BIOLOGICAL CHARACTERIZATION OF OVULATION-INDUCING FACTOR (OIF) IN LLAMA SEMINAL PLASMA

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“It is the mark of an educated mind to be able to entertain a thought without accepting it.”

- Aristotle -

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ABSTRACT

The purpose of the studies reported in this thesis was to provide a better understanding of the effects of purified ovulation-inducing factor (OIF) from llama seminal plasma in reflex ovulators (*lama glama*) and spontaneous ovulators (*Bos taurus*). The objective of the first study was to determine if the dose of OIF of llama seminal plasma required to elicit ovulation is physiologically relevant, and to test the hypothesis that CL form and function is affected by OIF in a dose-dependent manner. Llamas were treated with four different doses (500 μg, 250 μg, 125 μg and 60 μg) based on knowledge that for every ejaculate there is approximately 3 mg of OIF. Results supported the hypothesis that OIF affects ovulation and CL form and function in a dose-dependent manner. The high dose of OIF (500 μg) was associated with the highest incidence of ovulation, maximum CL diameter, plasma progesterone concentrations and plasma LH concentrations. The low dose of OIF (60 μg) was minimally effective for induction of ovulation and associated with smaller CL diameter and lower plasma concentrations of progesterone and LH.

The second study was carried out to test the hypotheses that OIF will induce ovulation and affects CL form and function in cattle (Experiment 1), and that OIF given at different stages of development of the first follicular wave will induce atresia of the dominant follicle and hasten emergence of a new follicular wave (Experiment 2). Heifers were treated on Day 5 (Day 0 = wave emergence; Experiment 1) or on Days 3, 6 and 9 (Experiment 2) with a) 1ml of saline, b) 100 μg of GnRH, or c) 1.0 mg purified OIF per 100 kg of body weight. Results of Experiment 1 demonstrated that OIF did not induce ovulation in cattle but it did induce atresia of the dominant follicle and earlier emergence of a new follicular wave. Results from the second study suggested that the effect previously demonstrated could be accomplished in sexually mature females after treatment on Day 6 corresponding to the late growing phase of the dominant follicle.

In summary, the minimum dose of OIF necessary to induce ovulation in llamas was between 60 μg and 250 μg. This dose is physiologically relevant and represents less than $1/6^{th}$ of what is normally present in a single llama ejaculate. In cattle OIF induced regression of the dominant follicle and early emergence of a new follicular wave in pre-

pubertal heifers and had a similar effect in sexually mature heifers after treatment on Day 6 of the estrous cycle.

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DEDICATION

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TABLE OF CONTENTS

PERMISSION TO USE ..... **I**

ABSTRACT ..... **II**

ACKNOWLEDGEMENTS ..... **IV**

DEDICATION ..... **V**

TABLE OF CONTENTS ..... **VI**

LIST OF TABLES ..... **VIII**

LIST OF FIGURES ..... **IX**

LIST OF ABREVIATIONS ..... **XI**

CHAPTER 1 GENERAL INTRODUCTION **1**

CHAPTER 2 LITERATURE REVIEW **3**

2.1 Overview of the reproductive physiology in cattle (spontaneous ovulators) and camelids (induced ovulators) 3
2.1.1 Spontaneous ovulators: Cattle 5
2.1.2 Induced ovulators: Camelids 6
2.1.3 Hormonal regulation 8
2.2 Follicular growth patterns in cattle and camelids 9
2.3 Mechanisms of ovulation in cattle and camelids 12
2.3.1 Dogma on induced ovulation 14
2.3.2 Role of seminal plasma in induced ovulators and evidence of action in spontaneous ovulators 14
2.3.3 OIF – Summary of current knowledge on ovulation-inducing factor 17

CHAPTER 3 OBJECTIVES & HYPOTHESES **19**

CHAPTER 4 DOSE RESPONSE TO OVULATION-INDUCING FACTOR (OIF) IN FEMALE LLAMAS **20**

4.1 Abstract 20
4.2 Introduction 21
4.3 Material and methods 22
4.3.1 Seminal plasma collection 22
4.3.2 Protein purification 22
4.3.3 Animals and treatments 24
4.3.4 Statistical analyses 26
4.4 Results 26
4.5 Discussion 30

CHAPTER 5 EFFECT OF PURIFIED LLAMA OVULATION-INDUCING FACTOR (OIF) ON OVARIAN FUNCTION IN CATTLE.....32

5.1 Abstract 32
5.2 Introduction 34
5.3 Material and methods 35
5.3.1 Experiment 1 35
5.3.1.1 Seminal plasma collection 35
5.3.1.2 Protein purification 35
5.3.1.3 Animals and treatments..... 37
5.3.1.4 Statistical analyses 39
5.3.2 Experiment 2 39
5.3.2.1 Seminal plasma collection 39
5.3.2.2 Protein purification 39
5.3.2.3 Animals and treatments..... 40
5.3.2.4 Statistical analysis 41
5.4 Results 42
5.4.1 Experiment 1 42
5.4.2 Experiment 2 45
5.5 Discussion 52
CHAPTER 6 GENERAL DISCUSSION.....54

6.1 Dose effect of llama seminal plasma on ovulation and corpus luteum form and function in llamas 54
6.2 Effect of ovulation-inducing factor on ovulation and follicular wave dynamics in cattle 56
CHAPTER 7 GENERAL CONCLUSIONS.....59

CHAPTER 8 BIBLIOGRAPHY 61

LIST OF TABLES

Table	Legend	Page
Table 4.1	Effect of dose of OIF on ovulation in llamas (mean ± SEM)……	26
Table 4.2	Effect of dose of OIF on CL development in llamas that ovulated in response to treatment (mean ± SEM, Day 0 = day of treatment)……...	27
Table 5.1	Effect of OIF on ovulation in pre-pubertal heifers (mean ± SEM; Experiment 1)…….	42
Table 5.2	Effect of OIF on follicle wave dynamics in heifers that did not ovulate in response to treatment (mean ± SEM; Experiment 1).……	43
Table 5.3	Follicle wave and CL dynamics (mean ± SEM) in heifers treated on Day 3, 6 or 9 after emergence of the first follicular wave of an interovulatory interval with GnRH, OIF, and in non-treated controls (Experiment 2)….	46

LIST OF FIGURES

Figure	Legend	Page
Figure 4.1	CL diameter (a) and plasma progesterone concentrations (b) in llamas given a single intramuscular dose of OIF (60 µg *, 125 µg □, 250 µg ▲, 500 µg ■) or PBS (○); n=10 per group. In panel (c), data from llamas that did not ovulate (i.e., no CL detected) were excluded; i.e., n=3, 7, 9, and 9 in groups treated with 60, 125, 250, or 500 µg OIF, respectively. (mean ± SEM)…….....	28
Figure 4.2	Plasma LH concentrations (mean ± SEM) in female llamas that ovulated following treatment with OIF (500 µg ■; 250 µg ▲, 125 µg □, 60 µg * and PBS ○). [a] Within group, the maximum concentration (P<0.05). [b] Within group, the first increase (P<0.05). [c]Within group, the first decrease from maximum (P<0.05). *Within group, the last value higher than pretreatment levels (P<0.05)……..	29
Figure 5.1	Dominant, first subordinate follicle diameter (a) and plasma FSH concentration (b) in pre-pubertal heifers (mean ± SEM) given a single intramuscular dose of OIF (■, ●) or PBS (□, ○) on Day 5 (Day 0 = wave emergence; n=10 per group; Experiment 1). *Between groups, values are different (P<0.05). [a,b,c] Values with no common superscripts are different (P<0.05)…….	44
Figure 5.2	Plasma LH concentrations (mean ± SEM) in pre-pubertal heifers treated with OIF (■), GnRH (*) or saline (□) on Day 5 (Day 0= follicular wave emergence; n = 11 per group; Experiment 1)…..	45
Figure 5.3	Diameter (mean ± SEM) of the dominant follicle of the first wave in heifers that did not ovulate after a single intramuscular dose of OIF or GnRH on Day 3 (a), 6 (b) or 9 (c), and in untreated controls. In panel (b), data from the single heifer that did not ovulate in the GnRH group were excluded ……...	48
Figure 5.4	Plasma FSH concentration (mean ± SEM) in heifers that did not ovulate after a single intramuscular dose of OIF (■) or GnRH (∗) on Day 3 (a), 6 (b) or 9 (c)…….	50

Figure 5.5 CL diameter and plasma progesterone concentration (mean ± SEM) in sexually mature heifers given a single intramuscular dose of OIF (■) or GnRH (∗) on Day 3 (a), 6 (b) or 9 (c), (n=5 per group per day) and in untreated controls (□; n=10) (Experiment 2)……... 51

LIST OF ABREVIATIONS

AI	Artificial insemination
CL	Corpus luteum
CIDR	Controlled internal drug releasing device
FA	Follicular ablation
FPLC	Fast Protein Liquid Chromatography
FSH	Follicle stimulating hormone
GnRH	Gonadotropin releasing hormone
HA	Hydroxy-apatite column
hr	Hours
im	Intramuscular
iu	Intrauterine
µg	Microgram
kDa	Kilodalton
kg	Kilogram
pLH	Porcine luteinizing hormone
LSD	Least significant difference
ml	Milliliter
min	Minute
OIF	Ovulation-inducing factor
PBS	Phosphate buffered saline
PGE	Prostaglandin E
PGF	Prostaglandin $F_{2\alpha}$
SAS	Statistical analysis system
SD	Standard deviation
SEM	Standard error of the mean
vs	Versus

CHAPTER 1
GENERAL INTRODUCTION

"Although sexual activity does not actually supply the motive power for the rotatory motion of our planet, the ability to reproduce is perhaps the most fundamental characteristic of the living organisms which abide there. There can be no doubt that the great diversity of body forms and life histories of vertebrates has evolved as a result of the many different habitats they occupy. In turn, their ability to utilize such habitats is a function of their distinct and varied methods of reproduction." [1]

For many years we have attempted to divide species by classifying their characteristics. One very popular way of classification has been the nature of their reproductive physiology. With better research techniques and introduction of ultrasonography in the 1980's, understanding of animal reproduction has improved and reproductive knowledge has been allowed to expand from more than just the mere observation of reproductive behavior. Although it may seem we have come a long way, especially in understanding the reproductive physiology of traditional livestock species, there are still many questions to be answered.

South American camelids have been the subject of reproductive studies for the past 15 years but still our understanding of their reproductive physiology seems very small when compared to species such as cattle. Llamas and alpacas have become of interest in the last few years in North America, and are of great economic importance for communities in the High Andes. One of the many interesting features of this species is the nature of their ovulation, characterized by Conaway in the 1970's as induced ovulators contrary to most of our domestic species, which are considered to be spontaneous ovulators. We have come a

long way since then, and know there is more to ovulation than just mere external stimulation of the genitalia in these species.

Reproductive management has not been developed as much in new world camelids as in other species, partially due to the lack of reliable information about their basic physiology. Now it is known that follicular development in camelids occurs in waves, ovulation does not occur at regular intervals, and camelid "cycles" can be managed with more than just natural mating of females when they adopt sternal recumbency. Various hormonal preparations have been used to induce ovulation in llamas and alpacas and intervals to ovulation, wave emergence and hormonal profiles of the treated animals have been reported. Recently the use of a biological factor present in the seminal plasma of llamas and alpacas has revolutioned the understanding of the mechanism of ovulation in these species. Studies from our laboratory have shown that more than 90% of test animals treated intramuscularly with seminal plasma ovulated, and isolation of the ovulation-inducing factor has been accomplished not only from llamas, but also from other species, including spontaneous ovulators such as cattle.

Ovulation-inducing factor (OIF) is a protein produced by the accessory sex glands and found in the seminal plasma of llamas, alpacas and cattle. From previous studies, we know that 1 to 2 ml of llama seminal plasma, containing approximately 1.5 to 3 mg of protein, is capable of inducing ovulation in more than 90% of the female llamas, and has luteotrophic effects as evidenced by a longer lasting preovulatory LH surge, larger CL diameter and double the amount of secreted progesterone. It is yet to be determined if the amount of this protein in the seminal plasma of a single ejaculate is sufficient to have the same effect on reproductive activity in the llama and if such protein, highly conserved amongst mammals, has any useful function in cattle.

The following literature review is focused on the differences in basic reproductive characteristics of spontaneous ovulators versus induced ovulators, and our knowledge of ovulation-inducing factor (OIF).

CHAPTER 2
LITERATURE REVIEW

2.1 Overview of the reproductive physiology in cattle (spontaneous ovulators) and camelids (induced ovulators)

"In all species of domestic animals that have reached puberty there is a definite physiologic functional rhythm of the reproductive system, called the estrous cycle." [2]

The physiological rhythm of reproduction mentioned by Roberts (1986) has been subject of many studies throughout the years and animals have been classified according to their capacity of dealing with non-pregnant cycles which in natural populations where species preservation is key, may represent a real disaster. Since the 1970's when Conaway classified species into spontaneous and induced ovulators [3], many studies have been developed to shed light over the mechanisms involved in the occurrence of such phenomenon responsible for the rupture of a dominant follicle and releasing of the egg into the uterus.

There are many theories that suggest that spontaneously ovulating species evolved from induced ovulators [3-5]. Induced ovulation appeared early in Orders *Lagomorpha and Rodentia* [5], and emerged later in closely related orders like Insectivora and *Carnivora* [6]. The existence of induced ovulation in the distant Order, *Artiodactyla* (camelids), however, suggests that the phenomenon may not be such an ancestral trait [7]. One would suspect that if induced ovulation evolved at such an early division of the animal kingdom, that it would also be prevalent in non-mammalian species. This is not the case; the red sided garter snake has so far been the only non-mammalian vertebrate identified as induced ovulator [4]. Some hypothesize that induced ovulation evolved in certain species as a way

to assure preservation through a state of constant receptivity that would allow maximum possibilities of conception in a dramatically changing environment [8]. This could be the case in camelids, which originated in North America but were forced across Berring and Panamanian land-bridges during the last glacial advance [9] evolving into today's old world camelids (Dromedary and Bactrian camel) and new world camelids (llama, alpaca, guanaco and vicuna), all of which still inhabit harsh environments such as high altitudes and deserts. Evidence from studies in our laboratory [7] suggest that vestiges of induced ovulation exist in cattle - which suggests that this phenomenon preceded the division of *Tylopoda* from the rest of the *Artiodactyls*.

There is current evidence of induced ovulation in several species. For example, rabbits [10-12], alpacas [13] and ferrets [14-16] are thought to ovulate after a single intromission and ejaculation, whereas voles [17, 18] and cats [19-21] require several intromissions and ejaculations. In some of these species, such as the domestic cat, induction of ovulation after the external stimulation of the genitalia with a glass rod or cotton swab has been reported [22]. Other species such as koalas [23] have been reported to be induced ovulators, and the influence of seminal plasma on ovulation has been studied in them. There are, however, numerous examples of coitus-enhanced ovulation in spontaneous ovulators. The best example of both ovulation mechanisms integrated into one species is the laboratory rat [4, 24] where ovulation may occur spontaneously but copulation seems to be necessary to fully develop a competent corpus luteum. An early study in cattle from the 1950's [25], reported an earlier ovulation in dairy heifers mated to a vasectomized bull during the first 6-8 hours of behavioral estrus. The effect of copulation and insemination in pigs has also been reported and are believe to shorten the interval between the onset of estrus and ovulation [26, 27].

The following is an overview of the two types of mechanisms involved in ovulation. Cattle were chosen as representative of spontaneous ovulators and camelids, mostly llamas, as an example of induced ovulators.

2.1.1 Spontaneous ovulators: Cattle

"Es'trus, oes'trus -G. oistros, mad desire- Heat; the period of sexual excitement in the female of the lower animals." [28]

Spontaneously ovulating species are those that will ovulate at regular intervals without the need of any external stimulation. These include most of our domesticated species such as horses, cattle and dogs. In cattle, the duration of the estrous cycle ranges from 17 to 25 days and can be divided into two defined periods: the follicular phase and the luteal phase [29]. The follicular phase (proestrus and estrus) extends from the regression of the CL to ovulation. The main structures present in the ovary are the growing follicles and the dominant hormone is estradiol. The luteal phase (metestrus and diestrus) extends from ovulation to regression of the CL. The main structure present in the ovary is the corpus luteum and the dominant hormone progesterone [29].

Puberty in domestic animals is generally defined as the first estrus accompanied by ovulation and followed by a normal luteal phase [30]. Events that lead to the first estrous cycle involve an increase in frequency of GnRH pulses that will induce the secretion of enough FSH and LH to initiate follicular growth, oocyte maturation and ovulation [31]. Follicle development occurs in waves in pre-pubertal heifers and mean serum concentrations of LH, estradiol and LH pulse frequency increases as the time of the first ovulation approaches [32, 33b, 34a]. After an abbreviated first cycle, heifers will continue with the same patterns associated with follicular wave emergence, regular inter-wave intervals and luteal phases throughout the entire reproductive life of the animal [34]. Onset of puberty is influenced by several environmental factors such as breed, weight and season, but in general cows reach puberty between 6 and 24 months or when they have reached 60% of their mature body weight [31].

The follicular phase in cattle is characterized by recruitment of a cohort of follicles from which one will emerge and become dominant [35]. This dominant follicle will

suppress the remaining subordinate follicles and grow until ovulation occurs [36-40]. Once luteolysis is complete, and the dominant follicle has reached a preovulatory size (>8 mm), cows undergo estrus which lasts an average of 12 to 18 hours . This phase is characterized by the expression of typical estrous behavior: cows stand to be mounted by bulls or other cows, cows in heat will come together in the so-called sexually active group - licking and smelling of the genitalia and a general increase in ambulatory activity [41]. In general, the first signs of estrus in the cow are associated with the beginning of the preovulatory surge of LH and ovulation occurs 24 to 30 hours after the LH surge [42]. Development of a corpus luteum characterizes the luteal phase in which the female will no longer receive the male, progesterone becomes the main hormone involved and it starts immediately after ovulation to Day 16 or 19 depending on whether it is a 2-waves or 3 waves cycle [38, 43, 44]. The corpus luteum in the cow can be detected immediately by ultrasonography and reaches its maximum size by Day 9 to 10 after ovulation [45, 46]. Plasma progesterone concentrations exceed 2 ng/ml by day 4 or 5 and are maximal by days 8 to 10 [46]. Luteolysis in cattle is a mechanism regulated by oxytocin and progesterone secreted by the CL itself and by PGF2α produced by the uterus [47-49]. It is believed that exposure to high levels of progesterone eventually induces the development of oxytocin receptors on the endometrium that will induce the production of prostaglandins that will in turn reach the CL through the utero-ovarian vascular countercurrent diffusion system and cause luteolysis [29, 49]. Total or partial hysterectomy causes prolongation of the lifespan of the CL, indicating the important role of PGF produced by the endometrium in luteolysis [49-51].

2.1.2 Induced ovulators: Camelids

Induced or reflex ovulators are those animals in which ovulation will not occur unless stimulated by coitus [13, 52, 53]. Most of the available information on induced ovulation in camelids is based on studies carried out in domesticated species of old (Bactrian camel) and new world camelids (llamas and alpacas). In general, in the absence of a male, female alpacas may remain receptive for over 36 days and undergo very short periods of non-receptivity no longer than 48 hours [52]. Llamas appear to have similar

reproductive behavior as reported by England et al in 1971. During the period of sexual receptivity, follicular development occurs in waves [54-57]. If ovulation occurs, females enter the luteal phase characterized by the presence of a corpus luteum, elevated levels of progesterone and non-receptivity to mounting [54].

Puberty in llamas and alpacas has not been studied critically, but traditionally these species are bred the age of 2 or 3 years, or when they have reached approximately 60% of their adult body weight [58], although some females may be receptive and capable of ovulating at 12 months of age [59].

Sexual behavior in llamas and alpacas is quite characteristic. Receptive females, in presence of a male, adopt sternal recumbency as an indication of their receptivity to mounting. Mating may last from 10 minutes to an hour [60] in which the male emits guttering sounds while the female remains on the ground silent [60, 61]. It has been reported that during this period, other females adopt the prone position near the mating couple [52, 61]. The advances of an aggressive dominant male llama or alpaca may also elicit the prone position in submissive females that are not otherwise sexually receptive [62], and this is why receptivity is not always associated with the presence of a mature dominant follicle [63, 64]. The presence of a CL is definitely associated with non-receptivity and females reject the males by spitting, kicking and screaming [65-68].

As for seasonal effect on the reproductive pattern of llamas and alpacas, it has been reported that in the northern hemisphere they breed throughout the year [69, 70], but in animals inhabiting Peru, Bolivia and Argentina matting is generally restricted to the summer, warm, rainy season from December to March [67, 71]. In New Zealand, alpacas seemed to be more receptive in autumn [72] compared to spring but conception rates did not differ from one group to another [73].

The sexual cycle of camelids can also be divided into two phases: follicular and luteal phases. During the follicular phase, like in cattle, the dominant structures in the ovary

are follicles that grow in a wave-like pattern, with selection of a dominant and regression of the subordinates (reviewed in Adams 2006). Llamas and alpacas are capable of ovulating a dominant follicle of 7 mm or larger but will not ovulate if follicles are smaller than 6 mm or regressing at the time of mating [54, 74] . During the luteal phase [54-56, 75], females remain non-receptive until, in the absence of pregnancy, luteolysis occurs. Corpus luteum can be first detected by ultrasonography 3 days after ovulation and reaches maximum diameter by 8 days [56]. The first significant decrease in CL diameter can be detected on 10 or 11 days after ovulation, and is non-detectable by 12 days. In the pregnant female, the CL remains throughout the whole of pregnancy [65]. Corpus luteum regression is controlled by the secretion of prostaglandins from the uterus into the veno-arterial system that communicates with the ovaries around 8 to 10 days after ovulation in non-pregnant animals [56, 76]. It has been hypothesized that each of the uterine horns have different mechanisms of inducing luteolysis [65, 77]. It appears that the right uterine horn acts through a more local effect on the ovary and that the left uterine horn has a local and a systemic luteolytic effect on the CL [77]. In the case of successful mating, pregnancy is maintained by the same corpus luteum and lasts about 325-361 days in alpacas [52] and 331-361 days in llamas [65]. Although synchronization in the release of gametes from the male and female achieved by induced ovulation seems to be a very effective way to assure pregnancy, embryo mortality in llamas and alpacas is relatively high (25-50% during the first 30 days in alpacas) [73, 78] and it has been hypothesized that the reason for such high incidence may be the ovulation of an old egg or alterations within the uterus [79].

2.1.3 Hormonal regulation

Regulation of the reproductive cycle involves interaction between the three main organs: the hypothalamus, anterior pituitary and the ovary. After luteolysis in spontaneous ovulators, concentrations of progesterone decline rapidly and the negative feedback with the hypothalamus is reduced. As progesterone drops, FSH and LH secretion increase in response to GnRH causing the increase in production of estradiol from the dominant follicle (from 1 pulse of LH every 4-6 hours to 1 pulse every hour) [80, 81]. Estradiol in

presence of low levels of progesterone, has a positive feedback on the surge center of the hypothalamus inducing the release of the preovulatory LH surge and initiate the cascade of events leading to ovulation [81]. The mechanism and hormones involved in ovulation are better described in the next section of this review. A surge in FSH is also observed as a result of GnRH release from the hypothalamus and a new follicular wave emerges. After ovulation, LH induces the luteinization of the theca and granulosa cells of the ovulated follicle and the CL is formed. Secretion of progesterone over 2 ng/ml can be detected by day 4 to 5 after ovulation [80]. In presence of high levels of progesterone, LH pulse frequency decreases and estradiol concentrations rise periodically with emergence of successive waves (reviewed in Adams 1999). Oxytocin receptor numbers in the endometrium increase after approximately 14 days under the influence of progesterone, and estradiol production from the dominant follicle of the ovulatory wave activates oxytocin receptors in the uterus to produce PGF, which in turn causes an immediate decline in progesterone production by the CL by 6 to 10 hours after the beginning of luteolysis [47, 49]).

In induced ovulators, estradiol does not appear to be the primary trigger for the pre-ovulatory surge-release of LH. In rabbits and ferrets, LH secretion was not induced after administration of exogenous estradiol, with or without progesterone [82, 83]. Physiological increases in follicular size have been positively correlated with an increase in the levels of estradiol in llamas and alpacas, but was not associated with a positive feedback on LH or ovulation [84-87].

2.2 Follicular growth patterns in cattle and camelids

Follicular growth in cattle occurs in wave-like patterns in which each follicular wave is preceded by a surge in FSH that recruits a cohort of follicles from which one will grow larger than the others and induce regression of the rest [29, 88]. Introduction of ultrasonography allowed the extensive characterization of wave patterns in cattle [36, 38a,

43, 44b, 89-91]. Basically, follicular dynamics involves four processes: recruitment, selection, dominance and atresia. Recruitment consists of the development of a cohort of small follicles 1-2 mm in diameter that continue growing until one is selected (selection). The largest follicle becomes dominant (dominance) inducing atresia of the subordinates and will ovulate in presence of low levels of progesterone [92]. Follicular waves occur throughout the life of the animal, from puberty and even during pregnancy [32, 33, 89]. Over 95% of estrous cycles in cattle consist of two- or three- wave patterns of development [35]. The first follicular wave emerges on day 0 (day of ovulation), the second on day 9 and the third on day 16 after ovulation [37]. All follicles in the cohort grow for about 2-3 days to about 4 mm [93]. The largest follicle at the time of emergence of a follicular wave, at a diameter of 1-2 mm, is destined to become the dominant follicle [94], and follicular dominance becomes apparent by a deviation in growth rates between the dominant and subordinate follicles at a diameter of about 8.5 mm 3 to 4 days after wave emergence [95] when only the dominant follicle continues to grow.

Each follicular wave is preceded by a surge in FSH [88]and according to the FSH/follicle coupling hypothesis suggested by Ginther [94, 96], the growing follicles are responsible for the decrease in levels of FSH after the surge because of the increasing levels of estradiol that are being produced. Only the largest follicle is capable of surviving with low levels of FSH and this becomes the only one involved in the coupling. Although subordinates undergo atresia once dominance is established, there is evidence that administration of FSH early in the follicular wave induces continued growth of the subordinate follicles and a delay in the establishment of dominance in cattle [97, 98] and co-dominance in mares [99, 100]. Eight hours before the beginning of deviation, granulosa cells of the largest follicle increase gene expression for LH receptors and allow it to become LH dependent [101]. Dominance is established once the largest follicle can utilize LH to produce estradiol and induces atresia of subordinates by suppressing plasma FSH concentrations. In the presence of a functional CL, the dominant follicle will undergo atresia like the subordinates, due to the continued lack of circulating FSH and LH. If luteal regression were to occur during the growing or early static phase, the dominant follicle will

ovulate [92, 102, 103] and in both cases new wave emergence occurs [80, 104, 105]. Mechanisms of recruitment, selection and dominance appear to be highly conserved among monovular species [35, 106].

In camelids follicular development also occurs in waves and patterns of follicular development have been well studied in non-mated (non-ovulatory), mated (ovulated and non-pregnant) and pregnant llamas [54]. In this study, the wave pattern of follicular growth was demonstrated by detection of periodic growth of groups of follicles, the appearance of one follicle that grew larger than 7 mm and an inverse relationship between the diameter of the largest follicle and the number of follicles detected. Reports on intervals between waves and the life span of the dominant follicle have been variable. It appears that llamas have a longer inter-wave interval and a longer-lasting dominant follicle. Adams et al (1990) reported a period of 19.8 ± 0.7 days and 14.8 ± 0.6 days between successive waves in non-mated (non-ovulated) and pregnant llamas, respectively. The lifespan of the anovulatory dominant follicle was of 20 to 25 days. Also, in this study lactation and pregnancy were associated with a smaller dominant follicle and wave emergence was shortened by 2.5 ± 0.5 days in lactating females. In other reports on llamas and alpacas the dominant follicle appeared to last around 12 to 14 days and the inter-wave interval was of 11.1 and 15 for llamas and alpacas respectively [85, 107]. More recent studies in llamas agree with the results reported by Adams et al in which the life span of the dominant follicle was of 22.6 ± 2.5 and the inter-wave interval of 18.0 ± 2.6 days [108]. In a more recent study of alpacas, the reported inter-wave interval was 12 to 16 days [74].

Hormonal regulation of follicular wave dynamics has not been studied as much as it has been in cattle, but levels of estradiol have been positively correlated to follicular size in llamas and alpacas [85, 87]. Exposure to progesterone (i.e., CL in pregnant and non-pregnant llamas) also decreases the size of the dominant follicle and shortens the inter-wave interval in llamas [54].

2.3 Mechanisms of ovulation in cattle and camelids

"Ovula'tion. The escape of an ovum from the graafian follicle. O'vum – L. Egg – The egg or female sexual cell from which, when fecundated by union with the male element, a new individual is developed." [28]

The preovulatory LH surge is the main factor responsible for the cascade of events that lead to ovulation. In spontaneous ovulators, ovarian steroids are responsible for the positive feedback with the surge center in the anterior region of the hypothalamus. In the presence of low plasma progesterone concentrations, estradiol secreted by the dominant follicle incurs in a positive feedback with the hypothalamus inducing the secretion of more frequent LH pulses [81] that in turn will surge and initiate the cascade leading to the release of the oocyte from the dominant follicle into the uterus [109]. Estradiol together with the secretion of inhibin from the dominant follicle inhibits the production of FSH from the pituitary without affecting secretion of LH. The dominant follicle becomes LH-responsive and reaches preovulatory size. Once estrogens reach a threshold level in plasma, the hypothalamus is stimulated to secrete increased pulses of GnRH that in turn will increase LH pulsatility and provoke the preovulatory LH surge [81]. This peak of LH is responsible for several events that will lead to the rupture of the follicle and release of an egg [109]: 1) hyperemia, is thought to be controlled by the secretion of PGE and histamine induced by LH. Histamine also increases the vascular permeability and the theca interna becomes edematous. 2) After the LH peak, the theca interna starts to produce progesterone locally in the follicle. Progesterone stimulates the production of collagenase that in turn weakens the tunica albuginea that surrounds the ovary. As the surrounding of the ovary weakens, the apex of the follicle called the stigma begins to be apparent. 3) Production of PGF from the ovary is also stimulated by the LH surge and induces contractility of the smooth muscles of the ovary and release of lysosomes from the granulosa cells that will further deteriorate the connective tissue of the apex. All of these mechanisms, plus gap-junction breakdown between granulosa cells and the oocyte, lead to the final rupture of the follicle and release of the oocyte [109].

In induced ovulators, the mechanisms involved in stimulation of the pre-ovulatory LH surge are unclear. Administration of exogenous estradiol in rabbits and ferrets did not induce LH secretion [82, 83]. Like in spontaneous ovulators, GnRH secreted from the hypothalamus controls LH pulses. GnRH has been measured in rats [110], sheep [111], rabbits [112] and rhesus monkeys [113, 114] mainly through the insertion of a push-pull canulae in the median eminence of the brain or collection of portal blood. It was demonstrated in rabbits that GnRH release increases 40 to 100 fold within 20-60 minutes after coitus, followed by an increase in LH concentrations [112]. A previous study in llamas demonstrated that LH increases 15 minutes after copulation, peaks at 2 hours and declines to basal levels by 7 hours [86]. The rabbit and the monkey were compared to characterize differences between spontaneous and induced ovulators (reviewed in [115]. In this review evidence was presented that certain brain peptides and catecholamines such as noerepinephrine (NE) released from the mediobasal hypothalamus function in activating the hypothalamohypophysial-ovarian axis during spontaneous and induced ovulation. But the difference in time between the stimulus responsible for ovulation (coitus in rabbits and estrogen in monkeys) and the activation of this neuroendocrine system implies that different mechanistic steps are involved in the surge release of NE, GnRH and ultimately LH in spontaneous and induced ovulators.

Thus, what is it that induces ovulation in reflex ovulators?

2.3.1 Dogma on induced ovulation

In induced ovulators "ovulation is triggered by the physical stimulation of the genitalia." [13]

Correlation between natural matting and ovulation in camelids has been the subject of many studies. Ovulation rates and intervals from treatment to ovulation have been described for natural matting and different ovulation inducing drugs. In the 1970's a study carried out in alpacas [13] reported significantly higher number of ovulations when females were mated by intact or vasectomized males and when hCG was used compared to unmated females, but the interval to ovulation was not reported. In another study on alpacas [52], the earliest ovulations were detected 26 hours after mating and 24 hours after hCG treatment, but the mean interval to ovulation was not reported. Bourke [116] reported an interval of 28 hours from GnRH treatment to ovulation. Daily ultrasonography monitoring of llama ovaries determined that ovulation occurred 2.1 ± 0.1 days after natural mating [54, 66], but more frequent ultrasonographic examination (every 4 hours) revealed a mean interval from first mating (or GnRH or hCG treatment) to ovulation of 29.5 hours [57]. Although this supports the notion that ovulation is coitus-induced, this dogma has been challenged since the 1980's when researchers in China reported 87% of females ovulating after injection of seminal plasma into the vagina through a rubber inseminating tube used for horse artificial insemination in Bactrian camels [117].

2.3.2 Role of seminal plasma in induced ovulators and evidence of action in spontaneous ovulators

Seminal plasma has traditionally been thought to aid in the transport of sperm through the male and female reproductive tract during mating. It is now known that it also contains substances such as proteins, cytokines, growth factors and hormones that affect sperm and reproductive functions in the female.

Since initial studies done on Bactrian camels [117], the role of semen and seminal plasma in induction of ovulation has been tested in different species [7, 23]. Secretion of LH from rat pituitary cells after treatment with alpaca seminal plasma suggested the existence of a GnRH-like factor in the seminal plasma of alpacas [118]. Systematic studies carried out in our laboratory clearly document the existence of an ovulation-inducing factor (OIF) in the seminal plasma of llamas and alpacas [120]. Females were treated with alpaca seminal plasma, llama seminal plasma or saline. Ovulation was detected in 13/14 alpacas (Experiment 1), 9/10 llamas (Experiment 2) and 6/6 llamas (Experiment 3) when treated with a single intramuscular injection of 1 to 1.5 ml of alpaca or llama seminal plasma. Ovulations were preceded by an elevation in plasma LH concentrations that were higher and lasted longer than that elicited by GnRH treatment. Also, the CL formed after seminal plasma treatment tended to be larger and grow longer than CL induced by GnRH. Plasma progesterone concentrations were twice as high in the seminal plasma- vs GnRH-treated llamas, and mean interval from treatment to ovulation was 29.3 ± 0.7 hours. This report clearly documented the existence of an ovulation-inducing factor in the seminal plasma of llamas and alpacas, and suggests that OIF and GnRH are different molecules and affect the pituitary in different ways.

Subsequent studies determined that the effect of seminal plasma was exerted systemically rather than locally [121]. In this study, alpacas were treated with 2 ml of seminal plasma intramuscularly, by intra-uterine infusion or by intra-uterine infusion followed by endometrial curettage. The proportion of ovulation was highest for the intramuscular treated group (93%) compared to the intra-uterine treated group (41%), and intermediate for those treated with intra-uterine infusion and endometrial curettage (67%). This study suggested that endometrial curettage may facilitate the absorption of ovulation inducing factor.

The Bactrian camel was the subject of initial studies on OIF [117, 122-124]. Levels of LH increase 4-6 hours after coitus, AI or intramuscular injection of seminal plasma and ovulation occurs within 30-40 hours. In a study of koalas, a luteal phase was induced in 7/9

and 4/9 koalas artificially inseminated via the urogenital sinus with or without subsequent glass rod stimulation of the genitalia, respectively [23]. Authors concluded that since stimulation of the genitalia alone did not result in a luteal phase, that semen was necessary for development of a normal luteal phase.

The presence of OIF in spontaneous ovulators such as bulls and boars has also been suggested. Studies in our lab demonstrated the presence of an ovulation-inducing factor in the semen of bulls [7]. In this experiment, llamas were injected with 2 ml of saline or bull, llama or alpaca seminal plasma. The proportions of ovulations for each group were 0%, 26%, 100% and 100% for saline, bull, alpaca or llama seminal plasma treated groups respectively. An earlier study in Bactrian camels also suggested the presence of a factor in the seminal plasma of bulls that could elicit ovulation in camels [122]. Females were treated with seminal plasma intramuscularly or by uterine deposition of bull seminal plasma. Treatment induced ovulation in 3/5 females. The effect of mating in cattle had already been suggested in the 1950's when a study reported an earlier preovulatory LH surge and consequent ovulation in those animals mated during the first 6-8 hours of behavioral estrus [25]. Effects of seminal plasma of boars deposited in uterus on ovulation are controversial, but studies suggest that the effects of seminal plasma on ovarian function may contribute to the reproductive success in gilts and sows [125, 126]. In one of the experiments, seminal plasma treatment did not affect the number of ovulations but weight of the CL and plasma progesterone concentrations were higher for this group [126]. Insemination or mating at the beginning of estrus resulted in ovulation 14 hours before expected in gilts and sows [26, 127].

In most of the experiments carried out in llamas and alpacas, the dose of seminal plasma injected or infused into the uterus was relatively conservative (1-2 ml) [7, 120, 121]. Differences between studies from our laboratory in the ovulatory response of females treated by intrauterine deposition of semen (0%, [120] compared to 41%,[121]) provided impetus to examine the effect of dose and distribution over the endometrial surface. This

provided rationale for the hypothesis that OIF may have a dose dependent effect on ovulation and CL form and function.

It is also important to mention that in all of these studies, females were treated with seminal plasma. To the date, purification of OIF has only been attempted from the seminal plasma of Bactrian camels [128, 129] but the bioactive fraction was not tested in-vivo. Purification of OIF has been carried out from llama seminal plasma in our laboratory (Ratto, 2008; unpublished data). In camels, partial purification was accomplished by ion-exchange chromatography and authors concluded that the bioactive portion of OIF was a sub form (F3-3) composed of two components that were difficult to separate by FPLC because of their molecular similarities [129]. One of these studies also suggested the similarity of OIF in camel seminal plasma and bull seminal plasma, further suggesting its presence in spontaneous ovulating species [128]. In the experiments described in this thesis, purified OIF from llama seminal plasma was used. Purification was accomplished by applying llama seminal plasma to two different protein separation columns (HA and FPLC) and diluted properly into the corresponding treatment concentrations.

2.3.3 OIF – Summary of current knowledge on ovulation-inducing factor

In summary, we know that ovulation-inducing factor (OIF):

- Is a protein molecule (ref) found in the seminal plasma of induced and spontaneous ovulators (Ratto, 2008; unpublished data)

- Is conserved across species; it is found in the seminal plasma of Bactrian camels, llamas alpacas and cattle

- Is produced by the accessory sex glands of the male and it is not known if it is also produced by the testis

- represents 60% of the total amount of protein present in the seminal plasma of llamas and alpacas (Ratto, 2008; unpublished data)

- acts through a systemic rather than a local route [120]

- has a very powerful ovulatory effect; a conservative dose of 1 to 2 ml of seminal plasma injected intramuscularly in camelids induced high proportion of ovulations and higher levels of LH compared to GnRH [120, 121]

- is luteotrophic, evidenced by larger CL diameter and plasma progesterone concentrations [120]

CHAPTER 3
OBJECTIVES & HYPOTHESES

The overall goal of this thesis research was to elucidate the biological characteristics of ovulation-inducing factor in seminal plasma, and thereby contribute to our understanding of the ovulatory mechanism in induced and spontaneously ovulating species.

The specific objectives of the first study (Chapter 5) were to:

- determine the minimum dose of ovulation-inducing factor necessary to elicit ovulation
- test the hypothesis that the ovulatory dose of OIF is physiologically relevant – that is, a fraction of that normally present in an ejaculate
- test the hypothesis that corpus luteum form and function is affected by OIF in a dose-dependent manner.

The specific objectives of the second study (Chapter 6) were to:

- determine if OIF from llama seminal plasma would induce ovulation and affect CL form and function in pre-pubertal heifers and
- test the hypothesis (based on initial results in prepubertal heifers) that administration of purified OIF at different stages of development of the dominant follicle of the first follicular wave would induce atresia of the dominant follicle and earlier emergence of a new follicular wave.

CHAPTER 4
DOSE RESPONSE TO OVULATION-INDUCING FACTOR (OIF) IN FEMALE LLAMAS

4.1 Abstract

The objectives of the study were to determine if the dose of ovulation-inducing factor (OIF) of llama seminal plasma required to elicit ovulation is physiologically relevant, and to test the hypothesis that CL form and function is affected by OIF in a dose-dependent manner. Female llamas were assigned randomly to five groups (n=10 per group) and given a single intramuscular dose of 500 μg, 250 μg, 125 μg, or 60 μg of purified OIF (representative of the amount present in $1/6^{th}$ to $1/50^{th}$ of a normal ejaculate) or 1 mL PBS (controls). Ovulation and CL development were monitored by transrectal ultrasonography. Blood samples were taken every-other-day from Days 0 (day of treatment) to 16 for measurement of plasma progesterone concentrations. More frequent blood sampling (16 samples in 12 hours) was done immediately after treatment on four animals per group to determine changes in plasma concentrations of LH. Results supported the hypothesis that OIF affects ovulation and CL form and function in a dose-dependent manner. The high dose of OIF (500 μg) was associated with the highest incidence of ovulation ($P<0.05$), largest CL diameter ($P<0.05$), highest day-to-day profiles of CL diameter and highest plasma progesterone concentrations (day-by-group interaction, $P<0.01$). A rise in plasma LH concentration was apparent in all llamas that ovulated and was highest in the high dose group (day-by-group interaction, $P<0.01$). The low dose of OIF (60 μg) was minimally effective for induction of ovulation ($P<0.05$), and the least luteotrophic as evidenced by the smallest maximum CL diameter, day-to-day CL diameter profile, and lowest plasma concentrations of progesterone and LH. End points were intermediate for the remaining two treatment groups of 125 and 250 μg. We conclude that OIF from llama seminal plasma has a dose-dependent effect on ovulation rate and CL form and function in llamas, and the effect occurred at physiologically relevant doses.

4.2 Introduction

Ovulation is the process preceded by an elevation in circulating plasma LH concentrations [81] that ends with the rupture and evacuation of the contents of the dominant follicle [109]. Mammalian species have been classified into “induced” or “spontaneous” ovulators [3]. In spontaneous ovulators, the preovulatory LH surge is the result of a positive feedback effect of rising circulating concentrations of estradiol, secreted mainly by the dominant follicle, in the absence of progesterone [81, 130]. In contrast, the trigger for eliciting the preovulatory LH surge in induced ovulators, such as camelids, has been attributed primarily to a neuro-endocrine response to the physical stimulation of the genitalia during copulation or other physical stimulation such as olfactory, auditory or visual factors [13, 53, 86]. However, the results of an early study on Bactrian camels, also considered an induced ovulator, suggest the presence of an ovulation-inducing factor in the semen of this species [117]. More recently, the presence of an ovulation-inducing factor in the seminal plasma of llamas and alpacas has been clearly documented [120, 121]. In these experiments, ovulation occurred in over 90% of llamas and alpacas treated intramuscularly with a relatively conservative dose of seminal plasma. Ovulation in seminal plasma-treated animals was consistently preceded by a rise in plasma LH concentrations. The magnitude of the LH response was greater in seminal plasma-treated animals than in GnRH–treated animals [120], and was associated with a subtle increase in CL diameter and a dramatic increase in plasma progesterone concentration, suggesting a powerful luteotrophic effect. A systemic rather than a local mode of action of OIF was deduced from the results a study in which more females ovulated after intramuscular treatment than by intrauterine deposition of seminal plasma (93% and 41%, respectively) and intermediate (67%) for animals treated by intrauterine deposition followed by endometrial curettage [121]. .This is consistent with results of a study in which alpaca seminal plasma stimulated secretion of LH from rat pituitary cells in vitro [118]. In most of the studies about OIF [7, 57, 117, 118, 120-122, 124], animals were treated with seminal plasma. In this study, OIF was previously isolated and purified by applying llama seminal plasma to two different protein separation columns and diluted properly into the corresponding treatment concentrations.

Based on the total amount of protein and OIF purified where 1 ml of llama seminal plasma contains approximately 1.5 to 1.8 mg of OIF, the objectives of this study were to determine if the minimum dose of purified OIF required to provoke an ovulatory response is physiologically relevant (this is what proportion of an ejaculatory dose), and to test the hypothesis that CL form and function are affected by OIF in a dose-dependent manner.

4.3 Material and methods

4.3.1 Seminal plasma collection

Semen was collected from four mature male llamas (5 to 7 years old) 2 to 3 times a week over a period of 2 months using an artificial vagina inserted into a wooden phantom [131]. Semen samples were processed according to procedures previously described by Adams et al, 2005. Briefly, semen samples were diluted 1:1 (v:v) with phosphate buffered saline (PBS, Invitrogen, Grand Island, NY), drawn back-and-forth through an 18-guage needle attached to a 10 cc syringe and centrifuged at 1500 x g for 30 minutes to decant spermatozoa. A drop of the supernatant was then evaluated by microscopy to confirm the absence of cells. If spermatozoa were detected, the sample was centrifuged and evaluated again in like manner until no spermatozoa were detected. Sperm-free samples were stored at -70^{o}C until further purification.

4.3.2 Protein purification

Hydroxyl Apatite Column Chromatography

Seminal plasma samples from the 4 males were pooled and purified in 12 replicates of approximately 12 ml of seminal plasma each. Seminal plasma was then sonicated to reduce viscosity. Sonification was done on ice using a Sonifier® Cell Disruptor with a microtip (Model W185, Heat Systems-Ultrasonics, Inc. ,Plainview,NY, USA) by applying five 15-second episodes at 70% of maximum power (35 watts) with 45-second rest periods between episodes. The sonicated sample was centrifuged at 10,000 x g for 20 minutes. Llama seminal plasma was then loaded onto a Macro-Prep ceramic Hydroxyapatite column Type 1, 20µm (Bio-Rad®, Ontario, Canada) which was previously equilibrated with 10mM Sodium Phosphate, pH 6.8. Approximately 24-30 mg of total protein for each replicate (n=12 samples) were loaded onto the column (1 x 10 cm), purification procedure was performed at room temperature and at flow rate

of 0.5 ml/min. Once the sample entered the column, the flow was stopped for 30 min to allow protein binding to the slurry. Elution was carried out using lineal gradient with 350 mM Sodium Phosphate, pH 6,8 and 2 ml fractions were collected. Their absorbance was measured at 280 nm and three peaks recovered. Fractions corresponding to each protein peak were pooled, concentrated and buffer exchanged into Phosphate Buffer Saline (PBS), pH 7.4 using 20 ml ultra filter device (Vivaspin, Vivascience/Sartorius, Goettingen, Germany) with a membrane cut off of 5 kDa.

Fast Protein Liquid Chromatography (FPLC)

After the Hydroxyl Apatite (HA) column, samples were filtered through a 0.2 μM syringe filter (Millipore; Bedford, MA, USA) and for each replicate (n=10) only the last protein fraction eluted from HA chromatography column and containing approximately 6-8 mg of protein was loaded onto a 320 ml size exclusion column (SEC, Hi Prep™ 26/60 Sepahacryl™ S-100) attached to the AKTA FPLC system (Amersham Biosciences Inc, Quebec, Canada). Elution was isocratically performed using PBS, pH 7.4. at a flow rate of 0.5 ml/min and 4 ml fractions were collected. Fraction absorbance was measured at 280 nm and those from the second eluted peak, corresponding to a protein of approximately 24 kDa MW, were pooled and concentrated in PBS, pH 7.4 using 20 ml ultra filter device (Vivaspin) with a membrane cut off of 5 kDa. Samples were then stored at -70^{o}C and diluted to 500 μg, 250 μg, 125 μg and 60 μg per ml of PBS before use.

All peaks from both the HA and FPLC columns were sampled and analyzed for the presence of OIF using SDS-PAGE through 12% polyacrylamide gels based on the protocol of Laemmli (1970). Protein concentration was determined using the micro-assay method (Bio-Rad®, Ontario, Canada) based on the method first described by Bradford [132].

Protein purification was carried out in 12 replicates of 12 ml of seminal plasma, each of which contained 24-30 mg of total protein before being applied to the HA column. The total amount of ejaculates collected from all four males was 72 (18 ejaculates per male). Thus, if the average volume per ejaculate in the llama is 2 ml, then the approximate concentration of total protein in undiluted ejaculate ranged from 5 to 6 mg/ml.

4.3.3 Animals and treatments

Mature non-lactating female llamas (n=10 per group), ≥4 years of age and weighing between 90 and 120 kg were selected from a herd of 53 llamas during July and August at the University of Saskatchewan, Canada (52°N, 106°W and 500 m above sea level). Llamas were examined daily by transrectal ultrasonography using a 7.5 MHz linear-array transducer [66] (Aloka SSD900, Tokyo, Japan) and when a follicle ≥7 mm that grew for 3 consecutive days was detected [54], they were assigned randomly to one of four treatment groups (10 llamas per group). Llamas were given a single intramuscular injection (semimembranosus or semitendinosus muscle) of: a) 1 ml phosphate buffered saline (PBS, negative control); or b) 500 μg, c) 250 μg, d) 125 μg purified OIF in 1ml PBS. Doses were based on the results obtained in previous studies carried out in our lab in which a relatively conservative im dose of 1-2 ml of unpurified seminal plasma (equivalent to 1.5-3 mg of OIF) resulted in 90-100% ovulation in female llamas [120, 121]. Thus, if the ejaculate has an average volume of 2ml [131], doses represent $1/6^{th}$ to $1/25^{th}$ of the amount of OIF normally present in a single ejaculate and allow us to determine the biological potency and importance of such molecule in causing ovulation.

The experiment was conducted in two replicates, n=6 per group in the first replicate and n=4 per replicate in the second. The second replicate involved frequent blood sampling to determine the endogenous LH response to treatment. To facilitate data collection in the second replicate, follicular wave emergence was synchronized among animals by giving a single intramuscular dose of pLH (5 mg Armour standard; Lutropin- V, Bioniche Animal Health, Belleville, Ontario, Canada)[57]. Llamas were re-examined 12 days later - enough time to allow CL regression in those that ovulated in response to pLH treatment and for all to have developed a new mature dominant follicle. Llamas with a follicle ≥7 mm and no CL were assigned randomly to the respective treatment groups. Based on the unexpectedly high ovulatory response to all doses of OIF in the first replicate, an additional group was included in the second replicate (n = 10) and treated with a lower dose (60 μg) of purified OIF representative of $1/50^{th}$ of the total amount of OIF present in a single ejaculate.

Ovaries were examined daily by transrectal ultrasonography until Day 3 (Day 0 = day of treatment) to detect ovulation and every-other-day thereafter until Day 16 to monitor CL form

and function [56]. Ovulation was defined as the disappearance of a large follicle (≥8 mm) from one examination to the next, and confirmed by the detection of a CL in subsequent examinations [66]. The onset of CL regression was defined as the first day the CL began a progressive decrease in diameter [120].

For measurement of plasma progesterone concentration in both replicates, blood samples were collected from the jugular vein into heparinized tubes (Vacutainer Systems; Becton Dickinson, Franklin Lakes, NJ, USA) every-other-day from the day of treatment. Plasma progesterone concentration was measured using a commercially available double-antibody radioimmunoassay kit (Coat-a-Count total progesterone; Diagnostic Products Corporation, Los Angeles, CA) [56]. Samples were analyzed in two consecutive assays with intra-assay coefficients of variation of 4.5%, 3.2% and 8.8% for reference concentrations of 1.9, 3.6 and 16.6 ng/ml, respectively for the first assay and 6.8%, 2.0% and 1.7% for reference concentrations of 1.6, 3.2 and 16.0 ng/ml, for the second assay. Inter-assay coefficients of variations were 9.2%, 6.5% and 6.9% for reference concentrations of 1.78, 3.49 and 16.36 ng/ml, respectively.

More frequent blood samples were collected from 4 animals per group in the second replicate for measurement of plasma LH concentration [120]. Samples were taken immediately before treatment, every 15 minutes until Hour 2, and again at Hours 3, 4, 5, 6, 7, 8, and 12 after treatment. A catheter was placed in the jugular vein on the day before treatment to minimize the effect of stress at the time of sampling. All samples were centrifuged within 2 hours of collection at 1700 x g for 20 min and plasma was stored at -20 ^{o}C. Plasma LH concentrations were measured using a double-antibody radioimmunoassay [120] and are expressed in terms of NIAMDDK-oLH-24. The minimum detectable limit of the assay was 0.1 ng. The range of the standard curve was 0.06 ng (80% ligand labeled LH) to 8.0 ng (20% ligand labeled LH). The intra- and inter-assay coefficients of variation for the low reference plasma LH concentration (0.13 ng/ml) were 10.3% and 8.3%, respectively. The intra- and inter-assay coefficients of variation for the high reference plasma LH concentration (0.99 ng/ml) were 11.1% and 10.0%, respectively.

4.3.4 Statistical analyses

Single-point measurements (i.e., follicle size at the time of treatment, day of appearance of the CL, maximum CL diameter, CL diameter on Days 8 and 14) were compared by 2-way analysis of variance to determine the effects of treatment and replicate. Serial data (i.e., plasma progesterone and LH concentrations, CL diameter profile) were compared among groups by 2-way analysis of variance for repeated measures to determine the effects of treatment and replicate. In the absence of a replicate effect, data from the two replicates were combined. Tukey's multiple comparison was used as a post-hoc test when a main effect of treatment or a treatment interaction was detected. Ovulation rates were compared among groups by chi-square analysis. Significance was assumed when the probability of values differing by chance alone was ≤0.05. Data were centralized to the day of treatment (Day 0) for preparation of figures. All statistical analysis was made using SAS statistical software (SAS, Statistical Analysis System Institute Inc., Calgary, NC).

4.4 Results

The diameter of the preovulatory follicle at the time of treatment did not differ among groups (P=0.3, Table 5.1). The proportion of llamas that ovulated was lower (P< 0.05) in the control and low-dose (60 μg) groups compared to the higher-dose groups (Table 5.1).

Table 4.1 Effect of dose of OIF on ovulation in llamas (mean ± SEM).

Group	Saline	60 μg	125 μg	250 μg	500 μg
Follicle diameter on day of treatment (mm) *	10.6 ± 0.6	10.6 ± 0.3	9.0 ± 0.2	9.1 ± 0.2	10.5 ± 0.1
Proportion of ovulations	0/10[a]	3/10[a]	7/10[b]	9/10[b]	9/10[b]
(%)	(0)	(30)	(70)	(90)	(90)

*No significant difference among groups (P>0.3)

[a, b] Proportions with different superscripts are different (P<0.05)

In those that ovulated, the CL was detected earliest (P<0.05) and reached the greatest diameter (P<0.05) in the high-dose group (500 μg; Table 5.2). Conversely, CL detection was

latest (P<0.05) and diameter was smallest (P<0.05) in the low-dose group (60 μg). By Day 14 after treatment, the diameter of the regressing CL did not differ among groups (Table 5.2).

Table 4.2 Effect of dose of OIF on CL development in llamas that ovulated in response to treatment (mean ± SEM, Day 0 = day of treatment).

Dose	60 μg (n=3)	125 μg (n=7)	250 μg (n=9)	500 μg (n=9)
Day of 1st detection of CL	3.3 ± 0.3^{a}	2.3 ± 0.2^{b}	2.5 ± 0.2^{b}	2.1 ± 0.1^{b}
Maximum CL diameter (mm)	10.9 ± 1.0^{a}	11.6 ± 0.7^{ab}	10.8 ± 0.7^{a}	13.5 ± 0.5^{b}
CL diameter on Day 8 (mm)	8.5 ± 2.0^{a}	11.3 ± 0.8^{ab}	10.4 ± 0.7^{ab}	12.8 ± 0.6^{b}
CL diameter on Day 14 (mm) *	7.8 ± 0.9	7.5 ± 1.2	7.2 ± 0.3	8.3 ± 0.6

[a, b] Values with different superscripts are different (P<0.05)

*No significant difference among groups

The day-to-day CL diameter profile was greatest in the 500 μg group, intermediate for the 250 and 125 μg groups and lowest for the 60 μg group (day effect, P<0.01; group effect, P<0.05; day-by-group interaction, P<0.01; Fig. 5.1a). Plasma progesterone concentrations in those that ovulated were significantly higher in the high-dose group and tended to be lower in the remaining treatment groups (day effect, P<0.01; group effect, P=0.03; day-by-group interaction, P=0.15; Fig. 5.1b). This effect was more evident when all animals, regardless of whether they ovulated, were taken into consideration (day effect, P<0.01; group effect, P<0.01; day-by-group interaction, P<0.01; Fig. 5.1c).

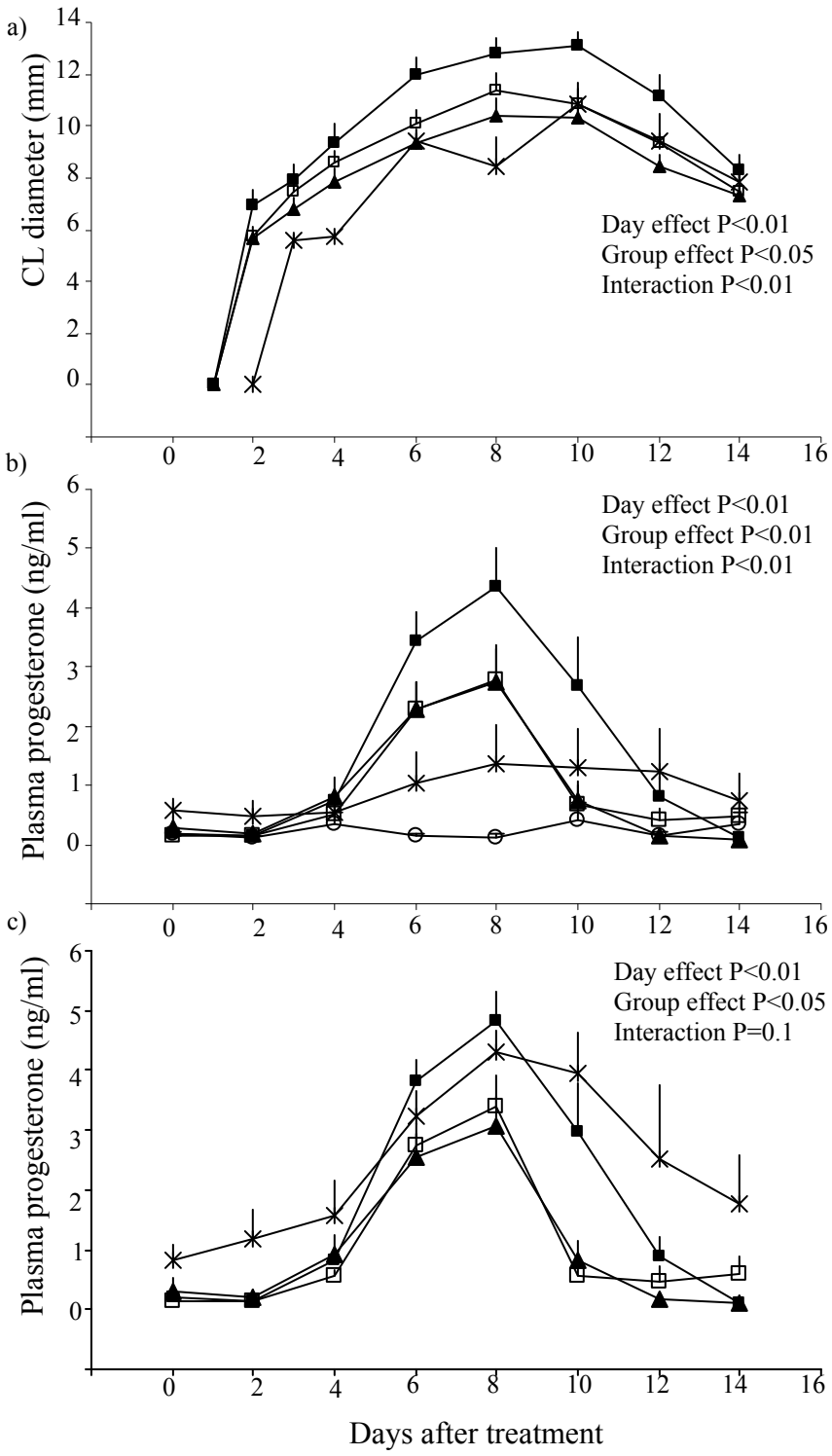

Figure 4.1 CL diameter (a) and plasma progesterone concentrations (b and c) in llamas given a single intramuscular dose of OIF (60 µg *, 125 µg □, 250 µg ▲, 500 µg ■) or PBS (○); n=11 per group. In panel (c), data from llamas that did not ovulate were excluded; i.e., n=3, 7, 9, and 9 in groups treated with 60, 125, 250, or 500 µg OIF, respectively (mean ± SEM).

A rise in plasma LH concentration was apparent in all llamas that ovulated, and absent in all that failed to ovulate. Data from all animals were included in the analysis of the day-to-day LH profile (Fig. 5.2) except for one llama from each of the 60, 250 and 500 µg groups that were excluded from the analysis because plasma concentrations of LH, averaged over all days, was more than 14 standard deviations above the average for the group. Despite the high LH values, ovulation was not detected in these individuals. Over the 12-hour sampling period, plasma LH concentrations tended to increase more rapidly, reached a greater maximum, and remained elevated longer in the high-dose group than in all other groups. The magnitude of the LH profile decreased in a dose-related manner.

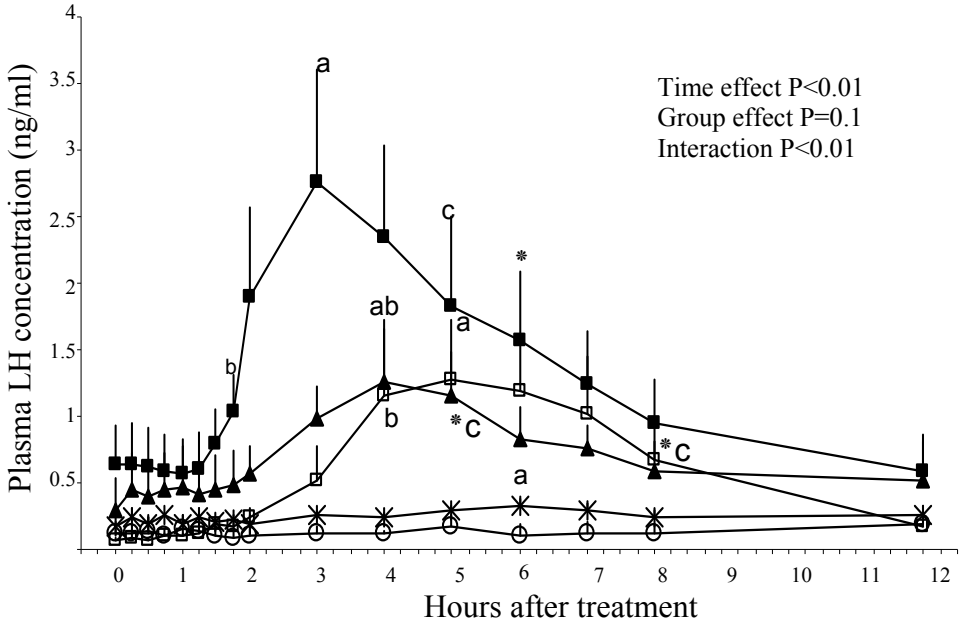

Figure 4.2 Plasma LH concentrations (mean ± SEM) in female llamas that ovulated following treatment with OIF (500 µg ■; 250 µg ▲, 125 µg □, 60 µg * and PBS ○).
[a] Within group, the maximum concentration (P<0.05). [b] Within group, the first increase (P<0.05). [c] Within group, the first decrease from maximum (P<0.05). *Within group, the last value higher than pretreatment levels (P<0.05).

4.5 Discussion

The presence of an ovulation-inducing factor in the seminal plasma of llamas and alpacas has been documented previously [120]. In the latter, not only did intramuscular injection of seminal plasma induce ovulation in more than 90% of the female llamas but also the preovulatory surge of LH appeared later and lasted longer when compared to animals treated with GnRH. The CL in these animals was larger and plasma progesterone concentrations were double of those present in GnRH treated females.

The present study was designed to determine if the dose of OIF necessary to induce ovulation in llamas is physiologically relevant and to characterize the dose-effect on CL formation and function. Results from the present experiment suggest that effects exerted by intramuscular injection of OIF are affected by dose. Four different doses were chosen based on the knowledge that for every ejaculate (of 2-3 ml) [131] approximately 3 mg of OIF were isolated with our purification process. Thus, the maximum dose in this experiment represented $1/6^{th}$ of what is normally present in an ejaculate (500 μg) and because an intermediate dose of 125 μg still induced ovulations in 70% of the females, a low dose representative of approximately $1/50^{th}$ of the normal amount present in a single ejaculate was added to the treatment groups. The dose effect was evidenced by higher ovulation rates (90% in the high dose group, $P<0.05$), earlier appearance of the CL and larger CL diameter and CL diameter profiles in the high dose group. On the other hand, ovulation rates and CL characteristics were lowest in the low dose group and intermediate for the 125 and 250 μg groups. As in previous studies, ovulation was preceded by an elevation in plasma LH concentrations. Plasma LH concentrations were highest in the high dose group and lowest in the low dose and saline-treated groups ($P<0.01$). Plasma progesterone concentrations were also higher in the high dose treated group, supporting the hypothesis of a luteotrophic effect of OIF. The lack of statistical difference with the low dose group for some end points such as plasma progesterone concentrations of ovulated animals, may have been the result of a low number of animals that ovulated (n=3), but was clearly evidenced when all animals, ovulated and non-ovulated, were included in the final analysis. A dose effect was also demonstrated when alpacas were treated with intrauterine deposition of seminal plasma [121]. In a previous study [120], the deposition of 1 ml of seminal

plasma into the uterine body of alpacas did not result in ovulations,. While 1 ml of seminal plasma deposited in each horn (2 ml total) resulted in 41% of ovulations in a next experiment [121],. This effect was probably a result of both dose and distribution over the endometrial surface.

Intramuscular rather than intrauterine treatment was chosen in the present experiment to minimize potential confounding effects of local stimulation of the genitalia. In addition, results of previous studies [121] documented a significantly higher incidence of ovulation following intramuscular administration of seminal plasma compared to intrauterine deposition in female alpacas (93% vs 41%, respectively). The effect of OIF mediated via a systemic (indirect) rather than a local (direct) route is evidenced by the observations that treatment with OIF is followed by a surge in circulating concentrations of LH, and that the incidence of ovulation was increased (i.e., to 67%) when intrauterine deposition was followed by endometrial curettage [121]. This was consistent with the findings in the same species where a dose of LH (5 mg, Lutropin) induced ovulation in >80% of alpacas when given intramuscularly [7, 133] failed to induce ovulation when given by intrauterine deposition [120]. Similarly, in rabbits, the dose of a GnRH analogue required to induce ovulation was ten times higher when infused into the uterus than when given intramuscularly (8 μg versus 0.8 μg Buserelin per female) [134]

It is evident, that the difference between spontaneous and induced ovulating species is not as clear as it once seemed. Not only does intramuscular injection of OIF induce ovulation, but it also exerts its effects at physiologically relevant doses. Ovulation can be induced in over 70% of female llamas with a dose as small as 125 μg ($1/25^{th}$ of an ejaculate) of purified OIF.

In summary, OIF is a very potent ovulation inducing factor found in high concentrations in the seminal plasma of llamas. A dose dependent effect of OIF was clearly demonstrated by: 1) higher ovulation rates, 2) larger CL diameters, and 3) higher plasma progesterone concentrations in those animals treated with a higher dose (500 μg) of purified OIF. The minimum effective dose appeared to lie somewhere between physiologically relevant concentrations of 60 μg and 250 μg of purified OIF, equivalent of that present in less than $1/6^{th}$ of a normal ejaculate.

CHAPTER 5
EFFECT OF PURIFIED LLAMA OVULATION-INDUCING FACTOR (OIF) ON OVARIAN FUNCTION IN CATTLE

5.1 Abstract

Systemic administration of ovulation-inducing factor (OIF), discovered recently in seminal plasma of llamas and alpacas (induced ovulators) and cattle (spontaneous ovulators), has been shown to induce ovulation in >90% of female llamas and alpacas. The objective of the present study was to determine the effect of purified OIF on ovarian function in cattle. In Experiment 1, pre-pubertal heifers (n=11 per group) were given an intramuscular injection on Day 5 of the first follicular wave after follicular ablation of a) 1ml of saline (negative control) b) 100 μg of GnRH (positive control) or c) 1.0 mg purified OIF per 100 kg of body weight. Ovulation occurred in 9/11 (82%) of GnRH-treated heifers, and 1/11 (9%) heifers in each of the OIF- and saline-treated groups ($P<0.05$). A surge in plasma LH concentration was detected in the GnRH group ($P<0.01$), but remained basal in the OIF- and saline-treated groups. The interval from treatment to follicular wave emergence was shorter ($P<0.05$) in heifers treated with GnRH and OIF than in those treated with saline (1.7 ± 0.3 days, 1.8 ± 0.2 days, 3.5 ± 0.4 days, respectively). Plasma FSH concentrations tended to be higher in the OIF treated group ($P=0.1$) and remained basal for the saline treated group. Follicle diameter profiles and maximum diameters of the dominant follicle were smaller in the OIF- treated group than in the control group (12.0 ± 1.0, 10.8 ± 0.4 and 12.4 ± 0.3 for the GnRH-, OIF- and saline- treated groups, respectively; $P<0.01$). Diameter profile of the first subordinate follicle of the same wave tended to be larger ($P=0.05$) and remained for a longer period of time in those animals treated with OIF compared to the control group. In the second experiment, sexually mature heifers (n= 5 per group, n=10 untreated control group) were given a single intramuscular injection on Days 3, 6 or 9 of the first follicular wave after ovulation of a) 100 μg of GnRH (positive control) or b) 1.0 mg purified OIF per 100 kg of body weight. Effects on follicular wave dynamics could not be clearly

confirmed due to differences in observation and blood sampling frequency with the untreated control group, but follicle diameter profiles tended to be smaller (P=0.1) and plasma FSH concentrations tended (P=0.1) to be higher in those animals treated with OIF on Day 6. These results provide the rationale for the hypothesis that OIF induces secretion of low levels of FSH that stimulate the largest subordinate follicle at the time of treatment to continue growing to a size where both the dominant and subordinate follicles compete for LH leading to a smaller dominant follicle, less secretion of estradiol and therefore, less negative feedback on FSH secretionwhich results in emergence of a new follicular wave.

5.2 Introduction

Spontaneous ovulators, such as cattle, are species capable of ovulating without the need of copulatory stimulation. In the presence of low plasma progesterone concentrations, estradiol secreted by the preovulatory follicle has a positive feedback effect on the hypothalamus leading to a surge release of LH [81] and the initiation of the ovulatory cascade [109]. Conversely, induced ovulators – such as llamas, alpacas and camels, are species that, in the absence of an neuro-endocrine response to copulation, will not ovulate or do so at a very low incidence (5% in alpacas) [4, 13, 54]. Several studies have been done to determine the role of semen or seminal plasma in the induction of ovulation in Old and New World camelids [117, 120-122, 124] and other induced ovulators such as koalas [23], but few reports were found regarding the importance of such factors in spontaneously ovulating species. In pigs, a reduction in the interval between LH peak and ovulation has been reported [26]. Another study carried out in pigs, failed to detect a difference in timing or incidence of ovulation but reported an increase in CL size and progesterone secretion after intrauterine infusion of seminal plasma in-vivo and increased secretion of progesterone and responsiveness to LH after seminal plasma treatment of granulosa and theca cells from preovulatory follicles in-vitro [126]. No references were found to studies on the effect of seminal plasma on ovulation in cattle.

Results of a recent study of the effects of bovine seminal plasma on ovulation in llamas, however, suggest that ovulation-inducing factor (OIF) may be widely conserved among induced and spontaneous ovulators [7]. After intramuscular treatment with an equivalent volume of seminal plasma from llamas, alpacas and bulls (i.e., 1 ml), ovulation was induced in 19/19 (100%), 19/19 (100%) and 5/19 (26%) female llamas, respectively, all of which were significantly higher than 0/19 (0%) in the control group. In a study done on Bactrian camels, 3/5 females ovulated after intrauterine or intramuscular treatment with bovine seminal plasma [122].

In both llamas and camels, the ovulation-inducing effect of seminal plasma was been attributed to a surge release of LH from the pituitary [7, 120, 121, 123]. Further, results of a recent dose-response study in llamas were consistent with the hypothesis that OIF affects

ovulation and CL function in a dose-dependent manner, and that this effect is at physiologically relevant doses. Doses of 1/6 to 1/50 of the amount of protein normally present in an ejaculate effectively induced ovulation and affected CL form and function [135].

The objective of the study was to determine the effects of purified OIF on ovarian function in cattle, as a representative species of spontaneous ovulators. We tested the hypotheses that OIF will induce ovulation and affect CL form and function in cattle (Experiment 1). Based on the results of Experiment 1, we subsequently tested the hypothesis that OIF, given at different stages of development of the first follicular wave, will induce atresia of the dominant follicle and hasten emergence of a new follicular wave (Experiment 2).

5.3 Material and methods

5.3.1 Experiment 1

5.3.1.1 Seminal plasma collection

Semen was collected from four mature male llamas (5 to 7 years old) 2 to 3 times a week over a period of 2 months using an artificial vagina and a wooden phantom mount [131]. To reduce viscosity, semen samples were diluted 1:1 (v:v) with phosphate buffered saline (PBS, Invitrogen, Grand Island, NY) and drawn back-and-forth through an 18-guage needle attached to a 10 cc syringe. Samples were then centrifuged at 1500 x g for 30 minutes to separate the spermatozoa from the seminal plasma. A drop of the supernatant was evaluated by microscopy to confirm the absence of cells. If spermatozoa were detected, the sample was centrifuged and evaluated again in like manner until no spermatozoa were detected [120]. Sperm-free samples were stored at -70^{o}C until further purification.

5.3.1.2 Protein purification

Seminal plasma samples from the four males were pooled and purified in 10 replicates of approximately 12 ml of seminal plasma each. Seminal plasma was sonicated on ice using a Sonifier® Cell Disruptor with a microtip (Model W185, Heat Systems-Ultrasonics, Inc.

,Plainview,NY, USA) to reduce viscosity. The sonicated sample was centrifuged at 10,000 x g for 20 minutes. For each of 10 replicates, llama seminal plasma (24-30 mg of total protein), was loaded into an Macro-Prep ceramic Hydroxyapatite column Type 1, 20 µm (Bio-Rad®, Ontario, Canada) which was previously equilibrated with 10 mM sodium phosphate at a pH of 6.8. Elution was carried out using lineal gradient with 350 mM sodium phosphate, pH 6.8. Absorbance of successive 2 ml fractions was measured at 280 nm and three peaks were identified. Fractions corresponding to each protein peak were pooled, concentrated and buffer-exchanged with phosphate buffered saline (PBS) at a pH of 7.4 using a 20 ml ultra filter device (Vivaspin, Vivascience/Sartorius, Goettingen, Germany) with a membrane cut off of 5 kDa. The last protein fraction eluted from each replicate through the HA chromatography column (i.e., peak C_2; Ratto et al., 2008) consisted of 6-8 mg of partially purified OIF protein, and was loaded onto a 320 ml size-exclusion column (SEC, Hi Prep™ 26/60 Sepahacryl™ S-100) attached to a AKTA FPLC™ system (Amersham Biosciences Inc, Quebec, Canada). Elution was performed using PBS at a pH of 7.4 and at a flow rate of 0.5 ml/min. Serial fractions (4 ml each) were collected and their absorbance at 280 nm was measured. Fractions corresponding to each protein peak were pooled, concentrated in PBS, pH 7.4 using 20 ml ultra filter device (Vivaspin) with a membrane cut off of 5kDa to a final concentration of 3 mg/ml. Samples were then stored at -70^{o}C.

All fractions from both the HA and FPLC columns were sampled and analyzed for the presence of OIF using SDS-PAGE through 12% polyacrylamide gels based on the protocol of Laemmli (1970). Protein concentration was determined using the micro-assay method (Bio-Rad®, Ontario, Canada) based on the method first described by Bradford [132].

Protein purification was carried out in 10 replicates of 12 ml of seminal plasma, each of which contained 24 to 30 mg of total protein before being applied to the HA column. The total number of ejaculates collected from all four males was 60 (15 ejaculates per male). Thus, if the average volume per ejaculate in the llama is 2 ml, then the approximate concentration of total protein in undiluted ejaculate ranged from 5 to 6 mg/ml.

5.3.1.3 Animals and treatments

Hereford crossbred heifers (n=33), 12.5 ± 0.5 months old (mean ± SEM) and weighing 350 to 370 kg were used during April and May at the University of Saskatchewan, Canada (52°N, 106°W and 500 m above sea level). Based on previous data [34], the heifers were approximately 1 to 2 month from reaching puberty (56.0 ± 1.2 weeks). Pre-pubertal heifers were chosen as experimental units to minimize the confounding effects of spontaneous ovulation and elevated plasma progesterone concentration in sexually mature animals. Transvaginal ultrasound-guided ablation of follicles ≥5 mm was done to synchronize follicular wave emergence among heifers [136]. Animals were assigned randomly to one of 3 treatment groups (n=11 per group) and treated on Day 5 (Day 0 = estimated day of follicular wave emergence, 1 day after follicular ablation; [137]) with a single intramuscular dose of: a) 1 ml phosphate buffered saline (PBS, negative control), b) 100 μg of GnRH (Cystorelin, Merial Can Inc., Victoriaville, PQ, Canada; positive control) or c) 1.0 mg purified OIF per 100 kg body weight. The effects of OIF appear to be mediated via a surge release of LH into circulation [120]; hence, GnRH was chosen as a positive control treatment over LH because of its similar effect on LH release. Day 5 of the follicular wave was chosen as the day of treatment, corresponding to the late growing phase of the dominant follicle [38], because previous studies demonstrated a better ovulatory response to GnRH treatment when heifers were treated on days 3 or 6 after wave emergence [137].

To moderate the ablation and examination schedule, the experiment was carried out in two replicates (n=17 for the first replicate and n=16 for the second replicate). The ovarian response was monitored daily by transrectal ultrasonography using a 7.5 MHz linear-array transducer (Aloka SSD900, Tokyo, Japan) from Day -3 to Day 0, and resumed from the day of treatment (Day 5 = day of treatment) to Day 9, and every 2 days thereafter for 17 days – enough time to detect ovulation and CL lifespan or to detect the emergence of the next follicular wave after treatment. Ovulation was defined as the disappearance of a large follicle (≥8 mm) from one examination to the next, and was confirmed by the detection of a CL in subsequent examinations. The dominant follicle of a wave was defined as the follicle that reached the greatest diameter. Wave emergence was retrospectively defined as the first day that the dominant

follicle was detected at 4-5 mm and was considered as the previous day if the follicle was first detected at 6-7 mm [37, 38]. The first subordinate follicle of a wave was defined as the second largest follicle at the time of treatment [38].

Blood samples were collected from the jugular vein into heparinized tubes (Vacutainer Systems; Becton Dickinson, Franklin Lakes, NJ, USA) from days 5 to 9 and every-other-day from there on for measurement of plasma progesterone concentration. Due to unexpected results, these samples were later used to measure plasma FSH concentrations and not plasma progesterone concentrations.

More frequent blood samples were collected from 5 animals per group corresponding to the total number of animals in the second replicate minus one (n=15) for measurement of plasma LH and FSH concentrations. Samples were taken at 0 min (immediately before treatment), 30, 60, 90, 120 min, and 3, 4, 5, 6, and 8 hr after treatment. Blood samples were centrifuged within 2 hours of collection at 1700 x g for 20 min and plasma was stored at -20 ^{o}C. Plasma LH and FSH concentrations were measured using a double-antibody radioimmunoassay [138] and are expressed in terms of NIAMDDK-bLH-24 and USDA-bFSH-I1. The minimum detectable limit of the assay was 0.1 ng. The range of the standard curves for LH and FSH were 0.06 ng (80% ligand labeled LH) to 8.0 ng (20% ligand labeled LH) and 0.13 (80% ligand labeled FSH) to 16.0 ng (20% ligand labeled FSH), respectively. The intra-assay coefficients of variation for the low and high reference plasma LH concentration (1.11 and 2.43 ng/ml) were 2.8% and 4.7%, respectively. The intra-assay coefficients of variation for the low and high reference plasma FSH concentration (1.39 and 3.96ng/ml) were 9.9% and 8.5%, respectively for the first assay. The intra-assay coefficients of variation for the low and high reference plasma FSH concentration (1.16 and 3.22 ng/ml) were 4.3% and 4.2%, respectively for the second assay. Inter-assay coefficients of variation were 12.2% and 12.4% for reference concentrations of 1.2 and 3.5 ng/ml, respectively.

5.3.1.4 Statistical analyses

Single-point measurements (i.e., follicle size at the time of treatment, interval from treatment to first and second wave emergence, maximum diameter of the dominant follicle and maximum diameter of the largest subordinate follicle) were compared by 2-way analysis of variance to determine the effects of treatment and replicate. Serial data (i.e., plasma FSH and LH concentrations, dominant follicle diameter profile) were compared among groups by 2-way analysis of variance for repeated measures to determine the effects of treatment and replicate over time. In the absence of a replicate effect, data from the two replicates were combined. Tukey's multiple comparison was used as a post-hoc test when a main effect of treatment or a treatment-by-time interaction was detected. Proportional data (i.e., ovulation) were compared among groups by chi-square analysis. Significance was assumed when the probability of values differing by chance alone was <0.05. All statistical analyses were made using SAS (Statistical Analysis System Inc., Cary, NC).

5.3.2 Experiment 2

5.3.2.1 Seminal plasma collection

Semen was collected and processed in the same way as for Experiment 1.

5.3.2.2 Protein purification

Seminal plasma samples from the 4 males were pooled and purified in 5 replicates of approximately 10 to 15 ml of seminal plasma each. Protein separation was achieved by applying the seminal plasma sample to a 7.5 ml 10/10 hydroxyapatite column (HA) attached to an AKTA FPLC™ system (Amersham Biosciences Inc, Quebec, Canada), and eluting at a rate of 0.2 ml/minute using sodium phosphate buffer. Unbound material was collected by washing the column at 0.5 ml/minute with the same buffer. After the unbound fraction was removed, a step gradient was applied at a rate of 0.75 mL/min of 10 mM sodium phosphate to a final concentration of 350 mM sodium phosphate. Two peaks eluted. The second peak was concentrated and buffer exchanged in to PBS using a Vivaspin 20 ml, 5kDa MWCO device

(Vivascience/Sartorius, Goettingen, Germany) centrifugated at 7000 x g and 18^{o}C to a final concentration of approximately 5 mg/ml. This second peak was applied to the size exclusion column as described before.

5.3.2.3 Animals and treatments

Sexually mature beef heifers weighing 400 to 450 kg were used during November and December at the University of Saskatchewan, Canada (52^{o}N, 106^{o}W and 500 m above sea level). The experiment was carried out in two replicates (n=20 in each replicate). To synchronize ovulation and wave emergence among animals, heifers were given a once-used intravaginal progesterone-releasing device (1.9 mg progesterone; CIDR; Pfizer Animal Health, Montreal, QC, Canada) [139] previously disinfected in a povidone-iodine solution (Betadine Scrub; Purdue Pharma, Pickering, ON, Canada) for at least 2 hours [140]. On the same day, heifers were given an intramuscular dose of 100 μg GnRH (Cystorelin). The CIDR was removed seven days later and heifers were given 500 μg of cloprostenol intramuscularly (Estrumate; Shering Plough Animal Health, Pointe-Claire, QC, Canada; PGF). Finally, heifers were given a second intramuscular dose of 100 μg GnRH (Cystorelin) 48 hs after prostaglandin treatment. Heifers were assigned randomly into six treatment groups (n=5 for treated groups and n=10 for the control group). On Day 3, 6 or 9 (Day 0 = ovulation, and emergence of first follicular wave of the interovulatory interval), corresponding to the early growing, early static and late static phase of the dominant follicle of the first follicular wave [38], heifers were given a single intramuscular dose of a) 100 μg of GnRH (Cystorelin, Merial Can Inc., Victoriaville, PQ, Canada; positive control), b) 1.0 mg purified OIF per 100 kg of body weight or assigned to the untreated control group.

The ovarian response was monitored twice daily using a 7.5 MHz linear-array transducer (Aloka SSD900, Tokyo, Japan) beginning on the day of the second GnRH treatment until ovulation (Day 0) and follicle wave emergence were detected. Daily ultrasonography was resumed on the day of treatment (Days 3, 6 or 9) for three consecutive days and every second day until ovulation of the next dominant follicle. Control heifers were monitored by transrectal

ultrasonography in like manner until ovulation and every second day for a complete interovulatory interval.

For measurement of plasma progesterone concentration, blood samples were collected from the jugular vein into heparinized tubes (Vacutainer Systems; Becton Dickinson, Franklin Lakes, NJ, USA) every-other-day from the day of treatment. Samples were centrifuged within 2 hours of collection at 1700 x g for 20 min and plasma was stored at -20 ^{o}C. Plasma progesterone concentration was measured using a commercially available double-antibody radioimmunoassay kit (Coat-a-Count total progesterone; Diagnostic Products Corporation, Los Angeles, CA) [56]. Samples were analyzed in four consecutive assays with intra-assay coefficients of variation for low (1.5 ng/ml) medium (2.9 ng/ml), and high (15.5 ng/ml) reference values were: 1) 2.0%, 3.7% and 6.5%; 2) 10.1%, 6.7% and 4.9%; 3) 3.8%, 4.5% and 10.9%; 4) 1.8%, 2.9% and 3.9%, respectively. Inter-assay coefficients of variations were 6.9%, 6.4% and 13.1% for reference concentrations of 1.6, 2.9 and 16.6 ng/ml, respectively.

For measurement of plasma FSH concentrations, blood samples were taken immediately before treatment with OIF or GnRH, and every 8 hours for a period of 3 days. Frequent samples were not taken from untreated control heifers. Plasma FSH concentration was measured using a double-antibody radioimmunoassay [138] and expressed as USDA-bFSH-I1. The minimum detectable limit of the assay was 0.1 ng. The range of the standard curves for FSH were 0.13 (80% ligand labeled FSH) to 16.0 ng (20% ligand labeled FSH). The intra-assay coefficients of variation for the low- and high-reference plasma FSH concentrations (1.58 ng/ml and 3.85 ng/ml) were 3.5% and 6.7%, respectively.

5.3.2.4 Statistical analysis

Single-point measurements (i.e., follicle size at the time of treatment, interval from treatment to first and second wave emergence, maximum diameter of the dominant follicle and maximum CL diameter) were compared by 2-way analysis of variance for a 3x3 factorial experimental design. Serial data (i.e., plasma FSH concentrations, dominant follicle diameter

profile, and CL diameter profile) were compared among groups by 2-way analysis of variance for repeated measures to determine the effects of treatment and replicate. In the absence of a replicate effect, data from the two replicates were combined and analyzed as a whole. Tukey's multiple comparison was used as a post-hoc test when a main effect of treatment or a treatment interaction was detected. Ovulation rates were compared among groups by Fisher's exact test. Significance was assumed when the probability of values differing by chance alone was <0.05. All statistical analyses were made using SAS statistical software (Statistical Analysis System Inc., Cary, NC).

5.4 Results

5.4.1 Experiment 1

The diameter of the preovulatory follicle at the time of treatment did not differ among groups (P=0.44, Table 1). The proportion of heifers that ovulated in response to treatment (i.e., within 3 days of treatment) was highest in the GnRH-treated group (P<0.05). The proportion that ovulated was minimal and did not differ between the saline- and OIF- treated groups.

Table 5.1 Effect of OIF on ovulation in pre-pubertal heifers (mean ± SEM; Experiment 1).

	GnRH	OIF	PBS
Follicle size at the time of treatment (mm) *	10.9 ± 0.26^{a}	10.2 ± 0.45^{a}	10.8 ± 0.44^{a}
Ovulation	$9/11^{a}$ (82%)	$1/11^{b}$ (9%)	$1/11^{b}$ (9%)

*No significant difference among groups (P>0.3)

[a, b] Within rows, values with different superscripts are different (P<0.05)

In a comparison among heifers that did not ovulate subsequent to treatment (Table 6.2), the interval from treatment to wave emergence was earliest (P<0.01), and the maximum diameter of the existing dominant follicle was smaller (P<0.01) in the OIF-treated group compared to the saline-treated group. The GnRH-treated group was excluded from further analyses involving heifers that did not ovulate subsequent to treatment because of low numbers; i.e., only 2 failed to ovulate in the GnRH group.

Table 5.2 Effect of OIF on follicle wave dynamics in heifers that did not ovulate in response to treatment (mean ± SEM; Experiment 1).

	GnRH (n=2)	OIF (n=10)	PBS (n=10)
Interval from treatment to 1st wave emergence (day)	1.7 ± 0.3[a]	1.8 ± 0.2[a]	3.5 ± 0.4[b]
Maximum diameter of the dominant follicle (mm)	12.0 ± 1.0[ab]	10.8 ± 0.4[a]	12.4 ± 0.3[b]
Maximum diameter of the 2nd largest follicle (mm)	6.0 ± 1.0	7.5 ± 0.4	6.9 ± 0.3

[a, b] Within rows, values with no common superscripts are different (P<0.05)

The day-to-day dominant follicle diameter profile in heifers that did not ovulate after treatment was smaller in the OIF-treated group compared to the saline-treated group (day effect, P<0.01; group effect, P<0.05; day-by-group interaction, P=0.49; Figure 6.1). Plasma FSH concentrations in non-ovulatory heifers tended to be higher in the OIF-treated group (day effect, P<0.01; group effect, P=0.1; day by group interaction, P=0.1) as a result of an increase in the OIF-treated group from Day 5 (day of treatment) to Day 7 (Day 0 = day of wave emergence) followed by a decrease to basal levels by Day 11 (Figure 6.1). Plasma FSH levels for the saline-treated group did not change over time. The maximum diameter of the largest subordinate follicle was recorded on the day of treatment (Day 5) and there was no difference between OIF- and saline-treated groups. However, the largest subordinate follicle remained large for a longer period in the OIF-than in the saline-treated group (day effect, P<0.01; group effect, P=0.05; day-by-groupinteraction, P=0.6; Figure 6.1).

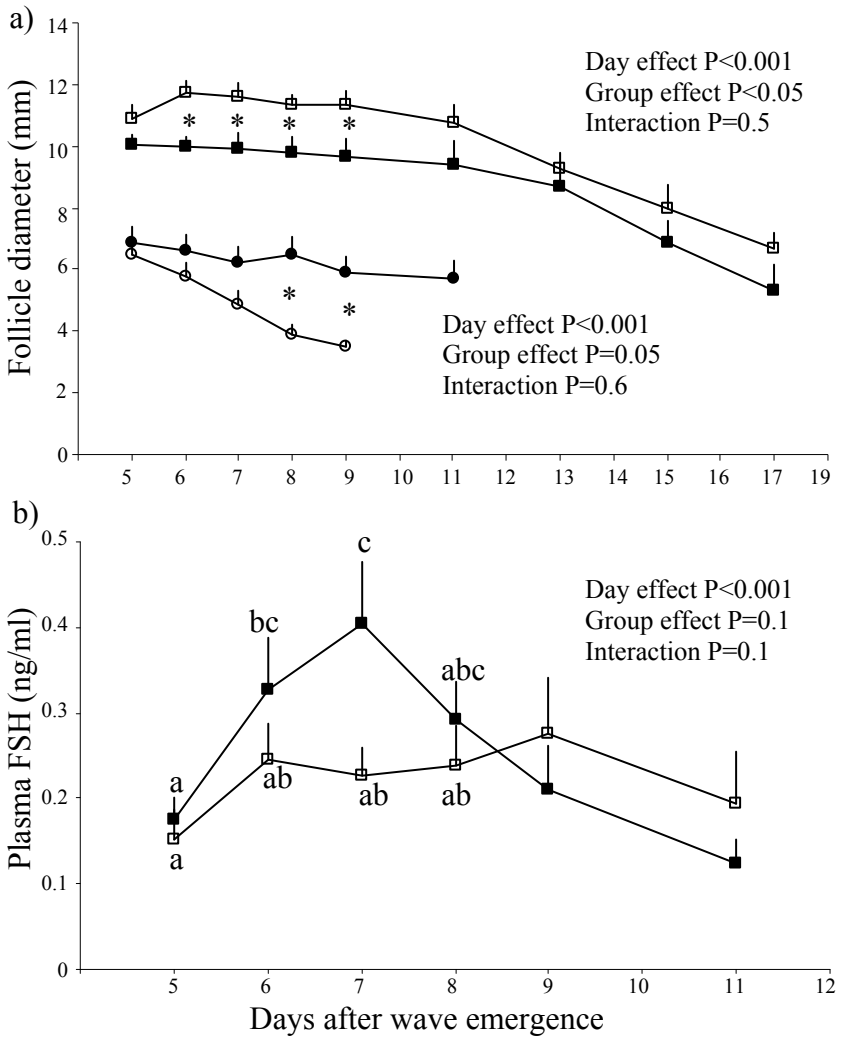

Figure 5.1 Dominant and first subordinate follicle diameter (a) and plasma FSH concentration (b) in pre-pubertal heifers (mean ± SEM) given a single intramuscular dose of OIF (■, ●) or PBS (□, ○) on Day 5 (Day 0 = wave emergence; n=10 per group; Experiment 1). *Between groups, values are different (P<0.05). ^{a,b,c} Values with no common superscripts are different (P<0.05).

A post-treatment surge in plasma LH concentration was detected in the GnRH-treated group, but LH remained unchanged in the OIF- and saline-treated groups (day effect, P<0.01; group effect, P<0.01; day-by-group interaction, P<0.01; Fig 6.2).

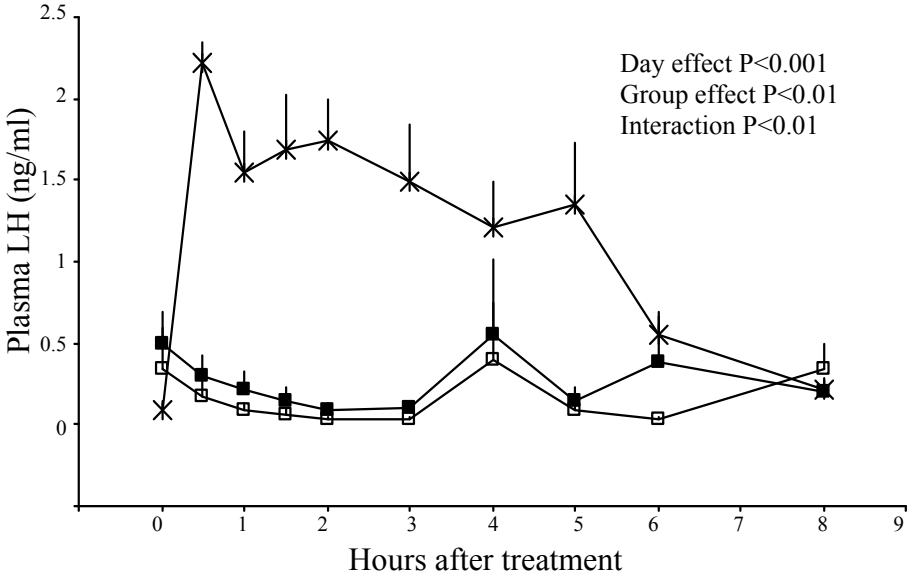

Figure 5.2 Plasma LH concentrations (mean ± SEM) in pre-pubertal heifers treated with OIF (■), GnRH (*) or saline (□) on Day 5 (Day 0= follicular wave emergence; n = 11 per group; Experiment 1).

5.4.2 Experiment 2

The diameter of the existing dominant follicle at the time of treatment was not different among groups on the same day of treatment (day effect, P<0.01; group effect, P=0.1; day-by-group interaction P=0.9; Table 6.3). The proportion of heifers that ovulated in response to treatment was significantly higher for heifers treated with GnRH on Day 6, but did not differ among the other treatment groups (day effect, P>0.05; group effect, P>0.05; day-by-group interaction P<0.05; Table 6.3).

Table 5.3 Follicle wave and CL dynamics (mean ± SEM) in heifers treated on Day 3, 6 or 9 after emergence of the first follicular wave of an interovulatory interval with GnRH, OIF, and in non-treated controls (Experiment 2).

End point	GnRH (n)	OIF (n)	Control (n)	Total
Follicle size at the time of treatment (mm)				
Day 3	7.3 ± 0.9 (5)	7.7 ± 1.0 (5)	9.0 ± 0.7 (10)	8.0 ± 0.9[a]
Day 6	11.6 ± 0.8 (5)	11.6 ± 0.4 (5)	12.2 ± 0.6 (10)	11.8 ± 0.6[b]
Day 9	12.7 ± 0.7 (5)	12.1 ± 0.7 (5)	13.6 ± 0.7 (10)	12.8 ± 0.7[b]
Ovulation (%)				
Day 3	2/5[a]	0/5[a]	1/10[a]	
Day 6	4/5[b]	0/5[a]	1/10[a]	
Day 9	1/5[a]	0/5[a]	1/10[a]	
Inter-ovulatory period (day) *				
Day 3	21.0 ± 0.0 (5)	22.2 ± 1.0 (5)	21.8 ± 0.6 (10)	
Day 6	22.4 ± 0.4 (5)	22.0 ± 1.2 (5)	21.8 ± 0.6 (10)	
Day 9	20.4 ± 0.9 (5)	21.4 ± 0.9 (5)	21.8 ± 0.6 (10)	
Interval from ovulation to 1st wave emergence (day) *				
Day 3	9.0 ± 1.2 (3)	9.6 ± 0.6 (5)	9.0 ± 0.5 (9)	
Day 6	7.8 ± 0.3 (1)	8.6 ± 0.6 (5)	9.0 ± 0.5 (9)	
Day 9	10.4 ± 0.4 (4)	10.6 ± 0.2 (5)	9.0 ± 0.5 (9)	
Maximum diameter of the dominant follicle (mm) *				
Day 3	11.8 ± 0.8 (3)	13.4 ± 0.7 (5)	13.0 ± 1.6 (9)	
Day 6	13.0 ± 0.0 (1)	12.6 ± 0.4 (5)	13.0 ± 1.6 (9)	
Day 9	14.3 ± 0.5 (4)	13.3 ± 0.3 (5)	13.0 ± 1.6 (9)	
Maximum CL diameter (mm)				
Day 3	20.6 ± 1.2 (5)	22.2 ± 0.5 (5)	19.5 ± 1.1 (10)	
Day 6	23.1 ± 0.9 (5)	24.5 ± 1.9 (5)	19.5 ± 1.1 (10)	
Day 9	24.3 ± 0.9 (5)	21.0 ± 1.5 (5)	19.5 ± 1.1 (10)	
Total	22.6 ± 1.0[a]	22.5 ± 1.3[a]	19.5 ± 1.1[b]	

[a, b] Proportion with different superscripts are different

* No significant difference among groups

In those animals that failed to ovulate, interval from treatment to first wave emergence was not significantly different (day effect, P=0.06; group effect, P=0.9; day by group interaction, P=0.3) nor did the existing dominant follicle reach a smaller diameter (group effect P=0.8) in the OIF treated group compared to the control group (Table 6.3).

Day-to-day diameter profiles of the dominant follicle in non-ovulated animals did not differ among groups treated on Day 3 (day effect, P<0.01; group effect, P=0.3; day-by-group interaction, P=0.5) or Day 9 (day effect, P<0.01; group effect, P=0.9; day-by-group interaction, P=0.2). The dominant follicle of animals treated with OIF on Day 6 tended to be smaller compared to the untreated control group (day effect, P<0.01; group effect, P=0.1; day-by-group interaction, P=0.2; Fig 6.3). The GnRH treated group was excluded from this analysis due to the low number of animals that did not ovulate and could have been included in the analysis (n=1).

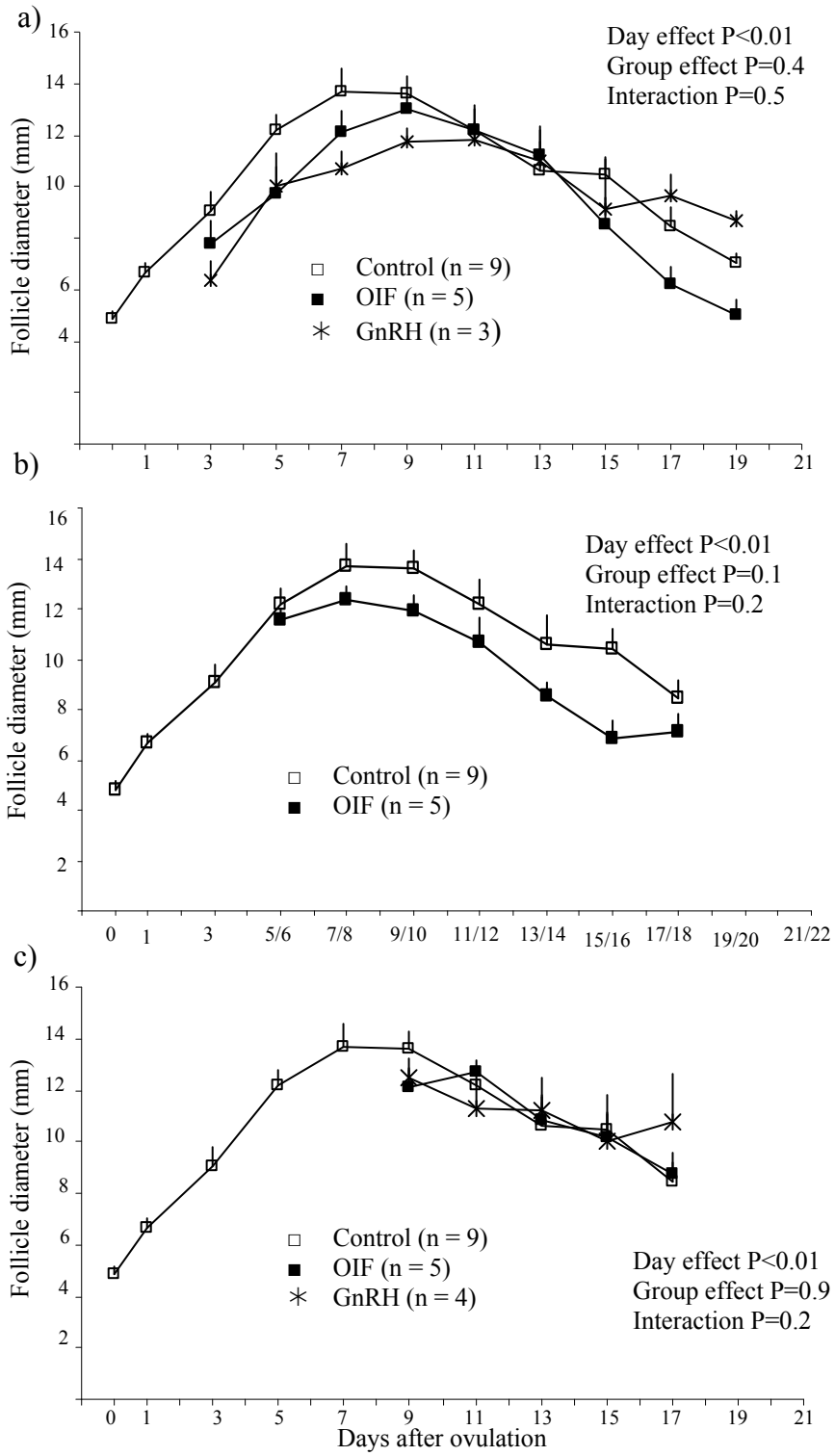

Figure 5.3 Diameter (mean ± SEM) of the dominant follicle of the first wave in heifers that did not ovulate after a single intramuscular dose of OIF or GnRH on Day 3 (a), 6 (b) or 9 (c), and in untreated controls. In panel (b), data from the single heifer that did not ovulate in the GnRH group were excluded.

Plasma FSH concentrations in unovulated animals treated on Day 3 with OIF remained basal and peaked for the GnRH treated group (day effect, $P<0.03$; group effect, $P=0.3$; day by group interaction, $P<0.05$). Plasma FSH tended to be higher and a rise was evident 24 hours after treatment in those animals treated with OIF on day 6 (day effect, $P=0.8$; group effect, $P=0.1$; day by group interaction, $P=0.6$) and 9 (day effect, $P<0.01$; group effect, $P=0.2$; day by group interaction, $P<0.01$). In those animals treated with GnRH, a rise was evident on day 9 but not in those treated on day 6 ($n=1$; Figure 6.4).

The maximum diameter of the CL was larger in the OIF- and GnRH-treated groups compared to untreated controls (day effect, $P=0.3$; group effect, $P<0.01$; day-by-group interaction, $P=0.3$; Table 6.4). Significant group effects or day-by-group interactions were detected in the day-to-day CL diameter profiles on treatment days 3 and 9 as a result of a larger diameter profile in the OIF- and GnRH-treated groups compared to untreated controls (Figure 6.5). Similarly, a group-by-day interaction was reflected in a larger plasma progesterone profile in OIF- and GnRH-treated groups compared to untreated controls on treatment Days 3 ($P=0.1$) and 9 ($P<0.05$; Figure 6.5). Significant group effects or day-by-group interactions were detected in the day-to-day CL diameter profiles and plasma progesterone concentrations in the OIF- and GnRH-treated groups compared to untreated controls on Day 6 of treatment but same size of the CL and same plasma progesterone concentrations on the day of treatment could not be confirmed due to differences in blood sampling frequency between treatment groups and untreated controls.

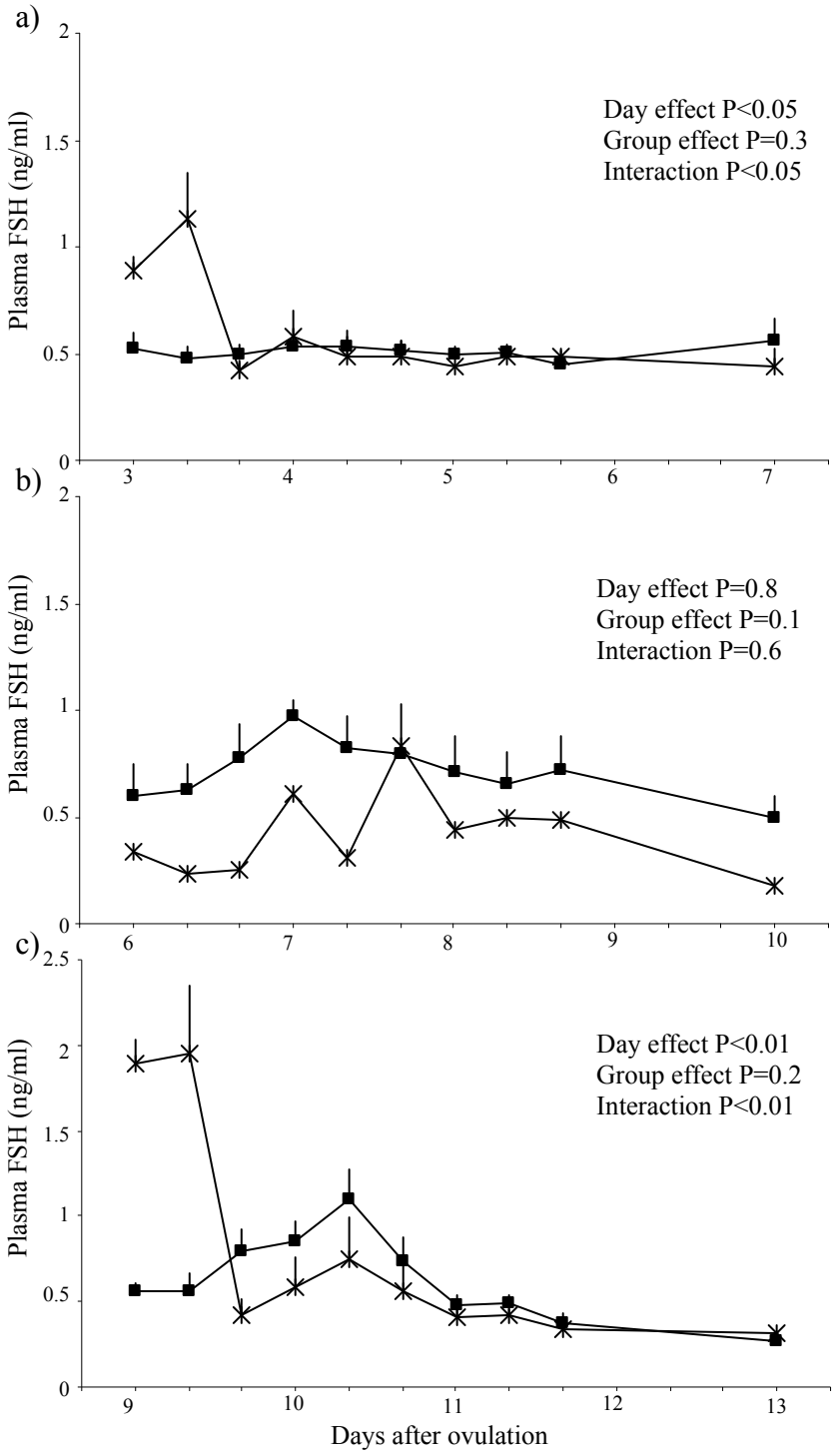

Figure 5.4 Plasma FSH concentration (mean ± SEM) in heifers that did not ovulate after a single intramuscular dose of OIF (■) or GnRH (∗) on Day 3 (a), 6 (b) or 9 (c).

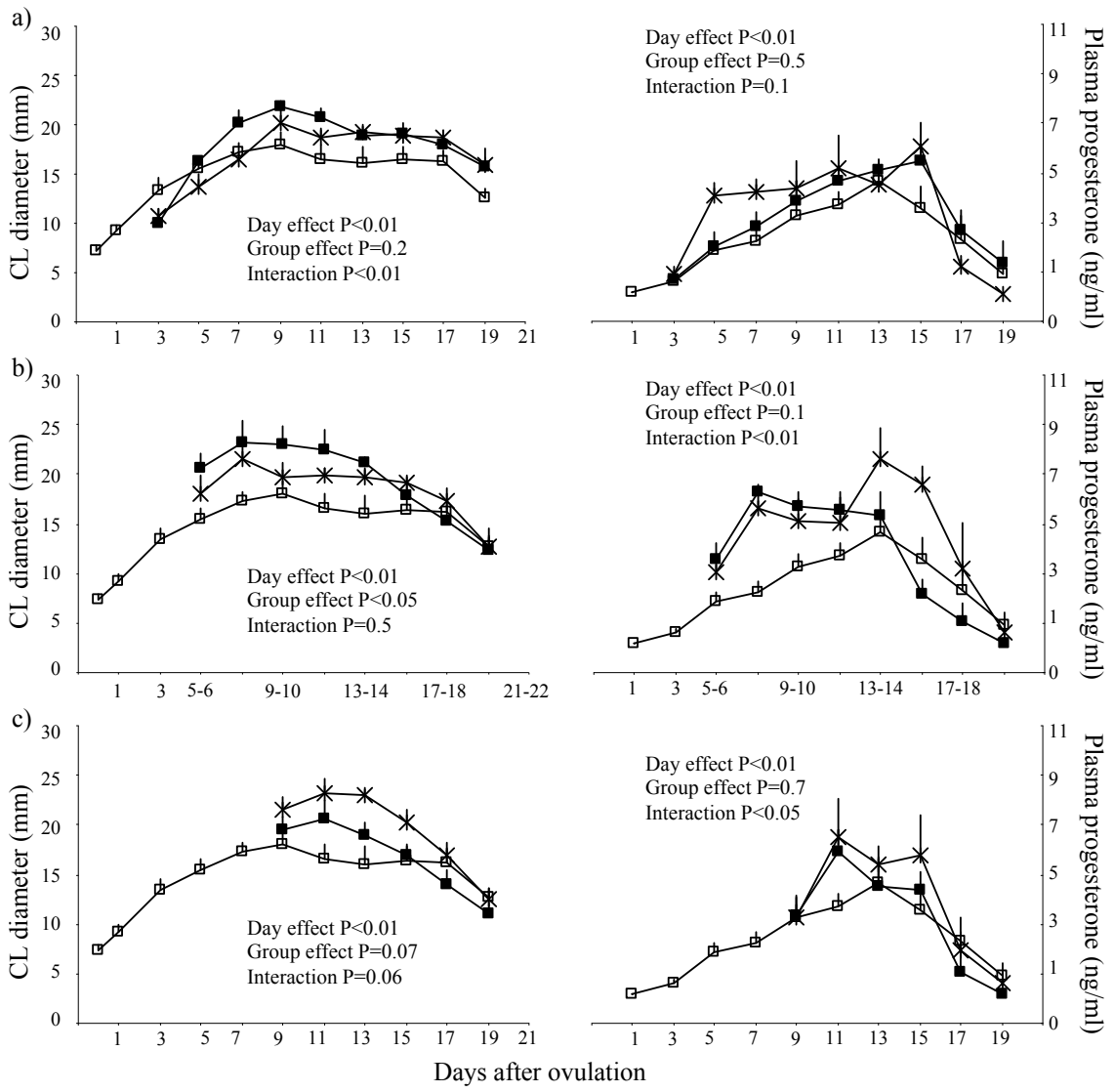

Figure 5.5 CL diameter and plasma progesterone concentration (mean ± SEM) in sexually mature heifers given a single intramuscular dose of OIF (■) or GnRH (∗) on Day 3 (a), 6 (b) or 9 (c), (n=5 per group per day) and in untreated controls (□; n=10) (Experiment 2).

5.5 Discussion

Two experiments were designed to determine the effect of OIF in cattle. Pre-pubertal heifers were used as a first approach to avoid confounding effects of spontaneous ovulations and circulating progesterone levels and as an attempt to imitate the physiological conditions of the unmated llama. Although no ovulations occurred, OIF produced an effect on follicular wave dynamics evidenced not only by an earlier wave emergence after treatment (1.8 ± 0.2 compared to 3.5 ± 0.4 for OIF and saline treated groups respectively; $P<0.01$) but also by a dominant follicle that stopped growing and remained smaller than in the control group ($P<0.05$). Also, the largest subordinate of the same wave remained large for a longer period of time compared to the PBS treated group. In cattle, wave emergence is preceded by a surge in FSH 12 to 24 hours before wave emergence [88]. In this first experiment plasma concentrations of FSH tended to be different and a rise 2 days after treatment could be observed in the OIF treated group but not in the saline treated group. Samples for measurement of FSH were not taken frequently enough to allow a better appreciation of this surge but it is important to point out that results on follicular wave dynamics were unexpected at the time.

Because of these results, a second experiment was conducted to confirm the effect on follicular wave dynamics and to determine if the day of the cycle in which animals were treated had any effect. The interval between wave emergences in cattle varies among individuals of two- or three-wave estrous cycles but the first follicular wave consistently emerges on the day of ovulation (Day 0) [32, 38, 43] or within the next 24 hours after follicular ablation [136]. Emergence of the second wave occurs between days 9-10 or 8-9 depending whether it is a 2- or 3-wave estrous cycle [141]. Dynamics of the dominant follicle of a follicular wave can be divided into basically three periods [38]: 1) growing phase (duration of approximately 4 to 6 days), static phase (duration of approximately 4 to 6 days), and 3) regressing phase, each of which have different circulating levels of FSH, LH and progesterone weather it is an ovulatory or non-ovulatory wave. Because of this dynamics, the dominant follicle is likely to respond differently at each stage of development and so three different periods were chosen to administer treatments in the second experiment. Effects of OIF could not be consistently confirmed in this

experiment due to differences in frequency of observations and blood sampling with the untreated control group, but there was a tendency for a smaller dominant follicle profile (P=0.01) and a rise in FSH concentrations (P=0.01) in those animals treated with OIF on day 6 when compared to the GnRH and control groups. This experiment also allowed us to compare the effects of treatment on the existent CL which apparently tended to increase in diameter and progesterone secretion after treatment with OIF or GnRH after treatment on Days 3 and 9.. In pigs, treatment could not be associated with higher incidence of ovulations or shortening of the time between the preovulatory LH surge, but it was associated to a luteotrophic effect on the CL [126].

A possible hypothesis may be that by means of affecting secretion of gonadotropins, OIF allows enough secretion of LH to exert a luteotrophic effect on the existing CL but not enough to induce ovulation. By inducing secretion of low levels of FSH, it stimulates the largest subordinate follicle at the time of treatment to continue growing to a size where both the dominant and subordinate compete for the little amount of LH present in the system. All of this leads to a smaller dominant follicle, less secretion of estradiol and therefore less negative feedback on FSH secretion and emergence of a new follicular wave.

It is also important to keep in mind that the exact molecular composition of OIF in cattle has not been determined and differences between llama OIF and bovine OIF may be sufficient to induce a different effect on ovarian dynamics.

In conclusion, treatment with OIF in pre-pubertal heifers does not induce ovulation, but it enhances wave emergence by affecting gonadotropin secretion. Treatment with OIF in sexually mature heifers also appears to affect the dominant follicle and secretion of gonadotropins. This effect appears to be more evident when treatments are given on day 6 after ovulation.

CHAPTER 6
GENERAL DISCUSSION

6.1 Dose effect of llama seminal plasma on ovulation and corpus luteum form and function in llamas

The belief that camelids only ovulate in response to copulation is no longer accepted as dogma. Previous reports have clearly documented the presence of a potent factor in the seminal plasma of llamas and alpacas capable of inducing ovulation without the need of external stimulation of the genitalia [7, 120, 121]. In these studies, the preovulatory LH surge and CL form and function have been characterized and compared to other ovulation-inducing hormones used in synchronization and AI protocols for llamas and alpacas. However, prior to the present study, no reports are available on the minimum amount of OIF necessary to induce similar effects and what proportion of an ejaculate it represents.

The purification process of OIF in the experiments described in this thesis (Chapters 5 and 6) was carried out in 10 and 12 replicates, respectively, of approximately 12 ml of seminal plasma for each replicate. The total amount of protein per replicate was 24 to 30 mg/ml, which made 2.5 to 3 mg of total protein per ml of seminal plasma. A total of 360 mg of seminal plasma proteins were purified using the described process in the first study from which 216 mg of OIF were isolated (60% of the total amount of protein). Considering that one ejaculate has an average volume of 2 ml [131], then the total amount of protein present in a single ejaculate is 5 mg (2.5 mg/ml * 2 ml of seminal plasma) from which 3 mg are OIF (60% of the total protein). This together with results from previous studies carried out in our lab where we concluded that 1 to 2 ml of seminal plasma was enough to induce an enhanced preovulatory LH surge and ovulation in over 90% of the females treated, determined the doses chosen to evaluate if OIF affected ovulation and CL form and function in a dose dependent-manner. Thus, the highest dose used in the first study represents less than half of the total amount of OIF used in previous experiments

and approximately 1/6th of that present in a normal ejaculate (0.5 mg out of 3 mg of OIF per ejaculate)

Results from this experiment demonstrated that the effects of OIF are influenced by dose. Of those animals in the high dose group (500 μg of OIF) 9/10 (90%) ovulated and had higher levels of LH two hours after treatment (interaction $P<0.01$) compared to those treated with the low dose of OIF (60 μg). Day of appearance of the CL ($P<0.05$), CL diameter ($P<0.05$), CL diameter profiles (group effect, $P<0.05$; interaction $P<0.01$) and plasma progesterone concentrations (group effect, $P<0.01$; day-by-group interaction, $P<0.01$) were also enhanced in the high-dose treatment group and provided more evidence on the luteotrophic effect of OIF mentioned in previous studies. In the low-dose group (60 μg of purified OIF) CL appeared 1.2 ± 0.2 days later than in the high-dose treated group and reached a smaller diameter. Although the low-dose group failed to demonstrate significant difference when only ovulated animals were included in the analysis, plasma progesterone concentrations were also lower regardless if they had ovulated. This lack of significance may have been due to a low number of animals that ovulated in this group (n= 3). End points were intermediate for the 125 and 250 μg treated groups in all cases.

The dose effect evidenced in this study was not unexpected and had already been suggested when in two other studies [120, 121] the proportion of ovulations were higher when treated with 2 ml compared to 1 ml of seminal plasma by intrauterine infusion. This difference was probably provoked by dose and distribution over the endometrial surface.

Elevation of LH concentrations in plasma after intramuscular treatment with OIF provided further support for the hypothesis that the effect of OIF is mediated through a systemic rather than a local route mentioned in studies carried out in llamas, alpacas and Bactrian camels [120-123].

Results clearly suggest that ovulation and CL form and function is affected by OIF in a dose-dependant manner and that the minimum dose necessary is physiologically relevant. This is, the concentration of OIF normally present in the seminal plasma of a single ejaculate in

llamas is enough to induce more than 90% of ovulations. OIF may be another way of assuring exact timing between the male and female gamete to make sure pregnancy is achieved like induced ovulation.

6.2 Effect of ovulation-inducing factor on ovulation and follicular wave dynamics in cattle

The release of an egg from the follicle of an ovary, a process commonly known as ovulation has much more to it than just a simple mechanical process that occurs at regular intervals. For the longest time the differences between those species that ovulated in response to copulation and those that ovulated spontaneously at regular intervals had not been challenged. Evidence of factors in the seminal plasma of reflex ovulators capable of inducing ovulation without physical stimulation of the genitalia challenged the dogma of ovulation in these species [117, 120-122]. The findings in llamas and alpacas begged the question if this factor could be found in cattle, a distant related ruminant. Previous experiments carried out in our lab where bull seminal plasma was injected into llamas confirmed the presence of an ovulation-inducing factor in this specie but the role in spontaneous ovulation still remained unclear [7].

Two experiments were carried out to determine if OIF had any effect on ovarian function in cattle. Conclusions from the first study in this thesis, where 500 μg per llama was enough purified OIF to induce ovulation, the fact that bulls have similar volume of ejaculate as llamas and from studies where 2 ml of bull seminal plasma induced ovulation in 26% of the females llamas, inspired the dose of 1 mg per 100 kg of BW used in both of the experiments carried out in cattle. To avoid confounding effects of spontaneous ovulations, the first study was carried out in pre-pubertal heifers. To further characterize the effect of OIF on follicular wave dynamics, sexually mature heifers were chosen for the second study.

Purified OIF from llama seminal plasma did not induce ovulation in cattle but it did affect follicular wave dynamics. Development of follicular waves in cattle has been extensively studied. Each wave is preceded by an elevation in plasma FSH concentrations that recruits a cohort of small follicles from which one will emerge and becomes dominant. The number of waves in the estrous cycle varies between 2 and 3 for most breeds of cattle. Each of these waves

can be divided into three periods, recruitment, selection and dominance. Once the largest follicle has begun to establish dominance, the rest of the follicles in the wave undergo atresia but previous experiments have documented that those follicles have not lost their capacity to respond to circulating gonadotropins [97, 98].

During the first experiment, the first follicular wave in animals that did not ovulate after treatment with OIF emerged earlier than in the control group (1.7 ± 0.3, 1.8 ± 0.2 and 3.5 ± 0.4 for the GnRH, OIF and saline treated groups; $P<0.01$). This fact was supported by an elevation in plasma FSH levels in the OIF-treated group that started one day ($P<0.05$) after treatment and reached highest concentrations 2 days after treatment. The maximum diameter of the dominant follicle was smaller for the OIF-treated group (12.0 ± 1.0, 10.8 ± 0.4 and 12.4 ± 0.3 for the GnRH, OIF and saline treated groups; $P<0.01$) and the first subordinate of that same wave grew for a longer period of time and tended to have a larger diameter profile than that observed in the control group. Although these effects could not be confirmed in the second experiment due to differences in observation and blood sampling frequency with the untreated control group, diameter profiles of the dominant follicle tended to be smaller and plasma FSH concentrations tended to be higher in animals treated on Day 6 of the estrous cycle with OIF corresponding to the late growing or early static phase of the dominant follicle.

Also dynamics of the existing CL in the second experiment showed that animals treated with GnRH or OIF had a larger maximum CL diameter ($P<0.01$) and day to day CL diameter profiles than control animals. Plasma progesterone concentrations for those animals were also higher.

These results provide rationale for the hypothesis that OIF induces enough secretion of LH to exert a luteotrophic effect on the existing CL but not enough to induce ovulation and that by inducing secretion of low levels of FSH, it stimulates the largest subordinate follicle at the time of treatment to continue growing to a size where both the dominant and subordinate follicles compete for LH leading to a smaller dominant follicle, less secretion of estradiol and therefore less negative feedback on FSH secretion and emergence of a new follicular wave.

Perhaps OIF has more of a luteotrophic effect in spontaneous ovulators than any effect on the ovulation mechanism itself. In the pig exposure to seminal plasma and local macrophage infiltration of the ovary was suggested to increase sensitivity to gonadotropins and enhance luteogenesis by inducing changes in LH receptors or steroidogenic enzyme expression in the granulosa cells. Perhaps this effect also reaches the subordinate follicle and allows it to become sensitive to small amounts of LH and grow in codominance with the existing dominant follicle. This fact would also explain the increase in size and progesterone secretion of the CL observed in the second study (Chapter 6).

Another theory may be that although we know the effect of OIF is mediated through a systemic rather than a local route, perhaps after absorption into the blood stream, it also reaches the ovary in the cow and affects the dominant follicle directly arresting its development, decreasing circulating levels of estradiol and allowing the emergence of a new follicular wave. This effect may be more evident on Day 6 in sexually mature animals because the dominant follicle at that time has mostly finished its growing phase and has not yet entered atresia and therefore is more responsive. The presence of a larger subordinate may be due to the fact that there are higher levels of gonadotripns in plasma now that the dominant follicle has been affected and is not using them for its own growth.

Based on these hypothesis, studies involving better coordination between observations and blood sampling amongst the different treatment groups, histology of dominant and subordinate follicles, image analysis of the animals ovaries and exact determination of quantity and quality of OIF in bull seminal plasma should be carried out to further test the hypothesis that purified OIF induces atresia of the dominant follicle and earlier wave emergence in sexually mature heifers.

CHAPTER 7
GENERAL CONCLUSIONS

- A single llama ejaculate of 2 to 3 ml contains approximately 5 mg of total protein from which OIF represents 50 to 60%

- OIF has a dose dependent effect on proportion of ovualtions, CL diameter and plasma progesterone concentrations in llamas

- The luteotrophic effect of OIF is dose-dependant

- The minimum effective dose of purified OIF to induce ovulation in more than 70% of female llamas lies somewhere between 60 and 250 μg

- This dose is physiologically relevant and represents less than 1/6th of what is normally present in a single ejaculate in llamas

- OIF has a very potent ovulatory and luteotrophic effect in llamas

- OIF did not induce ovulation in cattle

- Follicular wave dynamics in heifers is affected by treatment with OIF

- Treatment with OIF induces earlier wave emergence by affecting dominance of the largest and second largest follicle of a single wave in pre-pubertal heifers

- OIF appears to affect follicular wave dynamics in sexually mature heifers when treated on Day 6 after ovulation

- OIF affects size and progesterone secretion of the existent CL in sexually mature heifers

- OIF exerts its action on follicular wave dynamics by affecting dominance of the dominant follicle

- Studies with more frequent observations and blood sampling should be carried out to further confirm the effect of OIF in cattle

- Llamas represent an excellent bio-model for the study of the effects of OIF in induced ovulators and its existence in other species

CHAPTER 8
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