP2 is known to cleave following fertilisation due to *ovastacin*, a protease component of cortical granules, which could play a role in preventing polyspermy [123]. This theory is supported by the fact that 2-cell embryos lacking a cleavable ZP2 still bind sperm, while wild-type embryos do not [124]. The exact mechanism is still unknown, and even less is known about the system present in cattle. However, ZPs appear to be relatively well-conserved among species, presenting the possibility that the mechanism present in mice would be conserved or similar in cattle [122, 125].

6.2 - Acrosome Reaction

Binding with the zona pellucida triggers the acrosome reaction in the sperm head. The reaction releases specialised hydrolytic and proteolytic enzymes via exocytosis, which help the sperm penetrate through the zona, and promote plasma membrane fusion [125]. ZP penetration is believed to be a product of both sperm motility and enzymatic hydrolysis. *Acrosin*, an acrosomal serine protease, is believed to be an integral enzyme responsible for zona penetration [126]. However, doubt has been cast on the role of *acrosin*, since although knock-out mouse sperm exhibit delays in both the dispersal acrosome proteins and ZP penetration, they successfully penetrate and fertilise oocytes all the same [127, 128]. This suggests, other proteins play a significant role in the process or that there is a compensation mechanism in place for $Acr^{-/-}$ mice to be fertile [125].

6.3 - Fusion

Although, still poorly understood, two proteins in particular have garnered a lot of attention recently in the fusion process: *Izumo1* on the sperm and its counterpart on the oocyte, *Juno* [129, 130]. *Izumo1* is named for a Japanese shrine dedicated to marriage, while *Juno* is named after the Roman goddess of fertility and marriage. *Izumo1* is redistributed on capacitated sperm and knock-out male mice (but not females) are infertile since their sperm fail to bind to the oocyte [129]. In 2014, folate receptor 4 (*Folr4*) was identified as the *Izumo1* receptor on the oocyte and consequently renamed *Juno* [130]. *Juno* knock-out female (but not male) mice were

shown to be infertile despite being healthy and showing normal mating-behaviours [130]. Furthermore, *Juno* is believed to play a key role in preventing polyspermy, in that membrane-expression is quickly lost after fertilisation occurs [130]. With these recent findings in mice, it remains to be seen if the same mechanism is conserved in cattle. However, initial research indicates that the *Izumo1-Juno* interaction is conserved among all mammalian species, including distant relatives like marsupials [130].

6.4 - Oocyte Activation

Once sperm fuse with the oolemma, a series of rapid changes occur in the oocyte, in a process collectively known as oocyte activation. Activation is arguably one of the most important steps in fertilisation, since it is responsible for many important changes, including:

- Cortical granule exocytosis
- Meiotic resumption
- Extrusion of the second polar body
- Formation pronuclei
- Recruitment of mRNA
- Establishing a block to polyspermy

Oocyte activation in cattle is triggered by repetitive oscillations in the intracellular concentration of free Ca^{2+} ions relinquished from the endoplasmic reticulum. These wave-like pulses of calcium, trigger a cascade of events, such as protein kinase activation which eventually result in the destruction of cyclin B and down regulation of maturation-promoting factor (MPF).

6.5 - Calcium Channel Signalling, Down-regulation of MPF and Meiotic Resumption

Once the sperm has fused with the oocyte, there is still controversy over what happens next. One hypothesis is that the sperm releases an unknown soluble factor (generically known as sperm-oocyte activation factor (SOAF)) into the cytoplasm, which triggers activation. While others argue that the the sperm head incorporates or activates enzymes into/on the phospholipid bilayer itself. There is support for both arguments, with activation of a GPCR pathway leading to activation [131, 132], as well as injection of crude sperm extracts causing meiotic resumption in

the porcine model [133]. One molecule as a possible SOAF candidate that has garnered a lot of attention recently is Phospholipase C Zeta (PLCζ) [134, 135]. PLCζ is thought to activate the phosphoinositide pathway by interacting with the membrane-protein phosphatidylinositol 4,5-bisphosphate (PIP_2) and catalysing it into two secondary messengers: inositol 1,4,5-trisphosphate (IP_3), which is released into the cytosol, and diacylglycerol (DAG), which remains membrane-bound [136]. DAG plays a role in multiple signalling pathways, including protein kinase C (PKC), while IP_3 directly mediates Ca^{2+} release by binding to its receptor, IP_3R, a tetrameric ligand-gated ion channel on the endoplasmic reticulum [136]. Although this channel is well known to play a crucial role in the mouse, questions still exist over its functionality in bovine oocytes. For example, injection of an IP_3R-1 (a specific isoform) blocking antibody only prevented calcium release in 33% of oocytes tested [137], and some authors have suggested ryanodine receptor (RyR), which is known to be functional in bovine, may also play a role [138, 139].

Once released from the ER, intracellular levels of Ca^{2+} rise and fall in an oscillatory manner, in a species-dependant manner [136, 140]. In bovine, these oscillations occur every 19-20 minutes, beginning shortly after sperm-oocyte fusion and terminate as the first mitotic cell-cycle approaches [137]. Concurrently, DAG activates protein kinase C, which is active in several signal transduction pathways. Collectively, the activation of PKC with the calcium oscillations causes cyclin-B, a regulatory subunit of MPF to be broken down and disassociate from cyclin-dependant kinase 1 (CDK1). The union of CDK1 and cyclin-B, known as MPF, keeps the oocyte arrested in metaphase II. Therefore, the breakdown of MPF permits meiotic resumption in the cell and cortical granule release.

6.6 - Pronuclear Formation

Once the sperm head binds with the oolemma, the genetic contents will de-condense and two pronuclei will form, one maternal, and one paternal. The presence of two pronuclei are important markers that can be used to assess proper oocyte activation and absence of polyspermy *in vitro* [141]. Glutathione (r-glutamylcysteinylglycine; GSH), which is produced by the oocyte, is known to play an important role in the formation of the male pronucleus by breaking down

disulphide bonds in the sperm nucleus [142]. For example, GSH production inhibition has been shown to be deleterious to pronuclear formation [142], while culturing oocytes with cysteamine to promote glutathione production, or GSH itself, has been shown to be advantageous [143, 144]. Furthermore, the concentration of GSH was found to increase during oocyte maturation, and correlates with cytoplasmic maturation [143]. Finally, GSH has been found to be important in sperm, with sperm with a non-function glutathione-S-transferase exhibiting impaired motility, acrosome reaction and ability to fertilise oocytes [145].

6.7 - Cortical Granule Exocytosis and Block to Polyspermy

Once fertilisation has occurred, biochemical changes occur in both the oolemma and zona pellucida, making the oocyte unreceptive to additional sperm. This step is critical for reducing the chance of producing non-viable polyploid embryos in an event known as polyspermy. Collectively, these barricades in the oolemma and zona pellucida are known as the block to polyspermy [146]. There are two essential blocks to polyspermy, an oolemma-based fast block and a zona pellucida-based slow block. It has been found that some species tend to favour one block over another, based on the number of sperm that can be found in the perivitelline space of mono-spermic zygotes [146]. In cattle, however, evidence suggest that both blocks play a significant role in preventing polyspermy [146, 147]. This block to polyspermy is triggered by the cortical reaction, which occurs shortly after maturation. Cortical granules, filled with galactosyl-rich glycoconjugates and carbohydrates covalently linked to other types of molecules such as peptidoglycans or lipopolysaccharides, relocate from the cortex to just beneath the plasma membrane during oocyte maturation [148, 149]. This migration is typically complete by the M1 stage, until penetration, when the cortical reaction occurs and granules are released into the perivitelline space, where they are believed to interact with the zona pellucida to activate the polyspermy block [149].

6.7.1 - Membrane Block to Polyspermy

The fast-block to polyspermy is oolemma-based. In broadcast-spawning frogs and invertebrates, the fast-block to polyspermy is extremely quick (a few seconds), and is due to a

depolarisation of the oolemma after sperm penetration [150]. However, in mammals, the block occurs over a longer timeframe (30-45 minutes), and a significant membrane depolarisation has not been observed in several model mammalian species [146, 151-154]. Therefore, despite being described over 60 years ago, the exact mechanism behind this block has remained a mystery [146]. However, with the discovery of *Juno*, it was noted that the sperm receptor's expression is shed quickly after fertilisation and is lost completely in the correct timeframe, with levels significantly lower at the telophase stage and lost at pronuclear formation (**See Figure 2.7**) [130]. Furthermore, in parthenogenically- or ICSI-activated oocytes, expression is not lost, suggesting that *Juno* provides a possible mechanism for the membrane-block in mammals [130].

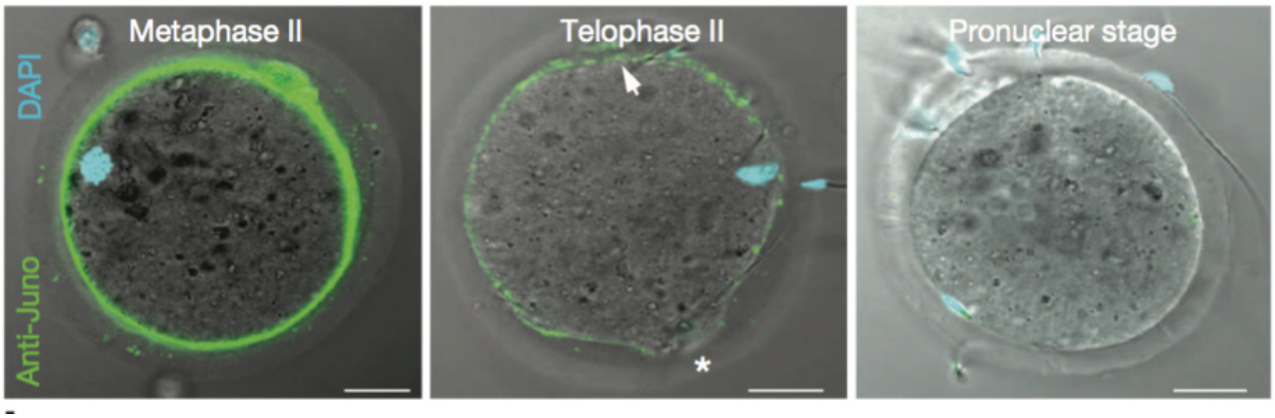


Figure 2.7: Juno Expression Following Fertilisation in a Murine Oocyte. Expression of *Juno* of the cell-surface is rapidly shed after fertilisation. *Juno* (in green) is expressed strongly in MII oocytes, but is barely visible in TII oocytes, and undetectable on pronuclear-stage zygotes. The arrow and asterisk indicate sites for the first and second polar body extrusion, respectively. Chromosomes are not within the plane of focus. Figure adapted from Bianchi and colleagues 2014 [130].

6.7.2 - Zona Pellucida Block to Polyspermy

The slow-block to polyspermy is zona pellucida-based and occurs relatively slowly (>1 hour) after fertilisation. This block is due to a hardening of the zona caused by the action of cortical granule enzymes after egg activation and is thought to be caused by the modification of ZP2 [119, 124]. Proteases within the CGs are thought to modify ZP2 into an inactive form,

altering the three-dimentional topography of the zona, preventing the binding of future additional sperm [119, 155]. The modification of ZP2 into its inactive form can be detected in the laboratory by a change in the electrophoretic mobility [155]. Another zona pellucida protein, ZP3, is also known to be modified after fertilisation, suggesting both ZP2 and ZP3 play a role in the block to polyspermy [155].

7.0 - Prepubertal Reproduction: Interest & Importance

Selective breeding, also known as artificial selection, is a phenomenon where humans select which animals to breed in order to proliferate a desirable trait. Hence, overtime, cattle can be bred in order to produce more milk, be more resistant to certain diseases, or have a better temperament. For example, in broiler chickens, it has been found that their average body weight at day 56, increased from 905g in 1957, to over 4.2 kg in 2005 [156]. The interval between generations is a major factor affecting the rate at which progress can be made, hence it is desirable to breed the best animals at the youngest age possible.

7.1 - Accelerated Genetic Gain

With the desire to have the best, and most profitable animals in their herd, there is continued pressure to increase the rate of genetic gain over time. This concept is presented in the following section, starting with a historical perspective and moving onto modern-day aspects.

7.1.1 - Historical Perspectives

The first record of livestock and poultry domestication is around 10 000 years ago [157]. This phenomenon played a critical role in human evolution and history since livestock allow for the conversion of non-edible materials such as grass into high-quality food suitable for humans in the form of meat, eggs and milk [157]. Cattle were also domesticated as a source of power for farming activities. Since then, selection of livestock for desirable traits has occurred continuously [157]. Charles Darwin himself noted that ‘our oldest domestic animals are still capable of rapid improvement or modification’, a statement which holds true to this day. For almost any trait, genetic variation still exists in modern livestock [157, 158].

The effects of selective breeding is evident across the agricultural sector. Most contemporary breeds of cattle are highly specialised in nature, with some breeds used exclusively for beef or dairy [159]. For example, in two studies comparing broiler chickens from 1957 to those in 2001, it was found that modern broilers reach market-weight in 1/3 the amount of time to that taken by 1957 strains, all while consuming less feed [160, 161].

Historically, desirable traits such as docility and temperament were selected for based on a visual examination. However, over the years, pedigree information was used and selection criteria shifted and became focused on production-related traits in order to maximise production. In recent years, however, many breeding programs have emphasised the importance of health, considering many traits including fertility.

Genetic gain through selective breeding has had a fundamental impact on the status of dairy farming across the world. This is evidenced by the fact that, in the United States, the number of cows on farms has steadily dropped annually since the 1950s, while the total milk production has steadily increased [162]. Hence, milk production per cow has increased, in part due to selective breeding and better management practices.

7.1.2 - Marker-Assisted Selection

The use of oocyte donor genomic analysis at a young age, combined with male selection, has fundamentally changed the management of livestock to improve future generations of dairy cattle [163]. Some traits are relatively easy to select for, such as milk production in cows. However, some traits are extremely difficult to select for based on a visual exam, for example, sex-specific traits such as milk production in bulls. The recent advent of genomic selection has made the selection of traits, especially obscure or difficult to screen for traits, much easier to select. Selection can now be made much more efficiently by analysing various genomic markers such as single-nucleotide polymorphisms (SNPs).

Specifically, genomic selection has allowed us to:

1. Assess the difference between the genetic value of the offspring compared to the mean parental value, without testing the progeny.

2. Evaluate more selection candidates than possible with progeny testing.
3. Efficiently select animals with traits that have low heritability [164].

7.1.3 - Formula

In order to calculate the rate of genetic gain, the following formula can be used.

$$\frac{\Delta G}{t} = \frac{h^2 x \Delta \mathrm{S}}{[GI]}$$

Where:

$\frac{\Delta G}{t}$ is the rate of genetic gain

h^2 is the heritability

$\Delta \mathrm{S}$ is the selection differential

$[GI]$ is the generation interval

Producers are always looking to maximise the rate of genetic gain. In order to do this, the generation interval plays an important role since heritability remains constant. Therefore, the larger the selection differential the better, and the shorter the generation interval the better.

7.2 - Typical Breeding Practices in the Industry

Although large variations exist among individuals and different breeds, most Holstein heifers reach puberty around 12 months of age. However, to avoid problems with dystocia at calving, animals are typically not bred until they reach sexual maturity at around 14-15 months

of age [165]. Therefore, heifers will generally give birth to their first calf at around 2 years of age, since gestation in Holstein cattle is nine months (283 days). Thereafter, although different farms have different management strategies, animals are bred once a year. Therefore, an animal will have her second calf at 3 years of age, and her third at 4 years of age, etc. With this in mind, it becomes clear just how much of an impact breeding animals at a pre-pubertal age could have on the industry. With ovum-pick up technologies, it is quite reasonable to be able to aspirate 30+ oocytes from a 2 month old calf. Therefore, by using embryo-transfer and recipient animals, multiple offspring could be born from an animal before they are 1 year of age, and sexually mature (**See Figure 2.8**).

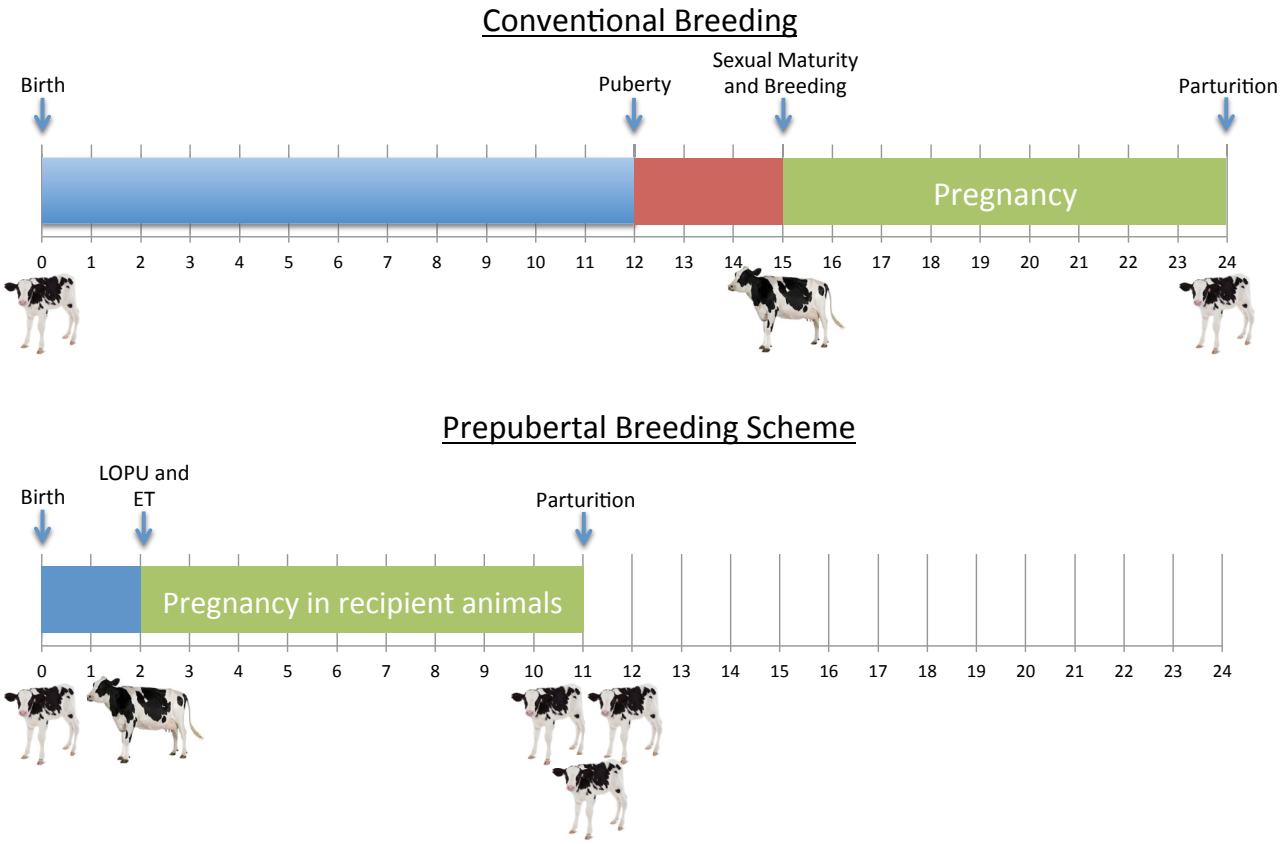


Figure 2.8: Timeline Between a Conventional and a Prepubertal Breeding Scheme. In a conventional breeding system, a Holstein calf will reach puberty around 12 months of age, and sexual maturity around 15 months, when she will be bred. Gestation in Holsteins is 9 months, hence, she is expected to give birth to her first calf at 24 months of age. In a prepubertal scheme, an animal can be bred using

LOPU/IVEP/ET at 2 months of age. Hence, multiple progeny can be born from an animal, 13 months before a single calf could be born using a conventional scheme.

7.3 - Present-day Considerations

With accurate genomic selection tools now available, genetically valuable animals can be identified at birth. The availability of modern *in vitro* embryo production techniques has placed an impetus on breeding animals as young as possible in order to maximise genetic gain. Using prepubertal donors has the potential to shorten the generation interval by at least 1 year [107]. The collection of oocytes and generation of young via IVEP and ET from prepubertal animals has the potential to dramatically improve rates of genetic gain by reducing the interval between generations [33, 78]. In 1995, Lohuis demonstrated in cattle that by using immature animals between 1-5 months of age, the annual rate of genetic gain for milk yield is predicted to increase by 22% over a 25 year period versus conventional progeny testing schemes [166].

With an efficient, repeatable technique to recover oocytes from the large ovarian pool present at young ages, genetically superior, unique, expensive or endangered animals could be rapidly proliferated [78]. LOPU is an effective and minimally invasive technique that permits repeatable oocyte collection [81]. Furthermore, it also provides a faster mechanism for the proliferation of lines from a particularly valuable genotype such as a transgenic founder animal [167].

8.0 - The Problem: Reproduction Before Puberty

LOPU combined with IVEP & ET would facilitate the breeding of prepubertal cattle. All three of these technologies are well developed and widely used, and therefore the infrastructure required in order to breed animals at such a young age is available. However, literature has consistently showed that although large numbers of COCs can be recovered from young animals, *in vitro* development of these oocytes lead to poor cleavage and blastocyst rates [11, 12, 34]. This topic will be explored in the following section.

8.1 - Historical Perspectives

Although animals cannot be bred naturally until after puberty, the prepubertal bovine ovary has been the source of attraction for a considerable amount of time. This is partially due to the fact that the pool of growing follicles is much larger, especially between 50 and 120 days of age, compared to adult cows [35]. In fact, calf ovaries are known to have larger pools of follicles of various sizes present on their surface than ovaries of adult cows [35, 163].

Despite identifying the potential of using prepubertal animals to increase rates of genetic gain early on, initial attempts to use MOET in calves predominately failed. In 1971, Seidel and colleagues super-ovulated prepubertal heifers aged 1 to 6 months, performed artificial insemination and collected embryos shortly thereafter, and found that the majority of ova collected failed to cleave further *in vitro* [168]. They also noted that embryos already possessing 4 blastomeres upon retrieval failed to cleave normally thereafter, and concluded that the prepubertal reproductive tract was detrimental and not conducive to normal early embryo development [51, 168]. In another similar study, Onuma and Foote noted that of 184 ova collected and cultured, none cleaved more than 2.5 times *in vitro* [169]. However, these studies were done in 1971 and 1969, respectively, when IVEP technology was in its infancy, and oocytes from adult cows rarely yielded superior results [169]. Further attempts in the 1990s, using conventional synchronisation, super-ovulation and insemination regimes, yielded similar results to previous trials and found that despite calves produced many follicles, few ovulated (means of 10.9 and 3.1, respectively), and of the oocytes recovered (mean 0.6 per animal), none were fertilised [170].

Early reports indicated that prepubertal animals could respond to exogenous gonadotropins, with many follicles developing in response, but LH treatment resulted in few ovulations [171, 172]. In fact, Casida and associates are believed to be the first researchers, who, in 1943, demonstrated that treating calves with anterior pituitary extracts would result in follicular development [171, 173]. In his research, Marden found conflicting results in that although most calves responded to FSH, and while most did not respond to LH, two calves in particular did as evidenced by high amounts of luteal tissue in the ovaries [171]. Furthermore, in calves that did respond to LH treatment and ovulated, insemination resulted in low fertilisation rates [172, 174]. And finally, those that were fertilised, resulted in poor development rates both

in vitro [169] and *in vivo* [168]. Therefore, with poor responses to LH, super ovulation regiments and subsequent embryo flushes resulted in low recovery rates and poor embryonic development [168, 169].

Following these poor results, interest was lost and little research was done until the mid 1990s, following increased use of OPU techniques. However, many of these studies also ultimately yielded poor results. Research during this period essentially showed that although OPU in prepubertal animals, resulted in oocytes that can resume meiosis, undergo germinal vesicle breakdown (GVBD) and reach metaphase II [16, 107, 175, 176]. Following IVEP, these oocytes resulted in development rates that were consistently lower than in mature control animals [11-13, 16, 176]. In 1995, Revel and associates worked with 3 month-old heifer calves, and found that although there was no significant difference in the fertilisation and cleavage rates between young and adult cattle. Prepubertal animals failed to produce similar blastocyst rates [34].

8.2 - Present-Day Considerations

Since these early attempts, advancements in marker-assisted selection, the improvement of IVEP systems and development of efficient laparoscopic procedures to gather oocytes resulted in renewed interest and the potential to circumvent these initial short-falls [177]. However, problems still exist. A reduced developmental competence during the prepubertal period has been noted in many species, including in the murine [178], ovine [179], caprine [180] and swine models [181], and is not limited to cattle. It has now been shown that calves respond well to FSH stimulation and produce many follicles, in some cases producing significantly more than mature cattle controls [163]. Consequently, young donors typically give more COCs and produce more 2-cell cleaved embryos [163]. However, oocytes that are recovered are less developmentally competent, with fewer oocytes reaching the blastocyst stage [13, 34, 168]. Despite this, multiple authors have demonstrated that these animals do have potential with a limited number of animals producing viable offspring following embryo transfer [34, 182]. Hence, the current challenge is learning how to prime these oocytes, both *in viv*o inside the follicle and *in vitro* during maturation and culture in order to improve the efficiency of breeding these animals. Furthermore,

prepubertal animals are also excellent negative models for the acquisition of developmental competence, meaning discoveries could lead to better medical treatments for infertility [183, 184].

9.0 - The Potential of Using Prepubertal Animals as Oocyte Donors

Our study was designed to look at several different aspects of reproductive competence in heifer calves, the scope of which is briefly reviewed in the following section.

9.1 - Follicular Responses to Gonadotropin Regimes

Before oocyte-retrieval, animals are typically primed using a combination of exogenous gonadotropins to stimulate the ovary. This gonadotropin priming regiment is known to affect the ovarian response, follicle number and oocyte quality upon collection [97]. Although sometimes mistakenly known as 'superovulatory stimulation', since follicles are punctured prior to ovulation, a better term is 'ovarian stimulation' (OS). The process of OS has three goals:

- Increase follicle size, and, consequently, number of follicles available for aspiration, as well as to improve recovery rates during collection.
- Facilitate the recovery of oocytes at a synchronous stage of maturation.
- Improve developmental competence of the oocytes following retrieval [15, 110].

9.1.1 - Gonadotropins and their use in Prepubertal Animals

Prepubertal animals respond well to exogenous gonadotropin stimulation, and most prepubertal protocol designs have been based on those used for adult animals, using either multiple FSH injections or single injections of compounds with a longer half-life such as pregnant-mare serum gonadotropin (PMSG), also known as equine chorionic gonadotropin (eCG) [110, 185]. Due to the short metabolic half-life of FSH, it is typically re-administered every 12 hours for 3-4 days. Stubbings and associates found that calves had a significantly better follicular response when calves were subjected to multiple (9 injections, 12 hours apart) rather than a single large dose of FSH [185]. In other work, Armstrong found similar results, in that animals subjected to multiple FSH injections (twice daily for 3 days) had better follicular

responses compared to a single 'one-shot' injection [107]. However, combining a single FSH injection with one of PMSG (which has a considerably longer half-life), resulted in a similar ovarian response to multiple injections [107]. This suggests that for purposes of convenience, the marginal improvements in recovery rate found in the multiple injection protocol may not be worth the time invested [110]. However, the gonadotropin priming regime does not appear to significantly affect the recovery rate [107]. Furthermore, with repeated good ovarian responses, it allows follicular aspiration to be performed repeatedly [185]. With prior exposure to sexual steroids or LOPU having negligible effects on the productivity of future LOPU sessions [167].

Sendag and colleagues suggested that FSH appears to be a better alternative to PMSG to maximise the response in tvOPU, and, to date, many studies have used FSH in their hormonal priming protocol before LOPU [97]. The source of FSH does not appear to be important since porcine-derived (Folltropin®) and ovine-derived (Ovagen®) appear to be equally effective in calves, yielding similar ovarian responses when combined with PMSG [107]. It is interesting to note that young calves generally require smaller doses of gonadotropins than adult cows [110, 167]. The reasons for higher sensitivity in calves in unknown; however, it is postulated that it could be due to a lower body mass, and/or a lack of a fully functional intra-ovarian regulatory mechanism, designed to limit ovulation rates [110, 167, 186].

Past studies have suggested that a single-dose of FSH is able to recruit but not sustain development of a follicle cohort [107, 185]. This data seems to be supported by the fact that combining a single-shot of FSH with a low dose of PMSG (which has a longer half-life) can result in a similar ovarian response to a multiple-injection regimes, with the FSH bolus able to recruit a cohort, and the PMSG able to sustain continued development. [107]. It could be possible that PMSG aids in follicle development from its inherent LH activity, which could act synergistically with FSH [107, 187]. It is unknown why a single-dose of FSH is inefficient for ovarian stimulation in calves, since numerous studies have reported effective stimulation when used in adult cows [188-190]. In prepubertal animals, two possibilities are that FSH is metabolically cleared faster in calves, or that endogenous levels of LH are inadequate for synergism with FSH, which may be required for follicular development [107].

9.1.2 - Follicular Responses

The follicular response upon gonadotropin stimulation is important since "*the history of the follicle determines the future of its oocyte*" [191]. In bovine, the ovarian response upon gonadotropin stimulation is widely variable among donors, with some donors routinely producing many follicles, while others produce very few [192]. The same variation has been found to be true with calves [167, 192]. The reasons for this high variability is unknown, however differences in FSH and LH receptors could possibly play a role, as has been postulated in sheep [193].

In early research, it was found that anterior pituitary extract injections can result in follicular development as early as 1 week of age, and that responsiveness remains relatively steady throughout prepubertal life, in that calves 3 weeks of age, exhibit the same response as those 6-8 months of age [51, 171]. Unsurprisingly, it has been shown that although follicles grow in response to gonadotropins, most calves fail to ovulate in response to LH or human chorionic gonadotropin (hCG) [171]. Intriguingly, It was also noted in early studies that 3-week old calves would sometimes ovulate spontaneously, although rarely, when stimulated with FSH but no LH [171]. Armstrong found that the number of follicles present after a three-day FSH treatment protocol was directly correlated with the number of follicles present prior to stimulation [110]. This indicates that is possible to select calves capable of strong responses to FSH, therefore making mean oocytes yields of >50 COCs more frequent [110].

Taneja and associates examined the ovary of 2 month-old animals prior to and after FSH stimulation and found that the mean number of antral follicles present in the ovary increased from 23 to 55 [167]. Similar results were found by Majerus and colleagues, noting lower numbers of follicles and oocytes collected in unstimulated calves [105]. However, Prescisse and colleagues found conflicting results when priming 5 month old animals with eCG, Synchro-mate B and FSH in a decreasing dose; commenting that hormonal priming does not appear to increase the number of follicles present on the ovary, just increases their size [36].

In mature cattle, there is a well-known relationship between the follicular diameter and oocyte competence [191, 194]. For example, in one study, it was found that oocytes from follicles 2-6 mm in diameter gave an average blastocyst rate of 34.3%, while oocytes from

follicles >6 mm in diameter increased rates to 65.9% [195]. This relationship has been the focus of several studies, and it is believed that oocytes acquire developmental competence in follicles around 3 mm in diameter, and this competence does not increase significantly until around 7mm. However, once follicles reach >8 mm in diameter, the developmental competence increases significantly in both untreated and gonadotropin-stimulated cows [191].

A clear understanding of the follicular fluid's properties is important since it may affect the oocyte's competence. Hence, the lower developmental potential in calves may be due to environmental deficiencies *in vivo* before retrieval [34, 95]. In cattle, it has been shown that mean levels of both LH and FSH decrease between 3 and 15 weeks of age [196]. Calf follicular fluid has been shown to contain approximately half the LH concentration compared to cow follicular fluid (2.0 +/- 0.2 ng/mL vs. 4.0 +/- 0.3 ng/mL) [183]. This is in accordance to plasma concentrations of LH, which has also been shown to be lower in younger animals [196]. Furthermore, cow follicular fluid has also been shown to contain approximately twice the FSH content to calf oocytes (12.7 +/- 5.5 ng/mL vs. 6.3 +/- 2.1 ng/mL) [183]. However, this may be a downstream effect of the reduced LH concentrations due to decreased androgen production by thecal cells resulting in a reduction of estradiol [183].

9.2 - Oocytes Quality responses to Gonadotropin Regimes

Preliminary studies by Onuma *et al.* (1970) and Seidel *et al.* (1971) found that even though young calves would respond to exogenous hormones, the developmental capacity of oocytes was limited to early cleavage [168, 197]. Multiple authors noted similar findings in the 1990s [11, 34, 176]. Calf oocytes, whether FSH-treated or non-treated, have a significantly lower developmental competence than those of mature adult cows [34]. However, this is not to say there is no differences among calves, with studies comparing hormonal stimulation vs. non stimulated calves indicating that OS increases the number of usable oocytes, cleavage rate and blastocyst rate [34, 36, 107]. For example, Presicce and colleagues found that stimulated calves produced significantly more morula than unstimulated calves of the same age, and that although they did not produce more oocytes, oocytes that were recovered were of better quality. However, the developmental competence of both groups were much lower than slaughter-house derived

cow-control oocytes, and failed to produce any blastocysts (**See Table 2.5**) [36]. Revel and associates (1995) found that stimulating calves with FSH, resulted in a much higher average COC recovery rate (39% vs 19%), but yielded similar results — with no statistical difference in fertilisation and cleavage (85% vs. 87% and 73% vs. 79% respectively) but a significantly lower blastocyst rate (11% vs. 27%) [34]. However, it should be noted that some papers have found that similar blastocyst rates can be obtained with or without FSH (22% and 19%, respectively) [105].

Table 2.5: Developmental Competence of Oocytes Recovered from Calves at 5 Months of Age.

Treatment	n	Follicles Aspirated	No. of Oocytes		% Developed at Day 7 to		
			Total	Usable (%)	2-16 Cells	Morulae	Blastocysts
Stimulated	5	121	91	84 (92)	24^{A}	10^{A}	0^{A}
Unstimulated	5	101	79	39 (49)	28^{A}	0^{B}	0^{A}
Cow Control	-	-	56	39 (70)	62^{B}	31^{C}	18^{B}

$^{A, B, C}$ Values within columns with different superscripts differ ($P < 0.05$). Table adapted from Presicce *et al.* 1997 [36].

There is a correlation between plasma anti-mullerian hormone (AMH) concentrations and IVEP embryo production rates (antral follicle >2 mm population, COCs retrieved, cultured and blastocysts produced) in both Nelore and Holstein calves [198]. It has also been shown that calves have higher circulating levels of AMH than sexually-mature cycling heifers, and treating cattle with FSH does not influence concentrations of AMH [198]. Thus, it has been suggested that AMH can be used as a helpful marker when selecting animals.

9.3 - Effect of Age

Oocyte quality and consequently, competence, is heavily influenced by the age of the donor female. Although it has been described broadly, the underlying mechanisms of why, remains largely unknown [95]. When hormonally stimulated and subjected to OPU, prepubertal

animals will give more COCs than their adult counterparts. However, the embryo yield reaching the 4-cell, morula and blastocyst stage is significantly reduced in relation to mature animals [95, 163]. The exact reasons for their reduced competence remains poorly understood, although some studies have pointed to differences in the make up and organisation of organelles within the cell, such as the distribution of cortical granules and the population of mitochondria [16, 199]. Furthermore, some studies have indicated differences in enzyme activity, mRNA transcription levels and metabolism of various compounds [95, 199, 200].

Developmental competence increases gradually and sequentially as oocytes increase in size, due to transcriptional activity during follicular and oocyte growth [195]. This is important since calf oocytes have been found to be significantly smaller than those of adult animals, despite originating from follicles of the same size [177]. Bovine oocytes range in size from around 110 μm to 130 μm in diameter [201]. With calf oocytes having a mean diameter of 118.04 μm +/- 1.15 μm, compared to a mean diameter of 122.83 μm +/- 0.74 μm for mature cattle [177]. Similar results have been found in other research, with the authors noting that although the ooplasm is homogenous in appearance, they are significantly smaller in diameter [33]. This is significant, since a small variation in diameter represents a larger variation in volume, and mRNA and protein synthesis required for developmental competence may be impeded in small oocytes [33]. Furthermore, it appears the capacity of bovine oocytes to mature to MII during IVM, is directly proportional to their diameter [202].

Despite an abundance of literature comparing prepubertal and mature cows, there is significantly less literature comparing different ages within the prepubertal age group, especially prior to 5 months of age. It has been shown that population of antral follicles present on the ovary rapidly increases during the first few weeks after birth, reaching peak values between 8 and 12 weeks of age [35, 38]. Mermillod and colleagues examined the reproductive competence of newly born animals, 8 to 15 days of age. There was a large variation among unstimulated calves, producing between 0 and 17 oocytes, with an average of 3 oocytes. This is in contrast to those that responded to FSH treatment which gave an average of 26.4 oocytes. However, it was found that only 5 of the 7 animals tested, responded to treatment. After IVEP, of all the oocytes

recovered (186), only one (0.5%) reached the blastocyst stage. Ironically, coming from an unstimulated animal, despite cleavage rate being significantly lower [203].

Tervit and associates worked with slightly older Hereford-Freisians and compared 4, 10 and 18 week old animals (**See Table 2.6**). In their work, they found that although there was no significant difference in the number of follicles, oocyte competence steadily grew with the predicted transferable blastocyst yield increasing from 0.3, 1.1 to 1.3 for the three age groups respectively [204]. Following these age groups, a recent research paper has outlined the reproductive animals aged 5 months to puberty. In their research, Landry *et al*. found that although the mean number of follicles aspirated steadily declined in the various age groups, the mean blastocyst rate steadily increased (**See Table 2.7**) [163]. It is not until around 11 months of age that oocytes from unstimulated heifers begin to show similar *in-vitro* developmental competence as compared to adult cows [36].

Table 2.6: Follicular and *In Vitro* Development Responses from Calves at 4, 10 and 18 Weeks of Age.

Calf Age (Weeks)	No (±SE) follicles aspirated	Percentage (±SE) of oocytes			
		Recovered per Animal	Cleaved	Morulae/ Blastocysts	Transferable Blastocysts
4	25.6 ± 6.3	68.1 ± 4.9	39.4 ± 5.6	10.8 ± 3.2	1.8 ± 1.2
10	38.4 ± 6.9	35.1 ± 4.1	49.1 ± 6.7	18.7 ± 4.3	6.3 ± 2.3
18	38.9 ± 5.9	45.7 ± 4.3	62.2 ± 5.6	18.5 ± 4.1	8.5 ± 2.7
	NS	P < 0.001	P < 0.05	NS	P=0.05

Table adapted from Tervit *et al.* 1997 [204].

Table 2.7: Follicular and *In Vitro* Development Responses from Heifers Aged Between 5 and 16 Months of Age.

Donor Age (Months)	n	Mean Aspirated Follciles (±SD)	Mean Blastocyst Rate (±SD)
5	22	25.74 ± 11.77	31.44 ± 20.22
6	57	23.70 ± 11.58	35.64 ± 22.30
7	102	21.71 ± 12.25	33.93 ± 22.01
8	131	17.23 ± 9.56	37.93 ± 22.90
9	189	16.15 ± 8.54	31.99 ± 23.92
10	190	15.11 ± 7.74	33.47 ± 22.80
11	131	13.11 ± 6.50	42.03 ± 23.37
12	103	12.32 ± 7.47	41.18 ± 22.79
16-18	106	13.30 ± 8.78	48.03 ± 28.73

Table adapted from Landry *et al.* 2016 [163].

9.4 - Polyspermy

During *in vitro* fertilisation, should an oocyte fail to initiate its block to polyspermy efficiently, an oocyte can be fertilised by multiple sperm, a phenomenon known as polyspermy,. Due to issues with polyploidy, polyspermy typically deems a zygote non-viable. The reorganisation and migration of organelles occurs during oopmasmic maturation and is known to be an important factor effecting the polyspermy blocking mechanisms present in the oocyte. Damiani *et al.* compared migration in calf and cow oocytes. In their work, they found that cortical granules did not migrate as efficient in calf oocytes, and that only 19% (17/90) of calf oocytes exhibited migration, while 71% (83/117) migrated in cow oocytes. This is significant, since 81% (73/90) of calf oocytes still possessed clusters of CGs following IVM, an important process in order to block polyspermy [16]. Furthermore, in 70% (19/27) of calf oocytes tested, CG migration was delayed, which was significantly larger than the 28% (7/25) of cow oocytes [16].

Following IVF, fertilisation rates (as measured by sperm penetration) appears to be normal, with no significant differences among prepubertal and adult animals [16, 33, 175]. However, the incidence of abnormally fertilised zygotes has been found to be significantly

higher in calf oocytes, compared to cow oocytes, 16% vs 7%, respectively (**See Table 2.8**) [16, 33]. In their study, Damiani and colleagues deemed abnormal fertilisation, as lack of sperm aster formation, asynchronous pronuclei development, and extrusion of maternal chromatin [16]. Furthermore, an IVF coincubation period of 8 hours showed that sperm head decondensation, aster formation, development of the female pronucleus and pronuclei migration all appear to be normal in calf oocytes [205, 206].

Table 2.8: The Development of Calf and Cow Oocytes Following *In Vitro* Maturation, Fertilisation and Culture.

Donor	Maturation (%)			Fertilisation (%)					Development (%)	
	N	MII	ABN	N	Normal	Polyspermic	Unfertilized	ABN	N	Blastocysts
Calf	65	75	25	299	51	25	8	16^{A}	615	6^{A}
Cow	143	86	14	360	58	26	9	7^{B}	1532	33^{B}

Data pooled from 13 replicates. MII is metaphase II oocytes at the end of 24 hours maturation. ABN is abnormal. $^{A, B}$ Values within the same column differ significantly ($P < 0.05$). Table adapted from Damiani *et al.* 1995 [205].

9.5 - Response to Activation

Parthenogenesis is a form of asexual reproduction, occurring naturally in many species across multiple orders. Although it has not been reported to occur naturally in mammals, parthenogenetic oocyte activation (PA) can be efficiently induced *in vitro*, using various stimuli [207]. In recent years, it has gained popularity as an experimental technique in order to produce embryos to study early embryonic development in the laboratory, partially due to its potential role in improving ICSI and SCNT. Various stimuli can be used and parthenogenetic oocyte activation has been induced in bovine using an electrical pulse, ethanol, calcium ionophores, cycloheximide, IP_3, and strontium [207]. A significant advantage of using PA over IVF is that it essentially rules out any possible sire-related effects [16]. In accordance with IVF results, Damiani and colleagues found that calf oocytes, when subjected to PA, had significantly lower cleavage and blastocysts than adult counterparts (**See Table 2.9**) [16].

Table 2.9: Induction of Parthenogenic Development of Calf and Cow Oocytes with Ionomycin and DMAP.

Donor	N	Cleaved	Blastocysts
Calf	92	40[a]	10[A]
Cow	202	56[b]	31[B]

Data is pooled from seven replicates. [A, B] Values within the same column differ significantly ($P < 0.05$). Table adapted from Damiani *et al.* 1996 [16].

9.6 - Response to Extended IVM

Maturation is a critical component of oocyte development, since it is during this time that the nucleus resumes meiosis, and cytoplasmic changes occur including reorganisation of cortical granules and mitochondria [15]. This is of interest since cytoplasmic deficiencies during maturation can lead to:

1. Sperm penetration and decondensation failure
2. Inability to form normal male pronuclei
3. Failure of polyspermy block
4. Early cleavage arrest
5. Failure to progress past embryonic genome activation
6. Developmental failure leading to a reduced blastocyst rate [15].

Finally, it has been suggested that cellular differences during the IVM period may account for the lower developmental competence in prepubertal animals [16, 177]. Hence, a potential need for IVM to be extended for a prolonged time [33].

Kajihara and colleagues compared IVM treatments of various lengths, on the developmental competence of oocytes obtained from calves (**See Table 2.10**). In their work, they found that although the cleavage rate was uniform across all treatments, blastocyst development was optimal when matured for 22 hours [11]. However, the maturation rate, i.e. the number of

oocytes reaching MII following IVM, has not been found to be different among cow and calf oocytes [16, 33, 176].

Table 2.10: The Influence of the Oocyte Maturation Time on *In Vitro* Development of Follicular Oocytes Collected from Heifer Calves.

Maturation Length	Cleavage Rate (%)	Morula Rate (%)	Blastocyst Rate (%)
22 h	48.1	28.4[A]	12.0[A]
24 h	48.2	16.5[B]	4.3[B]
26 h	48.8	23.5[A, B]	8.6[A, B]

[A, B] Values within the same column differ significantly ($P < 0.05$). Table adapted from Kajihara *et al.* 1991 [11].

Moreover, it has been noted that there are differences in the metabolism of calf and cow oocytes during maturation [177]. Gandolfi and colleagues found that in both cow and calf oocytes, glucose metabolism remains low and relatively constant throughout maturation [177]. The oxidative metabolism of both pyruvate and glutamate were significantly different among the two age groups, despite both groups using the two metabolites during the IVM period [177]. This difference could partially explain the differences in the arrangement of cytoplasmic structures [177]. Although it is not clear, whether differences in the developmental competence of calf oocytes is due to differences in the nucleus, cytoplasm, or possibly a combination of both [33]. Some authors have suggested that differences in the cytoplasm may be to blame [16]. This is evidenced by the fact that transferring the nuclei of adult oocytes into enucleated calf oocytes resulted in similar low development rates [208]. In addition to the failure of CG migration mentioned earlier (See Section 9.4 — Polyspermy), Damiani *et al.* also noted that other cytoplasmic organelles such as mitochondria, as well as lipids and membrane-bound vesicles, exhibited delayed migration in 44% (12/27) of calf oocytes. Furthermore, this would occasionally lead to “organelle-free domains”, a phenomenon which was not observed in oocytes sourced from adult donors [16].

9.7 - Cleavage and Development up to Block

When subjected to ovum pick-up, the increased number of follicles in young, compared to mature animals has been shown yield higher number of COCs following aspiration [163]. In other words, the recovery rate is comparable between the two age groups. Furthermore, there is no statistical difference in fertilisation and cleavage rates, hence more cleaved zygotes can be produced using prepubertal donors [34, 163]. Additionally, the maturation scheme used, either *in vitro* or *in vivo*, does not appear to affect cleavage rates [107]. However, cell-division does appear to be delayed in calf oocytes, as evidenced by the low proportion of 4-cell embryos 46 hours after insemination [209]. This is a significant finding since early-dividing embryos have been found to be more developmentally competent than late-cleaving embryos [194, 210]. For example, Majerus and associates found that on Day 3 of culture, the mean rate of embryos at the 5-8 cell stage from heifer-derived oocytes were 49% (40/81), while embryos from cow-derived oocytes were at 67% (35/52) [210]. However, it should be noted that heifers aged 6-8 months were used in this study.

Concerning the timing of developmental arrest, there is not an abundance of literature for young calves. In 6-8 month old heifers, however, it has been found that 67% (40/60) of the 74% of cleaved embryos that failed to reach the blastocyst stage arrested development between the 2-cell and 8-cell stage. This is significantly higher than the 18% (5/28) that arrested their development during those two-cell cycles in cow-derived embryos [210].

9.8 - Development to Blastocyst

Blastocyst development is known to be significantly lower than what is achieved with adult cows [2, 16, 34, 36, 167]. With the majority of immature bovine oocytes failing to reach the blastocyst stage *in vitro*, it is believed that the intrinsic quality of the oocyte is the key factor affecting embryo development rates [194]. However, the limited number of blastocysts produced from calves, appear to be normal — they have a visible inner cell mass, are the same size of cow-derived blastocysts, and reach the blastocyst stage at the same time [34, 211]. Prepubertal-derived blastocysts also had mRNA transcription levels comparable to adult-derived embryos for

a variety of genes critical for embryonic development and survival [211]. This is of interest since, proper embryonic genome activation and ensuing mRNA transcription patterns is known to be an important factor affecting early embryonic survival [212].

Armstrong and colleagues found no significant difference in blastocyst rate when comparing oocytes matured *in vivo* or *in vitro*, although it should be noted that IVM yielded slightly better rates [107]. It has also been found that large follicles in prepubertal animals yielded significantly more blastocysts than small follicles (16% vs. 6%). However, both remained significantly lower than blastocyst rates from cow oocytes (27%) [34]. Despite this, calf and cow oocytes from follicles >8 mm had similar blastocyst rates, suggesting that not only is developmental competence related to follicle size, but that the pre-maturation process occurring inside the follicle prior to aspiration is important in the acquisition of developmental competence [209].

Majerus and colleagues compared the blastocysts of heifer-derived (6-8 months) and mature cow embryos. In their work, they compared the inner cell mass (ICM), and the trophectoderm (TE). The ICM differentiates into the all tissues of the future foetus, while the TE develops into the placenta and extra embryonic membranes [213]. In recent years, several studies have compared the number of blastomeres present in the IVM and the TE, as it has been suggested to be a indicator of embryo quality and viability [214]. However, Majerus and associates found no difference in the TE/ICM distribution between hatched and unhatched blastocysts from cows and heifers alike [210]. Another indicator of blastocyst quality is the total number of blastomeres present. Majerus and colleagues found a slight difference in this between heifers and mature cattle, with day 8 blastocysts possessing 89 ± 20 cells for heifers, and 100 ± 30 cells for cows [210]. Finally, in their study, they looked at the accumulation of triglycerides in blastocysts. Triglycerides are one of the main lipids synthesised in the embryos, as they are a major energy store. However, once again, heifer-derived and cow-derived embryos were found to be very similar in lipid metabolism, with day 8 blastocysts accumulating 64 ± 15 ng vs. 65 ± 6 ng respectively per embryo [210].

9.9 - Pregnancy and Transfer

Established pregnancies and live births with full-term offspring have been reported by multiple authors using calf-derived oocytes following IVEP and ET [11, 34, 110, 175]. The average gestation length and birth weight of calves was also found to be the same as what is reported in adult cows [167]. However, upon embryo transfer of calf-derived embryos, Revel and associates found limited development in utero, and lower pregnancy rates (4% vs. 38%) [34]. Multiple authors have reported similar results [167, 215]. Interestingly, animals that have more follicles prior to stimulation gave oocytes which resulted in a higher pregnancy rate, after IVEP and ET [167].

Armstrong and colleagues performed embryo transfer with prepubertal-derived embryos on three recipient females, and found that two of them became pregnant [175]. Of these two animals that became pregnant, one suffered a miscarriage, while the other gave birth to a full-term female calf [175]. Revel and colleagues also assessed pregnancy rates following transfer. They found that untreated calves failed to produce any calves, and that in FSH-treated calves, although pregnancy rates were similar on day 21, 83% of the recipients lost their pregnancy, compared to 41% in the control cow group (**See Table 2.11**) [34].

Table 2.11: *In Vivo* Survival After Transfer of Blastocysts produced *In Vitro* from Calf and Cow Oocytes.

Donor	Recipients (Blastocysts)	Pregnancies		
		Day 21 (%)	Confirmed >6 months (%)	Lost (%)
Untreated Calves	14 (14)	36	0	100
FSH-Treated Calves	9 (18)	66	11	83
Control Cow	26 (26)	65	38	41

Table adapted from Revel *et al.* 1995 [34].

III. Rationale, Hypothesis and Objectives

The adoption of new genomic selection tools such as SNPs, allow genetically valuable animals to be identified at birth. Therefore, in order to minimise the generation interval between parent and progeny, it is desirable to reproduce animals at a prepubertal age to maximise the rate of genetic gain. Past literature has shown that oocytes derived from prepubertal donors are capable of developing to the blastocyst stage following IVEP, as well as establish pregnancy and live births following ET. However, these oocytes are less developmentally competent when compared to their adult-derived counterparts. Therefore, the objective of this study was to assess the follicular and *in vitro* embryo development dynamics of prepubertal Holstein heifer calves between the ages of 2 and 6 months. Specifically, two factors were investigated; the effect of age and the effect of gonadotropin stimulation prior to oocyte collection. Concerning the effect of gonadotropin stimulation, we hypothesise that prepubertal donors may require an extended gonadotropin stimulation for a prolonged period of time due to the reduced activity of the innate HPO axis in these animals. Furthermore, as animals get older, we hypothesise that the developmental capacity of oocytes from prepubertal donors should increase between 2 and 6 months of age as the ovary matures.

IV. Materials and Methods

4.1 - Animals

All experimental procedures using cattle were approved by the Animal Care and Use Committee of McGill University, in accordance with Canadian Council of Animal Care regulations. A total of 109 LOPUs were performed on a total of 14 Holstein heifer calves aged 2-6 months of age. Each animal underwent the procedure every two weeks for a total of between 7 and 9 sessions. Animals were divided into two successive groups of 6-8 individuals between May 2015 and June 2016. Animals were housed indoors in a large communal pen at the Large Animal Research Unit of McGill University on the Macdonald Campus in Sainte-Anne-de-Bellevue, Quebec, Canada (45.4252968 N, -73.9654065 W). Animals were fed good-quality second-cut hay and clean water offered *ad-libitum* and weighed weekly using a thoracic weighing tape. Animals were fed a milk-replacer substitute (Optivia®, Shur-Gain, Brossard, Quebec, Canada) until at least 2 months of age and consuming at least 1 kg of grain (Optivia®) daily along with *ad-lib* hay. Milk and/or grain medicated with a coccidiostat were fed twice daily, in measured quantities according to their body weight. During the winter, the barn was heated to 14°C, and, during the summer, animals were kept as cool as possible with large fans. Animals were housed in a well-ventilated room with windows, and artificial lighting was on a timer to deliver 13 hours of light. Animals also had access to various toys and environmental enrichment devices such as hanging hay dispensers and a soccer ball. Veterinary care was continuously available. Twenty-one sexually mature Holstein heifers were used as recipient females for embryo transfer, and were housed and cared for at L'Alliance Boviteq (St. Hyacinthe, Quebec, Canada). In order to assess the effect of age, the animal's age at the time of LOPU, was divided into one of three categories:

- **Young:** Animals less than or equal to 98 days old at the time of LOPU
- **Middle:** Animals between 99 and 130 days old at the time of LOPU
- **Old:** Animals greater than 130 days old at the time of LOPU

4.2 - Ovarian Stimulation

Animals were given intramuscular injections of FSH (Folltropin-V®, Bioniche Animal Health, Belleville, Ontario, Canada) at 12 hour intervals (7:00 am and 7:00 pm) using a 20G needle. Some animals were stimulated with eCG (Folligon®, Merck Animal Health, Kirkland, Quebec, Canada).

In order to assess the effect of ovarian stimulation, gonadotropin treatments were divided into one of three treatment:

- **No Gonadotropin Treatment:** Animals were not stimulated with any gonadotropins
- **Short-Treatment:** Animals were stimulated with FSH between 36 and 48 hours prior to LOPU
- **Long-Treatment:** Animals were stimulated with FSH for at least 72 hours prior to LOPU

Regardless of treatment, all animals had a vaginal progesterone implant (0.3g) in the form of an ovine CIDR (EAZI-Breed CIDR®, Zoetis, Kalamazoo, Michigan, USA) inserted five days prior to LOPU.

4.3 - Anaesthesia

Prior to laparoscopy, animals were fasted of solids for 24 hours and liquids for 18 hours. Anaesthesia was induced with intravenous (i.v.) administration of 0.05 m.g/KBW xylazine (Xylamax®, Bimeda, Cambridge, Ontario, Canada), followed 5 minutes later with i.v. administration of 0.01 mL/KBW telazol (Zoetis). Animals were then intubated and maintained under anaesthesia using 2% isofluorane (Isoflo®, Abbott, Montreal, Quebec, Canada).

4.4 - Chemicals and Reagents

Unless otherwise indicated, all chemicals and reagents were purchased from Sigma Chemical Company (St. Louis, Missouri, USA).

4.5 - LOPU

Ovaries were visualised under laparoscopic observation and follicular contents were aspirated in order to recover oocytes. The laparoscopy equipment used was composed of:

- 5 mm laparoscope with 5.5mm trocars
- light source and attached cable
- 3mm atraumatic grasping forceps
- Two additional 3.5 mm trocars

A puncture pipette and collection tube connected to a vacuum pump via flexible plastic tubing composed the follicle puncture equipment. In order to prepare the puncture pipettes, a short bevelled 20 G hypodermic needle was glued to the tip of 3mm acrylic pipette. The collection tube was a 50 mL Falcon centrifuge tube with inlet and outlet ports in the cap placed in an electronic warmer sleeve. The aspiration pipette as described above and a vacuum pump were then connected to these ports, respectively. A flow valve was used to regulate the vacuum pressure of the pump and measured as drops of collection medium per minute entering the collection tube. The pressure was adjusted to 60 drops of media reaching the collection tube per minute. Before use, all puncture equipment was rinsed with collection medium and between aspiration of different follicle size classes. The collection medium (Vetoquinol®, Lavaltrie, Quebec, Canada) was supplemented with 10U/mL of heparin (Fresenius Kabi Canada Ltd., Richmond Hill, Ontario, Canada), and 25µg/mL of gentamicin (Sigma-Aldrich, Saint Louis, MO, USA); the collection tube contained 0.5 mL of this medium to receive the oocytes.

After fully anaesthetised, animals were mounted and restrained on a cradled surgical table with an adjustable shoulder rest in the Trendelenburg position. The abdomen of the animal caudal to the umbilicus was shaved, cleaned and disinfected using 70% isopropanol and 2% chlorhexidine. Three small 0.5 cm incisions were made in the abdominal wall using a small scalpel blade. Trocars were inserted into the abdominal cavity through these incisions and a small volume of filtered air was pumped inside in order to facilitate visualisation of the reproductive tract. The ovaries were exposed by using the grasping forceps to pull the fimbria away in different directions. Once the ovary was grasped with the forceps, the number and size of each follicle was recorded, and follicular contents were aspirated by puncturing the follicle

with the aspiration needle. At the term of the procedure, in order to wash away any blood at the follicle puncture sites, the ovarian surface of both ovaries was flushed with warm saline solution using a pipette introduced through one of the 3.5 mm trocars. The incisions were sutured with Vicryl #1 (Ethicon Inc. Somerville, New Jersey, USA) and finally washed with hydrogen peroxide and covered with a generous amount of 2% iodine solution. Following the procedure, the CIDR was removed from the vagina and animals were given a long-lasting antibiotic, 1mL/ 10 KBW oxytetracycline (Oxymycine LA®, Zoetis, Kirkland, Quebec Canada) as well as 1mL/ 45 KBW flunixin meglumine (Banamine®, Merck Animal Health, Madison, New Jersey, USA), an anti-inflammatory analgesic administered via sub-cutaneous injection.

4.6 - Washing and Grading of Oocytes

After LOPU, the collection tube was transferred to the laboratory, its contents poured into a 50 mm petri dish and observed under a stereoscope with heating pad, in order to search for the cumulus-oocyte-complexes. The COCs were placed in HEPES-buffered Tyrode's medium (TLH) solution, supplemented with 10% bovine serum albumin, 0.2-mM pyruvate and 50 mg/mL gentamicin. They were then transferred into the IVM drops and graded based on their appearance.

- Grade 1: >3 layers of compact cumulus cells surrounding a uniform, evenly granulated ooplasm.
- Grade 2: 1-3 layers of cumulus cells and evenly granulated, uniform ooplasm.
- Grade 3: Absent cumulus oophorus.
- Grade 4: Expanded cumulus oophorus, and/or heterogeneous ooplasm, or degenerated.

The number of COCs in each class was recorded. Grade 1 and 2 COCs were selected for subsequent use, while grade 3 and 4 COCs were discarded.

4.7 - *In Vitro* Maturation

IVEP practices were the same as those described by Landry *et al.* 2016 paraphrased below. After grading and selection, healthy COCs were washed and placed in 50µL droplets of

maturation medium mounted on a petri dish under mineral oil. Maturation medium was composed of TCM 199 (Gibco, Invitrogen Life Technologies), 10% foetal bovine serum (Wisent Bioproducts), 0.2-mM pyruvate, 50-mg/mL gentamicin, 0.5 mg/mL FSH (Folltropin-V®), 5 mg/mL LH (Lutropin®, Bioniche Animal Health, Belleville, Ontario, Canada), and 1-mg/mL prostaglandin E_2. Maturation droplets were placed in an incubator for 24 hours at 38.5°C, with 5% CO_2 and 100% humidity.

4.8 - *In Vitro* Fertilisation

Following IVM incubation, COCs were washed twice in TLH medium before being transferred to a 48 μL droplet of IVF medium in groups of five under mineral oil. The IVF medium was composed of modified Tyrode's lactate medium supplemented with fatty-acid free BSA (0.6% w:v), heparin (2 μg/mL), pyruvic acid (0.2 mM) and gentamicin (50 mg/mL). After being transferred to the droplet, the oocytes were left to acclimatise for 15 minutes prior to IVF. In order to prepare the drops for IVF and promote sperm motility, each droplet was supplemented with 80mM penicillamine, 1 mM hypotaurine and 250 mM epinephrine. Frozen semen (Semex, Canada) was thawed by placing the straws in a water bath at 35.8°C for 1 minute. Following this, they were placed on a discontinuous gradient (45% over 90%) of BoviPure® (Nidacon Laboratories AB, Göthenborg, Sweden) and centrifuged at 600g for 5 minutes, and then at 300 g for 2 minutes. Between the cycles, the supernatant was discarded, and the pellet was resuspended in 1 mL of modified Tyrode's lactate. The supernatant was discarded again, and the pellet resuspended. In order to achieve a working concentration of 1 x 10^6 sperm/mL, spermatozoa were then counted using a hemocytometer, and diluted with a measured amount of IVF medium. Fertilisation was then allowed to take place by adding 2 μL of the sperm suspension to each droplet and incubating the plates at 38.5°C for 18-22 hours, with 5.5% CO_2 and 100% humidity.

4.9 - *In Vitro* Culture

After IVF, presumptive zygotes were washed and placed in 10 μL droplets of modified synthetic oviduct fluid (mSOF) plated under embryo-tested mineral oil. These droplets were

supplemented with non-essential amino-acids, 0.4% fatty acid-free BSA (ICP bio, Auckland, New Zealand) and 3 mM ethylenediaminetetraacetic acid (EDTA). The culture dishes were then placed in an incubator at 38.5°C, with 100% humidity with an atmosphere of of various gases (6.5% CO_2, 5% O_2 and 88.5% N_2) supplied by Praxair Inc. Forty-eight hours following fertilisation, cleavage rate was examined under a stereoscope and recorded. Seventy-two hours following fertilisation, embryos were transferred to new 10 µL droplets of mSOF containing both essential and non-essential amino acids. The process was repeated again at 120 hours post-fertilisation, but with larger droplets of 20 µL. At the end of the culture period, blastocyst development was examined using a warmed stereoscope on day 7 post-fertilisation.

4.10 - Embryo Transfer and Pregnancy Detection

In order to induce estrus in Holstein recipient heifers, 500 µg of cloprostenol (Modern Veterinary Therapeutics LLC, Miami, Florida, USA), a synthetic prostaglandin analogue was administered via an i.m. injection. Heat observation occurred 48 to 96 hours after cloprostenol. Seven days after heat-detection, a single blastocyst was transferred non-surgically into the uterine horn ipsilateral to the CL-bearing ovary. Pregnancy was monitored at regular intervals using trans-rectal ultrasonography.

4.11 - Maturation, Polyspermy and Embryo Analysis

In order to assess maturation rate, a small subset of oocytes were removed from their respective pools following IVM, but prior to IVF. Oocytes were then placed in a 4% paraformaldehyde solution for 15 minutes, and then transferred into a permeabilisation solution (phosphate-buffered saline (PBS) supplemented with 0.3% BSA and 0.2% Triton 100X) and refrigerated at 4°C until a time when staining could take place. Prior to staining, oocytes were permeabilised by warming them to 38.5°C on a warming plate for 30 minutes. Oocytes were then stained using 1 µg/mL DAPI diluted in permeabilisation solution and left to incubate at 35°C for 15 minutes protected from light. The oocytes were then washed three times using 500 µL of permeabilisation solution. After washing, oocytes were mounted onto microscopy slides using moviol warmed to 35°C. Maturation was assessed using a Nikon eclipse fluorescent microscope.

In order to assess polyspermy, zygotes were removed from their culture plate 15 hours post-fertilisation. In order to count the number of cells present in blastocysts, they were removed from culture on Day 7 or 8. Both zygotes and embryos were then fixed and stained using the same method described for the oocytes.

4.12 - Statistical analysis

All statistical analysis was performed in JMP software (SAS Institute Inc. Cary, North Carolina, USA). In order to test the normality of data, a Shapiro-Wilk W test was performed. If the data was not normal, then it was normalized. A one-way ANOVA followed by a Tukey-Kramer HSD test was then performed. Polyspermy and IVC data was analysed using a Chi-Square test using a contingency table. Differences were considered to be statistically significant at the 95% confidence level ($P<0.05$).

V. Results

In total, we performed 109 LOPUs, on 14 animals, with each animal undergoing the procedure between 7 and 9 times, during the course of this program. Cumulative results are presented in **Table 5.1**.

Table 5.1: Overall Follicular and *In Vitro* Development Responses of Oocytes Collected from Calves.

Procedure	Total #	Average # per Animal per LOPU	Efficiency (%)
Follicles Aspirated	2 092	19.2	-
COCs Recovered	1 635	15.0	78
COCs Deemed Usable	1 429	13.1	87
COCs Placed in IVM	1 069	9.5	75
Cleaved Zygotes	691	6.2	65
Blastocysts	170	1.6	16
Recipients Transferred (Pregnancies)	21 (13)	-	62

Calves aged 2-6 months of age, and underwent between 7 and 9 LOPU procedures.

5.1 - Effect of Gonadotropin Stimulation:

On average, animals produced a mean number of 19.2 follicles per LOPU, with a large variation ranging from a minimum of 3 to a maximum of 55. There was no significant difference among the three treatments: no, short and long treatment, in number of follicles produced/ aspirated (19.8 ± 9.2 vs. 20.7 ± 10.9 vs. 16.9 ± 9.7, respectively, $P > 0.05$). However, there were significant differences in the sizes of follicles among treatments. In general, the longer protocol resulted in a larger proportion of large follicles >5 mm in diameter. Furthermore, when animals were not subjected to gonadotropin stimulation, most follicles aspirated were small < 3 mm in diameter. These results are presented in **Figure 5.1**.

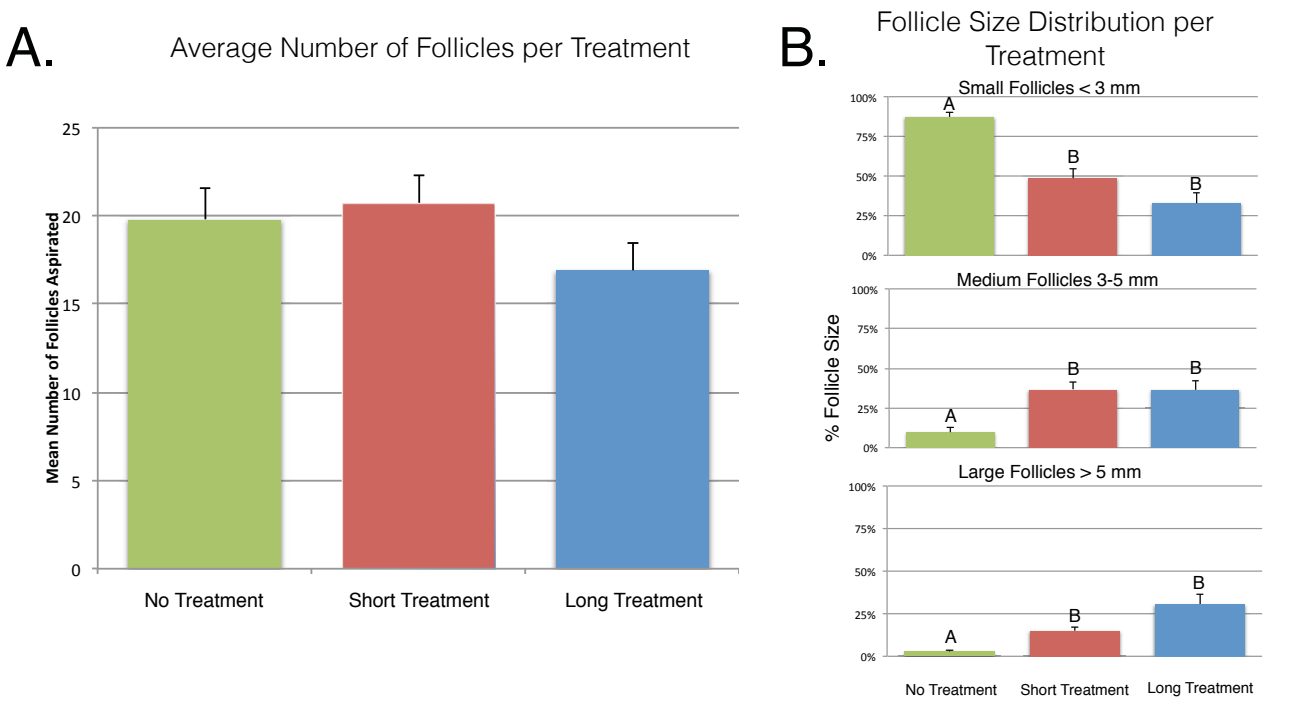


Figure 5.1: The Follicular Response to Gonadotropin Stimulation. A. The average number of follicles available for aspiration per treatment. There was no significant difference among treatments. B. Follicle size distribution per treatment. As gonadotropin stimulation got longer, the proportion of large follicles increased, and the proportion of small follicles decreased. [A, B] Values within the same follicle size category differ significantly ($P < 0.05$).

On average, 77.2% of the COCs from follicles aspirated were recovered, resulting in the number of COCs recovered per animal ranging from a minimum of 1 to a maximum of 51. Among treatments, long, short and no treatment, the short treatment yielded the most COCs (13.7 ± 6.6 vs. 18.1 ± 11.3 vs. 12.1 ± 7.7, respectively, $P < 0.05$). Concerning COC quality, 67% of COCs were found to be grade 1, 20% were grade 2, 3% were grade 3, and 9% were grade 4. On average, $87.4\% \pm 19.3\%$ of oocytes were found to be usable (grade 1 and 2). Furthermore, the long treatment yielded a significantly better rate of usable COCs than the other two treatments ($82.2\% \pm 13.8\%$ vs. $85.4\% \pm 22.1\%$ vs. $95.3\% \pm 18.4\%$, $P > 0.05$). Next, the normal fertilisation rate was assessed in a subset of fertilised zygotes. A zygote was deemed to be normal

with the presence of two pronuclei, while zygotes with three or more pronuclei were deemed polyspermic. There was no significant difference in the fertilisation/polyspermy rate among the three treatments (71% vs. 58% vs. 66%, respectively, $P < 0.05$) (**Figure 5.2**).

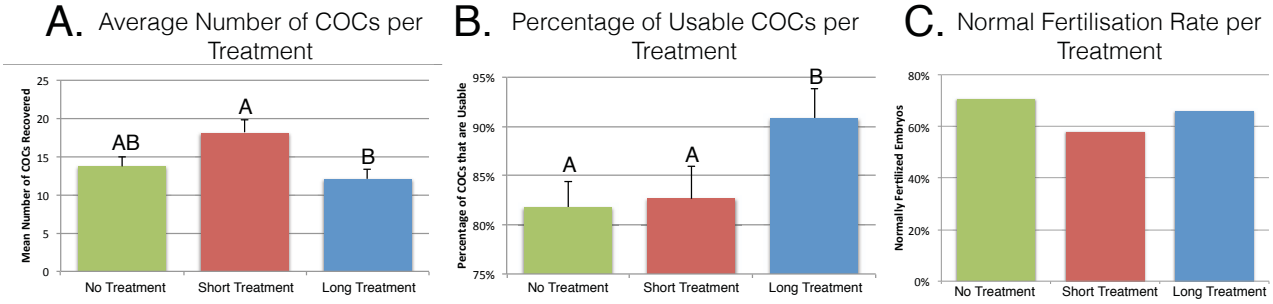


Figure 5.2: The Cumulus-Oocyte Complex Response to Gonadotropin Stimulation. A. The average number of COCs per treatment. B. The percentage of usable COCs per treatment. C. The normal fertilisation rate per treatment. Normal = 2 polar bodies and 2 pronuclei. [A, B] Values within the same section differ significantly ($P < 0.05$).

Overall, the average cleavage rate (checked on Day 2) was 65% ± 23.7%, the average morula rate (checked on Day 5) was 38% ± 25.7%, and the average blastocyst rate (checked on Day 7) was 25% ± 21.6%. The morula and blastocyst rate was calculated as a function of the number of oocytes entering IVEP, and not a function of the number of embryos cleaved. Among treatments, the long protocol consistently yielded superior results. There were small differences in the cleavage rate (66.0% ± 19.9% vs. 59.0% ± 22.5% vs. 72.7% ± 21.2%, respectively, $P < 0.05$), with the long treatment being significantly better than the short treatment. With embryo development, the long treatment resulted in morula rates and blastocysts rates almost double that of both the short and no treatment (Morula: 28.6% ± 14.1% vs. 28.1% ± 15.5% vs. 55.2% ± 27.4%, respectively, $P < 0.05$. Blastocyst: 16.7% ± 8.9% vs. 18.3% ± 15.2% vs. 36.7% ± 25.5%, respectively, $P < 0.05$) (**Figure 5.3**).

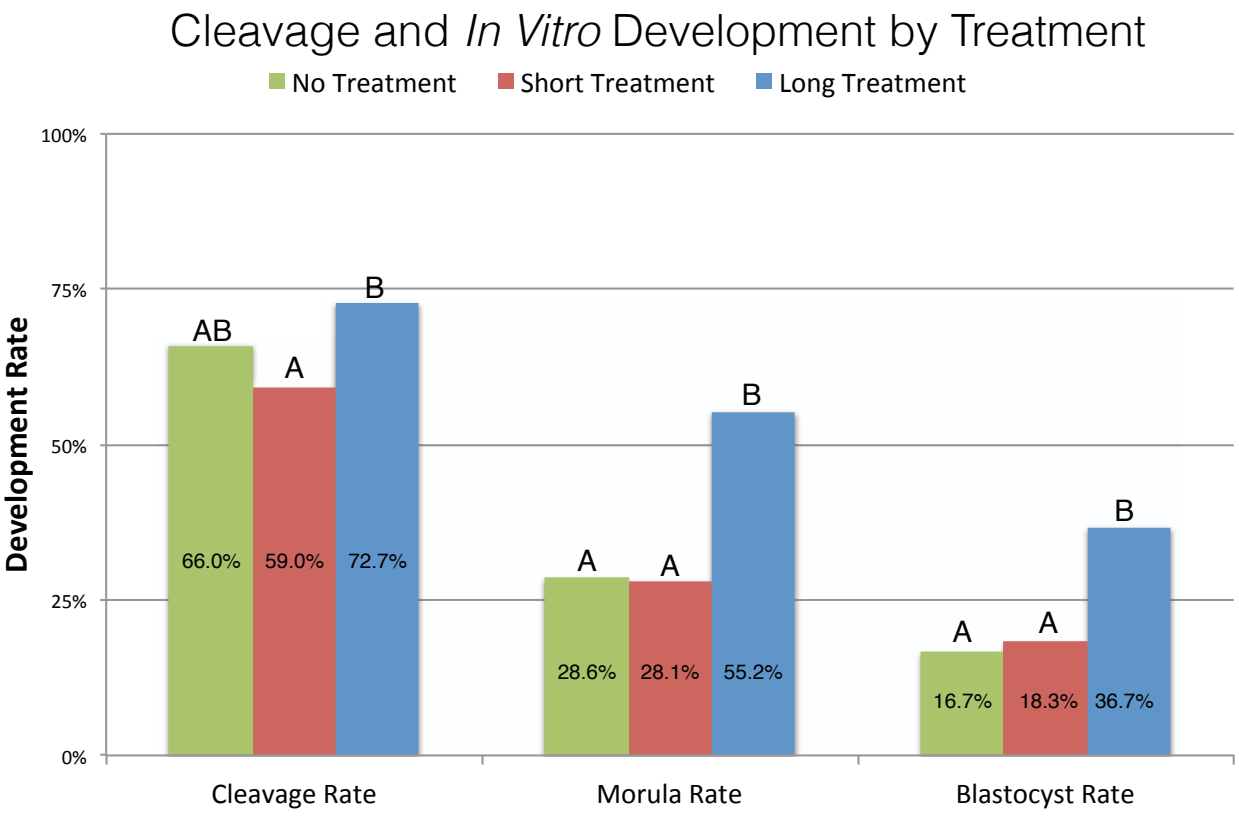


Figure 5.3: The Cleavage and *In Vitro* Development dynamics by Treatment. Green = No Treatment. Red = Short Treatment. Blue = Long Treatment. Morula and Blastocyst rates are calculated as a function of total number of oocytes placed in IVC. [A, B] Values within the same column cluster differ significantly ($P < 0.05$).

5.2 - The Effect of Age

In order to assess the effect of age, animals were divided into three categories, based on their age at the time of LOPU: ≤ 98 days of age, 99-130 days of age and ≥ 131 days of age. Animal age did not result in an increased number of follicles available for aspiration. In fact, animals between 99-130 days old produced the most follicles on average, with the older age group (≥ 131 days old) producing significantly less (19.9 ± 10.3 vs. 21.7 ± 9.2 vs. 16.1 ± 10.2, respectively, $P < 0.05$). Concerning the follicle size distribution per with age, all groups were relatively uniform (**Figure 5.4B**).

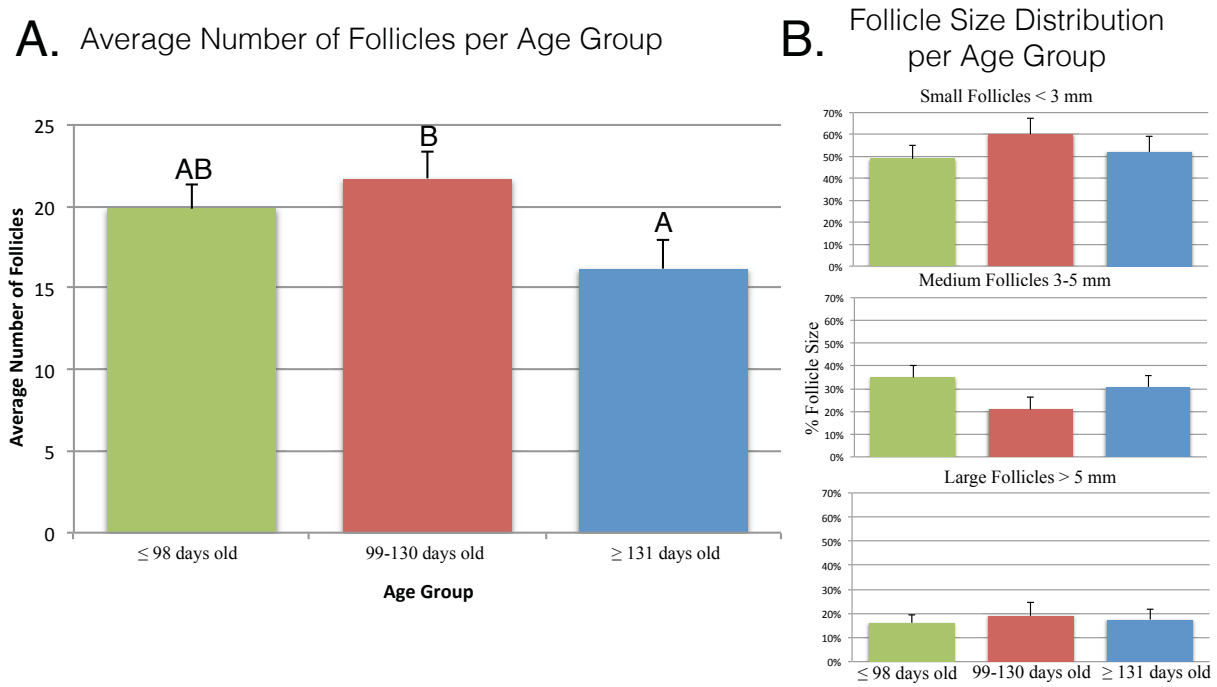


Figure 5.4: The Follicular Response to Age. A. The average number of follicles available for aspiration per age group. B. Follicle size distribution per age group. Green = ≤ 98 days of age. Red = 99-130 days of age. Blue = ≥ 131 days of age. $^{A, B}$ Values within the same column section differ significantly ($P < 0.05$).

As animals got older, a steady decline in the number of COCs recovered per LOPU was observed (17.5 ± 11.1 vs. 14.7 ± 7.0 vs. 11.9 ± 8.2, respectively, $P < 0.05$). However, as animals got older, there was a trend, although not significant, that the percentage of these oocytes which were deemed to be usable increased (84.2% ± 21.5% vs. 87.5% ± 13.3% vs. 93.6% ± 20.4%, respectively, $P > 0.05$). Furthermore, the normal fertilisation rate steadily increased with age (53.5% vs. 67.4% vs. 84.9%, respectively, $P < 0.05$). Results are shown in **Figure 5.5.**

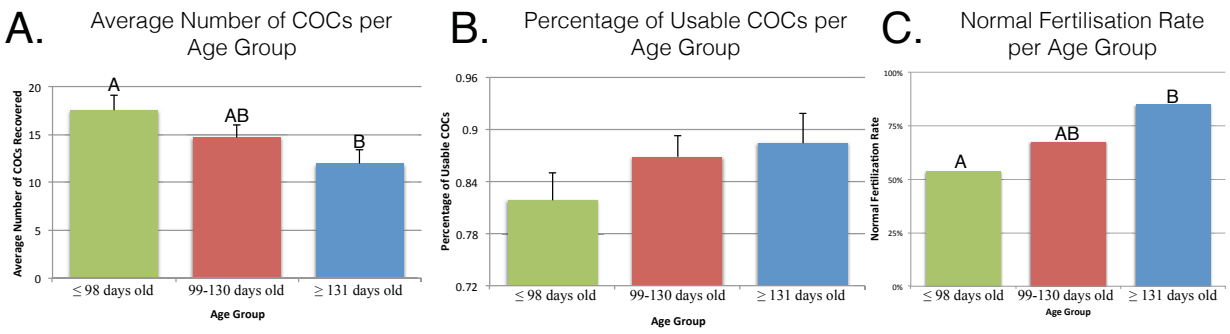


Figure 5.5: The Cumulus-Oocyte Complex Response to Age. A. The average number of COCs recovered per age group. B. The percentage of usable COCs per age group C. The normal fertilisation rate age group. Normal = 2 polar bodies and 2 pronuclei. [A, B] Values within the same section differ significantly ($P < 0.05$).

Consistent with the normal fertilisation rates, age did not have an impact on cleavage rates (66.3% ± 22.1% vs. 69.6% ± 23.8% vs. 65.0% ± 25.0%, respectively, $P > 0.05$). The *in vitro* development increased with age, with morula (checked on Day 5) and blastocyst rate (checked on Day 7) steadily increasing as animals became older (Morula: 18.0% ± 20.0% vs. 30.3% ± 24.6% vs. 33.3% ± 29.4%, respectively, $P < 0.05$. Blastocyst: 12.8% ± 19.9% vs. 17.1% ± 20.7% vs. 21.8% ± 24.9%, respectively, $P < 0.05$). These results can be seen in **Figure 5.6**.

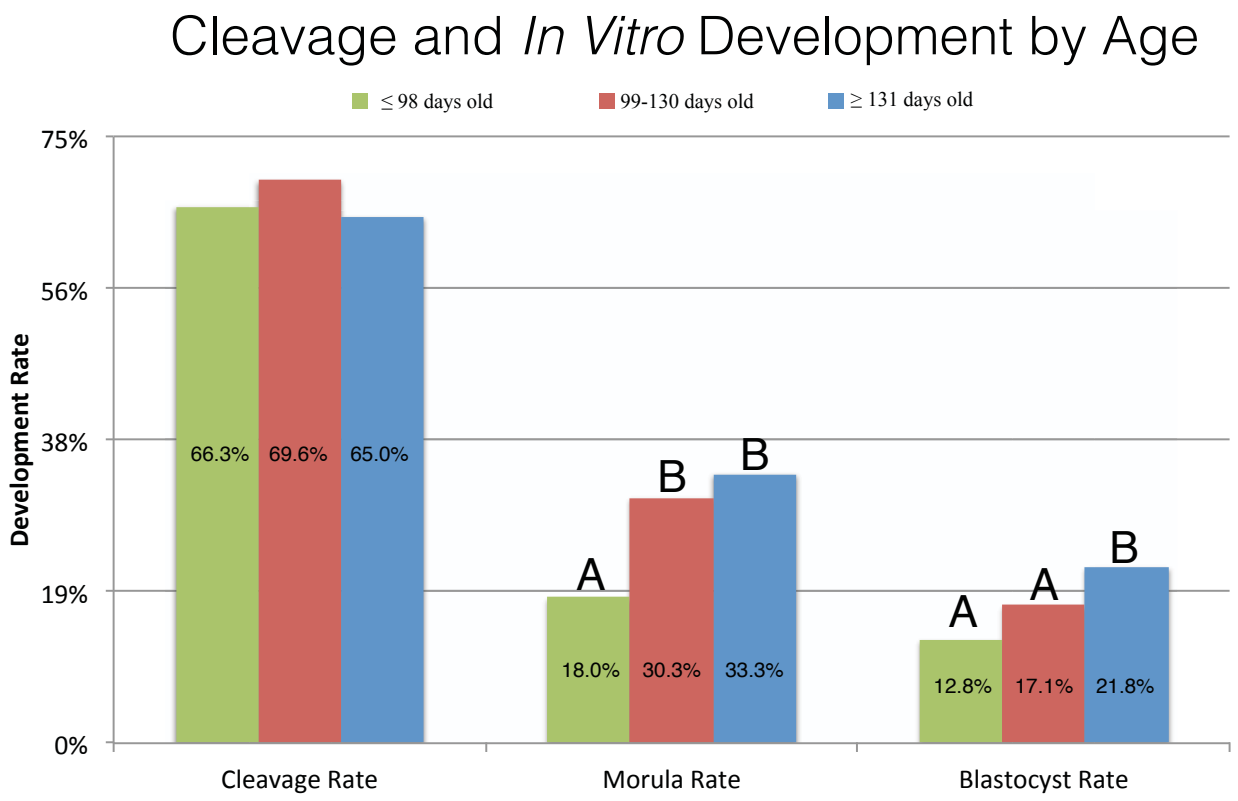


Figure 5.6: The Cleavage and *In Vitro* Development dynamics by Age Group. Green = ≤ 98 days of age. Red = 99-130 days of age. Blue = ≥ 131 days of age. Morula and Blastocyst rates are calculated as a function of total number of oocytes placed in IVC. [A, B] Values within the same column cluster differ significantly ($P < 0.05$).

5.3 - Developmental Arrest

In a subset of embryos that failed to develop to the blastocyst stage, we assessed at which point in development the arrest took place. Overall, it was found in the subset analysed that 57% of cells cleaved but failed to reach the blastocyst stage. Of these, we found that 51% arrested development prior to embryonic genome activation, which in bovine occurs at the 8-16 cell stage.

5.4 - Blastocyst Quality

A subset of blastocysts were stained in order to count the number of cells present. Each blastocyst had an average cell number of 76.9 ± 27.2 blastomeres (n=113). Between treatments there were variations in cell number, with no treatment yielding the most blastomeres (97.8 ± 27.3, 68.6 ± 20.7, 78.7 ± 28.9, respectively, $P<0.05$). Among age categories, the older animals (≥ 131 days old) had the most blastomeres present (67.2 ± 20.4, 72.8 ± 24.7, 92.0 ± 30.1, respectively, $P<0.05$). **(Figure 5.7)**

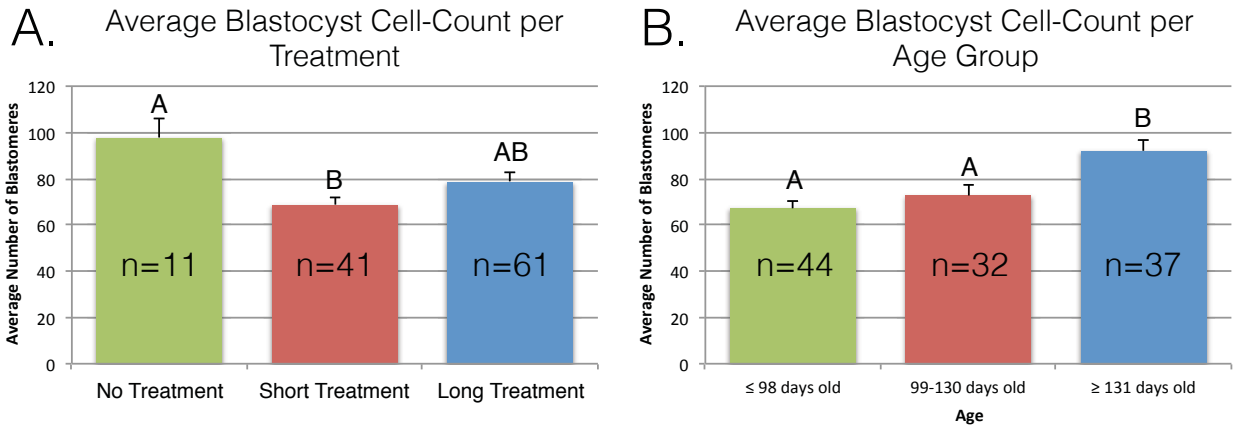


Figure 5.7: The Average Cell-Count of Blastocysts. A. The average blastocyst cell-count per treatment. B. The average cell-count per age group. n= the number of blastocysts in each group. $^{A, B}$ Values within the same column cluster differ significantly ($P < 0.05$).

5.5 - Embryo Transfer and Pregnancy:

A total of 21 embryo transfers were performed, with each recipient receiving a single, excellent-grade Day 7 blastocyst. Upon inspection on day 28, 13 became pregnant. Of the 21 blastocysts transferred, 13 were fresh, while 8 were frozen prior to ET. Overall, a 62% pregnancy rate was established, with a 46% pregnancy rate with fresh embryos, and an 88% rate using frozen embryos. Some pregnancies were purposely terminated, while 9 recipients were followed to term. Of the 9 animals that were followed to term, 8 delivered live calves, 6 bulls and 2 heifers. 7 of these animals were healthy and fed normally following birth, while one died approximately 2 hours post-parturition. One animal gave birth during the night, and upon arrival

by staff in the morning, the calf was dead. However, it is unknown if this calf was a stillborn, died shortly after birth, or if there were dystocia problems. The average weight at birth was approximately 55.3 kg.

5.6 - Safety:

Repeated LOPU in Holstein calves, in the hands of an experienced surgeon, appears to be safe, based on the following observations:

1. During the course of the experimental period, no animals exhibited any lesions in the ovary and/or fimbria that could be considered as sequels of manipulation, after undergoing the procedure up to 9 times.
2. After the last LOPU, animals did not show any signs of abnormalities in the reproductive tract by rectal palpation conducted by an experienced veterinarian.
3. After the experimental period, many animals became productive peri-pubertal OPU donors at L'Alliance Boviteq.
4. After reaching sexual maturity, animals became pregnant following ET.

VI. Discussion

It has been well established in the literature that embryos originating from calves, are less developmentally competent than those derived from adult cows [11, 34]. Even though, they do contain the potential to give rise to healthy offspring following LOPU/IVEP/ET [34, 175]. However, the underlying reasons for this remains poorly understood. Therefore, working under the assumption that oocytes have the potential to develop, we tested various gonadotropin priming protocols in order to assess what conditions can aid in increasing *in vitro* development rates, and allow these oocytes to develop to their full potential.

Our data appears to follow the observation by some authors that gonadotropin stimulation does not increase the number of follicles present on the ovary, rather just increase their size [36]. This is consistent with the knowledge that only follicles that have reached the antral stage of development are responsive to exogenous gonadotropins. However, it should be noted that some other authors found evidence to the contrary, finding that gonadotropin stimulation significantly increases the number of follicles available for aspiration [105, 167]. Therefore, activity of the HPO axis and how the ovary responds to exogenous hormones at such a young age remains unclear.

Past literature has shown that COCs recovered from larger follicles are more developmentally competent than those that originate from small follicles [209]. We could not directly confirm this with our data, since COCs originating from all follicle sizes were pooled together at the time of collection according to their donor. However, it was found that a long gonadotropin stimulation resulted in a high proportion of large follicles being present on the ovary, along with a significantly better morula and blastocyst rate. Suggesting that this is indeed the case, and that follicle size plays a critical role in predicting the future competence of the oocyte [191, 209].

The effect of age during the prepubertal period on the follicular response is less clear. Past literature has shown that the population of antral follicles present on the ovary rise rapidly after birth and peak between 8 and 12 weeks of age [35, 38]. Ages which fall into the youngest category (≤ 98 days of age) in our analysis. However, we found that although not significantly

different, animals between 99-130 days old tended to have more follicles available for aspiration , while older animals (≥ 131 days old) had less. The reasons for this decline remain unclear, however, it could possibly be an effect of a refractory response following repeated gonadotropin stimulation, and/or the loss of ovarian reserve.

Similar to follicular results, there was no significant difference in the number of COCs recovered among the three treatments. However, the long treatment resulted in significantly more COCs which were deemed to be usable based on a visual morphological assessment, of cytoplasm homogeneity and cumulus oophorus quality. We believe this was an effect of their follicular environment prior to collection, and that the long treatment, allowed the COC to develop and mature *in vivo* for a longer period of time prior to aspiration. Our data found that as animals get older, although they steadily yield less COCs, the developmental capacity of those oocytes *in vitro* steadily increases. This is in agreement with other work which showed the same trend at an older age [163].

Furthermore, although the rate of polyspermy decreased with age, polyspermy was still a significant problem. Although, not tested in our experiment, some authors have shown that cytoplasmic differences among oocytes could be a contributing factor. For example, Damiani *et al.* showed that only 19% prepubertal oocytes displayed cortical granule migration, a process which is known to be critical in initiating the block to polyspermy [16]. Therefore, future research could examine the potential that intra-cytoplasmic sperm injection (ICSI) could play in the reproduction of prepubertal animals, by effectively eliminating the polyspermy problem. However, bovine ICSI requires chemical activation following sperm injection, and it has not been effectively established in adults yet, which could potentially be even more problematic if applied to calf oocytes. Therefore, sub-zona insemination (SUZI) could be an alternative since chemical activation is not required.

The average blastocyst rate was lower than what can typically be found using adult oocyte donors [34]. For example, Landry and associates, using the same IVEP conditions as us, managed to obtain a 48% blastocyst rate using sexually mature heifers [163]. Although, it appears that a long gonadotropin treatment can significantly improve *in vitro* development performance. Since our results showed that for both morula and blastocyst rate, the long

treatment outperformed both the short and no treatment groups. However, the limited number of embryos which successfully reached the blastocyst stage appear to be fully competent, and normal. Since embryos which were transferred initiated pregnancy and resulted in live births at rates comparable to adult-derived embryos, and embryos that were stained had healthy blastomere populations. This is in agreement with past literature which has shown that calf-derived blastocysts have a visible inner cell mass, are the same size as those derived from cows, and reach the blastocyst stage at the same time [34, 211]. However, it should be noted that some authors have noted lower pregnancy rates following ET. For example, while we observed all 9 recipients maintaining their pregnancy past the 6-month mark, Revel and associates found that only 1/9 recipients maintained pregnancy following ET of FSH-stimulated calf embryos, and 0/14 when using unstimulated calf embryos [34].

Repeated LOPU appears to be safe in Holstein Calves. Following the procedure, animals were observed standing and feeding normally shortly following surgery and did not display any adverse reproductive lesions or sequels. LOPU is far less traumatic on the ovary compared to ultra-sound guided OPU, which is widely used worldwide in adult cows, since the the ovary can be visualised directly and only the theca is perforated [81]. This is in agreement with studies using LOPU in goats and sheep, which also observed no adverse fertility effects following repeated LOPU [81, 216].

VII. Conclusion

As a result of this work, several conclusions were established:

1. Prepubertal animals have the capacity to produce oocytes that can yield viable embryos, and offspring born following IVEP and ET.
2. On average, each LOPU resulted in 2 blastocysts per animal.
3. Longer gonadotropin stimulation protocols result in increased follicular size and improved oocyte competence based on *in vitro* development results.
4. The *in vitro* development capacity of prepubertal oocytes increases with age.
5. Upon ET, calf-derived blastocysts implanted and established pregnancy at a rate similar to what can be found in adult animals.
6. Repetitive LOPU is safe, since no adverse reproductive sequels or lesions were observed during or following the experimental period. Furthermore, after reaching sexual maturity themselves after the experiment, animals successfully became pregnant.

VIII. References

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