# INTERSPECIES COMPARISON OF THE EFFECT OF OVULATION INDUCING FACTOR (OIF) IN SEMINAL PLASMA

A Thesis submitted to the College of Graduate Studies and Research
in partial fulfillment of the Requirements for the Degree of Master of Science in the
Department of Veterinary Biomedical Sciences
University of Saskatchewan
Saskatoon

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**“…And I - I took the [road] less traveled by**

**And that has made all the difference”**

**-Robert Frost-**

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# ABSTRACT

The purpose of the studies reported in this thesis was to provide further evidence in support of the hypothesis that ovulation-inducing factor (OIF) is a component of seminal plasma which is conserved amongst mammals. Based on studies conducted *in vivo*, the results indicate that males ejaculate a substance during copulation which is responsible for the ovulatory and luteotrophic effect in female camelids. In our lab we have developed an *in vivo* llama bioassay to study the presence and biological effects of OIF in seminal plasma from different species.

The objective of the first experiment within the first study was to determine if llama seminal plasma would stimulate ovulation in prepubertal mice. Mice were treated with a single 0.1 mL intraperitoneal dose of 1) phosphate-buffered saline (negative control), 2) 5 µg gonadotropin-releasing hormone (GnRH), 3) 5 IU of human chorionic gonadotropin (hCG) or 4) llama seminal plasma. Results indicate that prepubertal mice treated with GnRH, hCG or llama seminal plasma stimulated similar proportions of mice to ovulate, which were all higher than the proportion of mice that ovulated after saline treatment. The number of oocytes observed under a stereomicroscope was also higher in all treatment groups than in mice treated with saline. However the number of oocytes observed was lower in mice treated with seminal plasma than those treated with GnRH, both of which were similar to the number of oocytes observed in hCG treated mice.

In a second part of this study the corollary that OIF is present in the seminal plasma of horses and pigs was examined. Seminal plasma from horses or pigs was administered intramuscularly to female llamas and ovulation was monitored using transrectal ultrasonography. Llamas were treated with an intramuscular dose of 1) phosphate buffered saline (negative control), 2) llama seminal plasma (positive control), 3) equine seminal plasma or 4) porcine seminal plasma. Ovulations were detected in llamas treated with seminal plasma while none were observed in saline-treated llamas. The proportion of llamas that ovulated when treated with equine seminal plasma was higher than llamas treated with saline. The proportion of llamas that ovulated after porcine seminal plasma tended to differ from negative control groups, but did not reach statistical significance. The proportion of llamas that ovulated after equine or porcine

seminal plasma treatment was lower than animals treated with llama seminal plasma which indicates that either OIF is not present in equal concentration among mammals, or that OIF is not structurally the same across mammals.

The second study was carried out to test the hypothesis that OIF stimulates LH secretion at the level of the anterior pituitary gland. The second objective was to determine if the degree of LH release was related to the dose of OIF treatment. Anterior pituitary cells ($2 \times 10^6$ cells/ well) from either llamas (reflex ovulator) or cattle (spontaneous ovulator) were incubated for 2 hours with either media containing no treatment (control), GnRH or OIF. In all experiments, GnRH and OIF stimulated more LH secretion than control groups. An effect of dose was evident in the llama pituitary cell culture where mean LH concentrations were greater in wells treated with a higher dose of OIF in comparison to wells treated with a lower dose, both of which were higher than in wells with no treatment. Although OIF stimulated LH release in bovine cell cultures, an apparent dose response was not detected. Results indicate that the preovulatory LH surge observed after OIF treatment in camelids may be the result of OIF directly stimulating LH release from gonadotrope cells within the anterior pituitary gland.

In conclusion these results illustrate that the presence and the response to OIF is conserved among species that share no relation or common reproductive strategy.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Gregg Adams for taking the time to listen my ideas whether they made sense or not, and providing me with constructive criticism, time and patience which without, I would not have been able to grow personally and professionally throughout the completion of this degree.

I would also like to extend my sincerest appreciation to Dr. Baljit Singh who, every day, demonstrated that words such as ‘professional’ or ‘scientist’ do not mean boring.

Appreciation is extended to other committee members for their guidance: Dr. Roger Pierson and Dr. Valerie Verge.

My sincerest gratitude is extended to Dr. Marcelo Ratto and Dr. Ximena Valderamma for all their support and guidance throughout the years.

To all my mentors and colleagues in the reproductive science and medicine group: Thank you for providing a welcoming working environment. During this degree, I have knocked on everyone’s door and have never been turned away. Thank you.

To Rhonda and Lynn, thank you for allowing me to adopt you as surrogate parents in Saskatoon.

To Dr. Larry Barcza, thank you for all your support and for never screening my abundant phone calls whenever I needed sage advice.

I would like to thank my family: Melissa Mendrek, Valerie Foley, Tara Brouwer, Rachel Stack and Holida Tek. The bond that we share goes beyond the constraints of friendship.

To my roommates Tatrina Tai, Logan Peters, Lynne Girardin and Mario Poulin. Thank you for providing me with a home in Saskatoon and for memories that can only be shared among roommates.

Finally, to Jimena Yapura, Luca Panizzi, Mahsa Abrashami, Behzad Toosi, Terri Bloski and Dion Hill: this journey would not have been possible without friends to laugh with, vent to, and cry on.

# DEDICATION

I dedicate this thesis to my parents, Evard and Joylyn Bogle and my brother, Ev for their unwavering support and guidance and for telling me that I was *smart* in times of failure and great stupidity. Thank you for providing me with a place that I can always recognize as home.

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| | |
|---|---|
| BW | Body weight |
| CL | Corpus luteum |
| ELISA | Enzyme-linked immunosorbant assay |
| eCG | Equine chorionic gonadotrophin |
| Egr-1 | Early growth response factor-1 |
| FPLC | Fast protein liquid chromatography |
| FSH | Follicle stimulating hormone |
| GnRH | Gonadotropin releasing hormone |
| HA | Hydroxyapatite column |
| hCG | Human chorionic gonadotrophin |
| h | Hours |
| im | Intramuscular |
| iu | Intrauterine |
| IU | International unit |
| LHRH | Luteinizing hormone-releasing hormone |
| LSD | Least significant difference |
| kDa | Kilodalton |
| kg | Kilogram |
| MMP-19 | Matrix metalloproteinase-19 |
| µL | Microlitre |
| µg | Microgram |
| mL | Mililitre |
| min | Minute |

| | |
|---|---|
| ng | Nanogram |
| pLH | Porcine luteinizing hormone |
| OIF | Ovulation-inducing factor |
| PBS | Phosphate buffered saline |
| $PGF_{2\alpha}$ | Prostaglandin $F_{2\alpha}$ |
| SAS | Statistical analysis system |
| SD | Standard deviation |
| SEM | Standard error of the mean |
| vs. | Versus |
| v/v | Volume to volume |

## 1.0. GENERAL INTRODUCTION

Ovulation, the event in which an oocyte is released from its enclosed follicle, is a process that is not completely understood. In animals that cycle and ovulate independent of the male, the elements and hormones which regulate the process have been well studied. In contrast, little is known about the other group of animals which depend on copulation-associated male input to ovulate. During copulation females are exposed to a variety of male-derived cues which have been shown to cause, or contribute to events leading to ovulation. Of the abundance of possible stimuli, it is commonly accepted that intromission is the principle incident leading to ovulation in induced ovulators. However, it has not been observed that physical stimulation of the female genitalia will invoke ovulation in all induced ovulators. For example, it has been documented that stimulation of the vagina and cervix induces ovulation in animals such as ferrets and cats, while no response is observed in llamas and koalas. The lack of a conserved response to physical stimulation suggests that another cue is responsible for oocyte release.

In 1986, investigators in China first proposed the idea that ovulation was triggered by a substance within male seminal plasma, the ovulation-inducing factor (OIF). Using the female camel as a bioassay, investigators reported that intravaginal inseminated of whole camel semen or seminal plasma caused ovulation in camels. In addition, it appeared that this substance spanned to both induced and spontaneous ovulators as seminal plasma from bulls also caused female camels to ovulate. Similar results have been generated using related camelids, the llama and alpaca, as bioassays.

More recently, OIF has been isolated from llama seminal plasma and is a 26 kDa protein. Following intramuscular administration, seminal plasma or purified OIF induced ovulation by way of a preovulatory LH surge. The duration of this surge was longer in alpacas treated with seminal plasma than those treated with GnRH. Ovulation-inducing factor has also been shown to have a luteotrophic effect; alpacas that ovulated after seminal plasma treatment produced larger corpora luteum and correspondingly more progesterone in comparison to those that ovulated following GnRH treatment.

In order to provide further evidence that OIF is the primary stimulus which triggers the ovulatory pathway in camelids, the conservation of the response to various sources of seminal plasma must be documented. In addition, in order to support the notion that OIF and the factors

contributing to its response is preserved among mammals, the ovulatory effect of OIF must be documented in other species.

The purpose of this thesis is three-fold. Firstly, to provide further evidence that OIF is a conserved constituent of mammalian seminal plasma, secondly, to demonstrate that its ovulatory-inducing effect can be seen in spontaneously-ovulating species, and thirdly, to determine if OIF elicits its ovulation-inducing effect by directly stimulating LH secretion from the pituitary gland.

The following literature review describes the events of induced ovulation with the focus of camelid reproduction.

### 1.1. Theories behind induced ovulation

Animals are divided into two groups based on their mode of ovulation: those that require copulation to ovulate and those that do not. Induced ovulators are those animals in which coitus or a coitus-derived stimulus is required for ovulation while spontaneous ovulators rely on endogenous hormones to ovulate in regular periodic intervals. Currently, our knowledge of induced ovulators is limited. The focus in literature tends to be skewed to spontaneous ovulators since this group encompasses humans as well as most common laboratory animals, such as rodents. It is estimated that 90-95% of animals used in biomedical research are mice and rats: both species are spontaneous ovulators [1]. For the remaining 5-10%, the USDA (United States Department of Agriculture) lists dogs (8%), cats (2%), nonhuman primates (6%), guinea pigs (19.8%), hamsters (16%), rabbits (23%), pigs (5.5%), sheep (1.3%), other farm animals (3.3%) and other species (13.9%) as the other species used in research. Of the animals listed above, only two (the rabbit and the cat) are induced ovulators. Nevertheless, increasing attention is being given to these animals in the breadth of two questions- How and why did two different reproductive strategies evolve?

The most prevalent theory suggests that mating-induced ovulation is the primitive form and spontaneous ovulation evolved due to various selection pressures. In support of this hypothesis, induced ovulation is seen in primitive Orders *Insectivora* and *Rodentia* [2-4], as well as the Infraclass *Marsupialia* which includes the koala and grey short-tailed possum [5-7]. Different from most nonmammalian species, the red-sided garter snake has also been shown to ovulate with mating [2, 8]. In 1971, Conaway proposed criteria to distinguish animals into three different groups: Type I-ovulation and pseudopregnancy is spontaneous; Type II-ovulation is induced, pseudopregnancy is spontaneous; and Type III- ovulation and corpus luteum formation is spontaneous, but pseudopregnancy is induced. Pseudopregnancy refers to any luteal function in a non-pregnant cycle [9]. Pseudopregnancy was deemed “spontaneous” if luteal function followed ovulation; if luteal formation did not follow ovulation, pseudopregnancy was considered “induced” [9]. Conaway used this classification to conclude that the phenomenon of induced ovulation is observed throughout most Orders and should not be considered as an opposition of spontaneous ovulation, rather, both traits should be viewed as two extremes of a single continuum [9]. In support of this idea, observations have been made that copulation influences ovarian behaviour in both reproductive types. Similarly, spontaneous ovulation is seen

in induced ovulators [10]. After a thorough review of literature, Jöchle made similar conclusions acknowledging that all mammals express some degree of induced ovulation [11, 12].

Without knowledge of the reproductive physiology of many of the existing animals or, animals from which present animals have evolved, it is difficult to ascertain which of the two strategies is primitive. Using phylogenetic trees constructed from large datasets of genomic sequences [13, 14], no clear pattern exists to shed light on evolutionary trends as induced ovulators occur in a variety of mammalian Orders. From these analyses, however, it appears that induced ovulation may have evolved multiple times at the individual species level which may also explain why there is not one single stimuli that induces ovulation [15].

Another theory derived to explain induced ovulation suggests that this ovulation type evolved in animals that undergo major population fluctuations [16, 17]. The benefit of ovulation synchronized with the presence of sperm is the increased likelihood of fertilization. Ergo, the chance of pregnancy is enhanced if the animal ovulates after copulation, thereby promoting a rapid recovery from low population levels [16, 17]. Another theory proposed that induced ovulation may have evolved in socially solitaire animals, where male and female encounters are unpredictable. Copulation-driven ovulation would ensure ovum release and subsequent fertilization would most likely occur when a male encounters a female [16, 18].

One study observed that this trait may have evolved in light of social and environmental pressures in carnivores that are induced ovulators. By retrospective analysis, induced ovulators tended to be seasonal breeders, have larger home ranges and longer estrous periods. The display of sexual behaviour was also different in induced ovulator carnivores who displayed multimale mating systems in 93% of cases in comparison to spontaneous ovulation which had a more even distribution of monogamous (42%), multimale (33%) and polygamous (25%) mating systems [19]. In addition, investigators found that the presence or size of the os penis could not be used as an indicator of induced ovulation as this structure was equally present in spontaneous ovulators [20].

### 1.2. Overview of the reproductive physiology in camelids

The evolutionary pathway of llamas and alpacas demonstrates their resilience. Fossils of *Pliauchenia*, the original ancestor of llamas and alpacas, were found in North America and were

dated approximately 9 to 11 million years ago (MYA) [21]. *Hemiaichenia*, evolved from *Pliauchenia* and migrated to South America during the transition from the Pliocene to Pleistocene era. After the travel to South America, the genus *Llama* and *Vicugna* evolved, and evidence suggests that they were the only two South American camelid species to survive the Pleistocene period [21]. More recent fossil evidence has been interpreted to mean that *Llama guanaco* and *Vicugna vicunga* resided in Argentina and Bolivia, while more domesticated forms roamed the Peruvian Andes [21, 22].

Camelids belong in the Order *Artiodactyla*, which is divided into three suborders: Suiformes, Pecora and Tylopoda [23]. Suiformes are the only non-ruminants and include pigs and hippopotami; Pecora include deer, cattle, goats and antelopes, while Tylopoda include animals that are camelids [23]. Camelids are further divided into New and Old World Species. Llama (*Lama glama*) and alpacas (*Vicugna pacos*) represent the two domesticated forms of New World camelids. The other two species, vicuna (*Vicugna vicugna*) and guanaco (*Lama guanicoe*) live in the wild Andes of South America, especially in Peru and Bolivia [24, 25]. The two species of Old World camelids include the Dromedary (one hump) and Bactrian (two humped) Camels [25].

Camelids do not share a uniform breeding period. Old World and the wild form of New World species (vicugna and guanaco) have a breeding period that is dependent on season. In contrast, llamas and alpacas are capable of breeding throughout the year. In the absence of a male, investigators monitored female alpacas for 36 days and found that at the end of their observational period, females were still sexually receptive with occasional periods of anoestrus no longer than 48 hours [26]. The 36-day estrus duration was determined by whether in the presence of a male, the female took the prone position. Not clearly outlined in this experiment was the duration of exposure to the male and the degree of contact the female had with the male. Females that are receptive may not immediately assume the copulatory position but enters recumbency after the male exerts force on her hindquarters [27]. Similar to observations in alpacas, in the absence of a male, female llamas remained in estrus for the entire 30 day observational period [28]. Whether or not a female is receptive to a male is not transpired at the level of the ovary. Researchers reported that follicle size does not influence sexual receptivity; alpacas with only 3 mm follicles were equally receptive to male advances as females with

follicle diameters ≥ 7 mm [29]. Results from a study conducted in Peru found no significant difference in the number and size of follicles among seasons [30]. It was further hypothesized that alpacas mate throughout the year. A compilation from zoos in North America and Europe reported that captive llamas bred year round, while other camelids, such as the Bactrian camel, appeared to imitate seasonal reproductive patterns as seen in the wild [31].

Camelids do not have an easily detectable estrous cycle therefore the time in which females reach sexual maturity is difficult to assess. Consequently, variation exists in the reported age that females enter puberty [32]. In alpacas, ovarian function has been detected as early as 10 months of age [33] and the females can be bred at 12-14 months of age [34]. Body weight (BW) has also been used as an indicator of puberty. Llamas and alpacas weighing 50-60% their total BW demonstrate signs of sexual maturity [35, 36]. In Peru, llamas and alpacas are not bred until they reach 2-3 years of age [32, 36].

Several factors are used to determine puberty in male camelids. The presence of mature spermatozoa in llamas has been detected as early as 10-12 months old using an artificial vagina as a semen collection method [36]. In male dromedary camels, however, mature spermatozoa have been detected only after 3 years of age [37]. Testicular size, sperm morphology, and the attachment between the penis and prepuce are used as other indicators of male puberty as full penile intromission cannot occur if this structure is present. The penile-preputial attachment in llamas, alpacas and dromedary camels is fully detached by 3 years of age [36, 37]; it is also at this age when males have sufficient libido to dominate females into breeding [25]. The concentration of testosterone has been correlated with age, testicular size and sexual drive. An increase in testosterone concentration in llamas and alpacas indicate that puberty occurs anywhere between 2-3 years [38]. The concentration of testosterone in llamas remain around 120-150 pg/mL at birth to 18 months of age [38]. Once the animal reaches the age of two, testosterone concentration increases to 500 pg/mL and is even higher by 36 months (800 pg/mL) [38]. The increase in circulating levels occurs earlier in alpacas and has been reported to increase from 213 pg/mL at 12 months to 1156 pg/mL at 18 months of age [38].

During copulation, the sexually receptive female immediately submits to the advancing male and adopts a prone (copulatory) position. The sternal recumbency position signals to the male that the female is sexually receptive and copulation occurs. Once the female is in the

recumbent position the male enters a half-sitting mounting position at her rear and adjusts his pelvis to facilitate intromission. During intromission the male moves his pelvis forwards and backwards, a process reported to last anywhere from 3 – 65 minutes depending on the species of camelid [26-28]. Ejaculation in llamas and alpacas occurs throughout the intromission period and does not occur in one final bout, which in some animals, concludes the copulatory period [38]. If the female does not immediately assume a recumbent position, the male will exert pressure on the female's hindquarters in attempt to force the female to lie down [27]. Non-receptive females flee when a male advances or resort to kicking, spitting or vocalization [27, 29, 39]. Females are non-receptive or reluctant to breed in the event of pregnancy, the presence of a corpus luteum, or lack of ovarian activity [40]. Females remain sexually nonreceptive until luteolysis occurs.

### 1.3. The effect of male associated copulatory stimuli on ovulation

"Reflex ovulation is brought about by afferent impulses from the genitalia and the eyes, ears and nose that converge on the ventral hypothalamus and provoke an ovulation-inducing release of LH from the pituitary" [41].

A number of male factors have been associated with stimulating ovulation, but none have been shown to uniformly provoke the ovulatory response in all coitus-induced ovulators. The report from an earlier study conducted in alpacas found intromission was necessary for ovulation in females [42]. However, these reports contradict studies in other camelids such as alpacas [43], where curettage of the endometrial lining followed by intrauterine infusion of saline failed to stimulate ovulation. Other proposed ovulation-inducing stimuli include treading of the male's forelimbs on the back and sides of the female, and guttural humming sounds emitted by the male [44]. One study showed that 2/13 female alpacas ovulated after they were mounted without intromission which was not statistically different from the unmated control group (1/20 alpacas ovulated); intromission-free mounting followed by artificial insemination also failed to drastically improve ovulation rates as only 3/9 alpacas ovulated [42]. Thus, researchers concluded that ovulation does not occur in alpacas if there is no intromission.

The role of intromission during copulation is also controversial. It is accepted that stimulation by the penis during copulation initiates the preovulatory cascade by activating sensory nerves in the vagina and cervix. As a consequence it is common veterinary practice to

induce ovulation mechanically in domestic cats displaying signs of estrus using a glass rod [45]. To the contrary, glass rod stimulation of the vagina and cervix was ineffective in producing luteal development in the koala [6], while in ovariectomized ferrets, glass rod stimulation of in the presence of a male increased LH concentrations from $\leq 0.5$ ng/mL to $2.4 \pm 0.43$ ng/mL [46].

Spontaneous ovulators have also been linked with mating-associated stimuli that influence ovarian function. One study was designed to determine the ovarian response in rats that were electrically stimulated, mechanically stimulated or naturally copulated [18]. When the vagina and cervix were stimulated with a glass rod rapidly for 15 seconds, ovulations were detected in 82% of rats. Following copulation, 77% of rats ovulated; placing electrodes directly on the surface of the cervix for 5 seconds caused half of the rats to ovulate. Rats were compared to positive control groups treated with pregnant mare serum (PMS) where all rats ovulated, and negative control groups where animals were treated with PMS followed by chlorpromazine hydrochloride, where ovulations were detected in only 8% of rats treated [18]. To illustrate the importance of the nervous system during copulation a group of rats were subjected to a pelvic neurotomy followed by the PMS, mechanical and electrostimulation treatment. While the neurotomy did not influence ovulation rates in PMS-treated rats, the ovulatory response was abolished in rats that were mechanically stimulated following neurotomy. It was concluded that the ovulatory response in rats was mediated by the nervous system [18]. A similar conclusion was made using cats. One study reported that after severing neurons that supplied the vagina and body of the uterus the proportion of cats that ovulated (3/9) after glass rod stimulation was lower in comparison to sham-operated females (7/10) [47].

### 1.3.1. The role of seminal plasma in ovulation

Traditional roles of seminal plasma were merely in relation to the transport and support of sperm. Currently, the importance of seminal plasma is elevated by the various factors it possesses including growth factors, proteins and cytokines which influence male fertility [48-50].

The male reproductive tract includes accessory glands which comprise the bulk of seminal plasma. In general, animals contain an arrangement of four accessory glands: ampullary, prostate, vesicular and bulbourethral glands. There is considerable variation among and within mammalian Orders with respect to the configuration of accessory glands that are present. That

is, apart from the prostate gland, the collection of accessory glands is inconsistent. For example, in bulls and stallions all four glands are present while boars do not have ampullae, and dogs have only a large prostate gland [51]. Nevertheless, the accessory glands play an important role in providing several factors which contribute to the reproductive success of sperm [52]. The importance of the vesicular glands was illustrated in mice where removal of these glands decreased pregnancy rates and delayed gestation periods; litter size, however was not affected [53]. Vesicular glands in rodents have a heightened importance as they contribute to the formation and function of the vaginal plug [54]. Removal of vesicular and prostate glands severely impaired pregnancy rates in mice, but contrary to other reports [53], litter size also decreased during pairings where vesicular glands were removed [55]. In rats, similar observations have been made. While removal of the ventral portions of the prostate had little effect on fertility, excision of the vesicular glands or dorsolateral lobes of the prostate resulted in complete infertility in rats [56]. Recently, an association between the components found in accessory glands and male fertility have been made in larger animals. One study in stallions found correlations between seminal plasma proteins and fertility, where fertility was measured by total number of conceptions [57]. In Holstein bull seminal plasma, a 26 kDa and 55 kDa protein were found in greater concentrations in bulls with higher fertility, while two other proteins (both 16 kDa) were found in bulls with lower fertility [49]. Results from a study designed to determine the influence of sterile copulation on ovulation found that the interval from the end of estrus to the release of the ovum was shortened by approximately 2 hrs in heifers [58].

In sows, mating has been reported to hasten the interval from estrus to ovulation by 3.9 hours [59]. In a similar study, seminal plasma hastened the onset of ovulation in the ipsilateral ovary in comparison to the contralateral ovary, and shortened the interval between the LH peak and ovulation in comparison to saline treated groups [60]. In addition to ovulation, seminal plasma appeared to have a luteotrophic effect in prepubertal gilts. Intrauterine infusion of seminal plasma produced heavier corpora luteum and higher concentration of progesterone, while the number of follicles that ovulated were similar to those observed in saline treated animals [61]. Results from this study also indicate that seminal plasma stimulated greater leukocyte recruitment into ovarian tissue than in those gilts treated with saline [61].

It appears that koala seminal plasma may have a similar ovulation inducing factor. Female koalas inseminated with koala seminal plasma ovulated and induced a luteal phase observed by an increase in progesterone concentration [6].

In camelids, seminal plasma stimulates the events which lead to ovulation. In the first reported study of its kind, investigators observed that insemination of 3 mL of undiluted camel seminal plasma into the vagina caused 6/8 camels to ovulate [62]. In the same study, ovulation was detected in camels treated with bovine seminal plasma but none were observed after goat or boar semen treatment [62]. Following insemination, animals treated with camel seminal plasma displayed an increase in LH concentrations by 4 hours [63]. In the previously mentioned experiments, ovulation was detected by rectal palpation. More recent studies conducted in New World camelids used transrectal ultrasonography to assess the effects of seminal plasma on llama and alpaca ovarian function. Intrauterine insemination failed to induce any ovulations; however, after an intramuscular dose of alpaca seminal plasma ovulations were observed in 13/14 animals [64]. Ovulation was preceded by a surge in luteinizing hormone concentration 30 hours after treatment and resulted in a corpus luteum which produced a greater concentration of progesterone in comparison to alpacas that ovulated after GnRH treatment [64]. Subsequent experiments designed to scrutinize if seminal plasma elicited its ovulatory effect via a local or system route showed a similar proportion of alpacas ovulated when treated intramuscularly or with curettage following intrauterine infusion, while the lowest ovulation rate was observed in animals where seminal plasma was administered by intrauterine infusion without curettage [43]. The detection of ovulations in animals after intrauterine treatment contradicts earlier results where no ovulations were observed [64] but it was suggested that this anomaly may be the result of increasing the seminal plasma dose and/or accidental curettage during infusion of seminal plasma into each uterine horn [43]. These studies illustrate that contact with the circulatory system is necessary for seminal plasma to elicit its effect, and thus, the factor responsible for ovulation does not apply its ovulatory effect directly at the level of the ovary.

Another ovulation-induction effect of seminal plasma is observed after camel seminal plasma stimulated ovulation in prepubertal mice and also influenced the gonadal weight in both prepubertal males and females [65]. An intramuscular dose of seminal plasma increased the weight of the testis in comparison to saline treated mice [65]. Similarly, ovarian weight had also

significantly increased from 0.039 ± 0.004 g in control groups to 0.057 ± 0.005 g after a 125 µL dose of seminal plasma [65]. In addition, seminal plasma produced a greater number of follicles <100 µm in diameter in comparison to control groups ($P<0.05$) [65].

Recently, the factor in llama seminal plasma responsible for inducing ovulation has been isolated as the ovulation-inducing factor (OIF). In *in vivo* studies using its purified form, there is a positive correlation between the concentration of OIF and the proportion of females that ovulate [66]. Others have also reported purifying OIF from camel seminal plasma using ion exchange chromatography and tested its biological activity in female camels and mice by rectal palpation [67]. In camels, researchers identified that OIF existed as a four-layered structure with the core consisting of amino acids with a molecular mass of 13 kDa [67]. Another group attempted to isolate the biologically active fraction of seminal plasma from Bactrian camel seminal plasma using anion exchange chromatography, but could not further separate components within fractions due to similarity in molecular structure [68]. The function of each biological fraction was tested *in vitro* using a rat pituitary cell culture as well as *in vivo* using female camelids [68].

### 1.4. Hormonal regulation

Regulation of the reproductive cycle involves an intimate relationship between the hypothalamus, anterior pituitary, ovaries and uterus. Depending on the stage of the ovarian cycle, the role of each hormone secreted from these organs is different. At any given time camelids are found in either one of two phases of ovarian development: follicular or luteal phase.

#### 1.4.1. Follicular growth pattern in camelids

In the past, assessment of follicular wave dynamics was obtained on post mortem specimens euthanized at various intervals [69]. Transrectal ultrasonography provides a method where more accurate, real-time assessment of follicular patterns can be made. Most of the information available regarding follicular wave dynamics has been conducted in cattle but the follicular pattern appears to be conserved in camelids. Follicular waves can be divided into four phases: recruitment, selection, dominance and atresia [70]. The wave-like follicular patterns exist as a cohort of ovarian follicles is recruited from a pool of primordial follicles and synchronously emerges and grows [71]. Follicles continue to grow until one is selected and becomes dominant.

In cattle, selection of the dominant follicle is associated with the induction of LH receptors on granulosa cells, an increase in circulating estradiol-17β and a decrease in plasma FSH [72, 73]. The dominant follicle continues to grow and it suppresses further growth of the subordinate follicles and as a result, they regress at various intervals [72, 74]. Similarly, in the absence of an LH surge the dominant follicle regresses and a new follicular wave emerges. Contrary to earlier reports that observed an alternating occurrence of dominant follicles between the right and left ovary in 81% of llamas [75], more recent studies observe no alternating pattern of dominant follicle emergence [76, 77].

The phenomenon of follicular waves has been documented in both spontaneous ovulators (cattle [74, 78], goats [79], mares [80] and women [81]) as well as induced ovulators (llamas [75, 76], alpacas [77], camels [82] and vicuna [83]). In spontaneously ovulating species such as cattle [84], women [81] and sheep [85] a surge in circulating levels of FSH precedes each follicular wave. In llamas periodic fluctuations in FSH concentration are noted but investigators reported no association between follicular wave dynamics and an increase in FSH; estradiol-17β concentrations, however, were positively related to follicle size [75].

The reproductive strategy of camelids ensures that llamas are constantly in a period of follicular growth [76]. Follicular activity persists in llamas regardless of reproductive status. Follicular waves occur in llamas that are anovulatory, pregnant, mated but non-pregnant, lactating or non-lactating and the rate at which the dominant follicle grows is also independent of reproductive status [76]. In llamas, selection of a dominant follicle with a diameter of 3 to 4 mm was retrospectively detected 3 days following ovulation [76]. In accordance with wave characteristics a negative correlation exists between the diameter of the dominant follicle and the number of subordinate follicles [76, 77]; subordinate follicles do not reach a diameter ≥7 mm.

Follicles ≥ 6 mm in diameter and growing are capable of ovulating in copulated llamas and alpacas [76] while follicles that are regressing tend to luteinize without ovulation [86]. The maximum diameter in anovulatory waves of the dominant follicle is influenced by reproductive and lactational status [76]. The maximum diameter of the dominant follicle is smaller in llamas that are pregnant or lactating in comparison to those that are not pregnant and non-lactating, respectively [76]. Using transrectal ultrasonography, the maximum diameter of nonovulatory dominant follicles ranged between 9 to 16 mm before it started to regress [76]. Another study

reported the largest diameter between 8 to 12 mm [75], however follicles ≥12 mm were considered cystic. In alpacas, the mean maximum diameter ranged from 6.4 mm to 11.2 mm [77].

The lifespan of a dominant anovulatory follicle spans between 20 to 25 days in llamas and successive dominant follicles emerge at an interval of 19.8 ± 0.7 days; dominant follicle lifespan and interwave intervals are shorter during lactation and in the presence of a CL in the event of mating-induced ovulation or pregnancy [76]. A shorter interval was reported in another study where the mean lifespan of the dominant follicle reported was 13.8 days, and a mean inter anovulatory wave interval of 11.1 ± 1.9 days [75]. A recent study conducted in alpacas reported an interwave interval that ranged from 12 to 22 days and associated the duration of the interval with the diameter and consequently lifespan of the dominant follicle [77]. A longer interwave interval of 18.2 ± 1.0 days is observed in Dromedary camels [82] while vicunas are reported to have the shortest dominant follicle lifespan of 7.2 ± 0.5 days and an interwave duration of 4.2 ± 0.3 days [83].

### 1.4.2. Luteal phase in camelids

Following ovulation, blood vessels from the theca interna invade the ruptured follicle and form the corpus hemoragicum [87, 88]. Subsequently, luteinization of the theca and granulosa cells of the ovulated follicle occurs and the corpora luteum is formed in response to high levels of LH produced during the preovulatory surge [89]. During the high-progesterone luteal phase, anovulatory follicular waves continue to emerge but the size of the dominant follicle is smaller in comparison to low progesterone environments [76]. Similar observations were made when exogenous progesterone in the form of a CIDR (controlled internal drug release) influenced the follicle size in llamas. A decrease in the follicular diameter was observed in ovaries where follicles ≥ 6 mm were present, and a suppression of follicular growth was observed in ovaries where dominance was not yet present (< 6 mm) [90].

Corpus luteum formation is detected by day 3 (day 0 = copulation) in llamas [91] and alpacas [87] and reaches its maximum diameter by days 8-9 (day 0 = day of treatment) after copulation or intramuscular injection of hCG in non-pregnant alpacas [87, 92]. Elevated progesterone levels are observed from the first day of CL detection to the day of maximum CL

diameter detection [83]. There is a strong correlation between the diameter of the preovulatory follicle and subsequent diameter of the corpus luteum diameter [91]. If mating successfully results in pregnancy, the CL is maintained throughout the entire gestation length [91] which lasts between 331 to 347 days in llamas [93] and 325-361 days in Huacaya and Suri breeds of alpacas [26].

Luteolysis, or the regression of the corpus luteum, is controlled by the uterus. Luteolysis has been associated with a pulsatile release of prostaglandin ($PGF_{2\alpha}$) from the uterus 8 to 10 days after mating [94]. Corresponding, the first significant decrease in CL diameter in nonpregnant llamas occurs on day 9 and was fully regressed by day 12 [91]. Partial hysterectomy studies conducted in alpacas where removal of the uterine horn ipsilateral to the ovary with the CL, showed that luteolysis was impaired and the CL diameter and progesterone concentrations did not regress and females were not receptive to males [95]. Removal of the oviducts or contralateral horn had no effect on luteolysis. Thus, it was apparent that the luteolytic factor from the uterus exerts its effect locally [95]. If a CL is present in both ovaries, removal of both uterine horns prolongs the lifespan of both CLs; removal of the left horn only showed regression of the CL in the right ovary while the CL in the left ovary persisted and progesterone levels remained high [95]. Removal of the right horn had no effect and luteolysis occurred, progesterone levels declined sharply by day 12 (day 0 = treatment) and the female was sexually receptive [95]. These results indicate that luteolysis pathways in camelids work both locally, as in the case of the right uterine horn and systemically, viewed by the left.

### 1.5. Mechanism of ovulation in induced ovulators

Induced ovulators are those animals which ovulate after receiving a copulation-associated stimulus. However, identifying an animal as an induced ovulator reveals little about their estrous behaviour as these species display a range of features. In animals such as camelids, rabbits and ferrets, ovulation occurs after a single copulatory event [16]. In camelids natural mating, interrupted mating with or without an intact male, single or multiple copulations all result in similar ovulation rates [42]. In ferrets, pairings interrupted after 1 minute were still sufficient in stimulating a preovulatory LH surge [96]. In animals such as cats [97] and musk shrew [3] multiple copulations are necessary to achieve maximum number of ovulations.

The LH surge is the accumulation of rapid pulses that precedes ovulation in both spontaneous and induced ovulators. In spontaneous ovulators, the determinants and characteristics of this surge has been extensively studied. In contrast, the pathways by which ovulation occurs in induced ovulators is poorly understood. In these animals there lacks homology of the factors responsible for initiating the ovulatory response as well as the duration in which the LH surge and subsequent rupture of the follicle occurs. Amongst induced ovulators, camelids and ferrets have been reported as having two of the longest copulation times (3-65 minutes [26-28] and 1-84 minutes, respectively [96]) which presumably, would result in a more latent pre-ovulatory LH surge. However, a prolonged LH surge is only seen in ferrets, with the first significant rise in LH occurring 2 hours after mating, peaking after 8 hours and a declines to basal levels after 12 hours [96]. In llamas, an elevated level of LH occurs within 15 minutes after mating and peaks after 2 hours before resuming basal concentrations by 7 hours [98]. Regardless of the duration of the LH surge, subsequent ovulation was similar in both animals which occurs 30 h post coitus [98-100]. Further variation in the time in which the first significant rise in LH is observed in reflex ovulators with times ranging from 3 minutes in rabbits [101] 5-10 minutes after mating in the short tailed field vole [102] and 24-32 hours in the koala [6].

The LH surge regulates several factors and genes involved in the ovulatory process, demonstrating its importance. LH regulates gene expression by turning off genes involved in folliculogenesis and stimulates the production of factors in granulosa cells, such as EGR-1 [103]. Mice expressing the null allele for this factor results in infertile mice and impaired development of the anterior pituitary gland [103, 104]. LH also stimulates prostaglandins and enzymes involved in follicle rupture, such as matrix metalloproteinase-19 (MMP19), in theca and granulosa cells [103].

In reflex ovulators, there has been little evidence that estradiol production contributes to the preovulatory LH surge. In rabbits, the concentration of estradiol-17β remained unchanged during periods of estrous through pseudopregnancy [105] and administration of exogenous estradiol had no effect on LH secretion in rabbits and ferrets [106, 107]. Reports from one study, suggested that a possible role of estradiol was to augment the sensitivity of LH to GnRH [108]. In camelids, the role of estradiol in the ovulatory process needs further examination. A clear relationship between estradiol concentration and follicle size is evident [82, 83, 98]. At the time

of coitus, a dominant follicle and elevated levels of estradiol are present. Estradiol levels declined and remained low 48h after ovulation while progesterone levels were high [98]. A similar relationship was evident in cats, where levels of estradiol-17β fluctuated during estrus with the growth of the follicle, but appeared to have no effect on the magnitude or duration of the LH surge [109] .

### 1.6. Role of gonadotropin-releasing hormone in induced ovulators

Gonadotropin-releasing hormone (GnRH) is viewed as the central regulator of LH pulses in mammals. In spontaneous ovulators, each LH pulse from the pituitary coincides with a GnRH pulse from the hypothalamus [110]. GnRH is a decapeptide secreted by neurons which are widely distributed in the hypothalamus. The number and location of GnRH neurons distributed within the hypothalamus varies between species, however, concentrated perikarya typically exist in the preoptic and medial basal region of the hypothalamus [16]. When released into primary portal capillaries located in the median eminence, GnRH is delivered to target cells in the hypophysis through the hypothalamo-hypophyseal portal system [111]. The importance of GnRH secretion has not directly been made in induced ovulators as it has been in spontaneous ovulators. It is generally perceived that stimulation of peripheral nerves during coitus is the primary signal responsible for the preovulatory LH rise and subsequent ovulation [2, 16]. These nerves relay coital signals to regions of the brain containing GnRH perikarya, stimulating GnRH release in the portal system [16]. Direct sampling of GnRH from portal blood has never been attainable in induced ovulators because GnRH is not secreted systemically and it is difficult to directly measure GnRH in the hypophyseal portal system [16]. However, using push-pull perfusion samples of the medial basal and anterior region of the hypothalamus, the concentration of GnRH increased from 1.15 ± 0.29 pg/mL in unmated female rabbits to 106.67 ± 37.42 pg/mL post copulation [112]. Immuno-labelled assays have also helped in determining the ovulatory role of GnRH in reflex ovulators. An ovulatory LH surge induced by stimulation of the vagina and cervix in ferrets, resulted in a 50% depletion of GnRH neurons and contributed to the hypothesis that the LH surge is mediated by an abrupt release of GnRH [46]. In addition, activation of GnRH neurons was observed in mated ferrets in which their nares were occluded to remove possible pheromonal stimuli [113]. A similar observation occurred in voles. After 5

minutes of mating, the mean content of residual immuno-reactive GnRH was 496 ± 111 pg/hypothalamus in comparison to 1138 ± 135 pg/hypothalamus in unmated female voles [114]. In contrast, after 1 h of copulation in the musk shrew, there was no change in GnRH immuno-reactive cells in the hypothalamus, however, an increase in proGnRH cell numbers was evident. Investigators hypothesized that mating induces either rapid translation and/or transcription of GnRH mRNA [115].

Nerve stimulation is one plausible stimulus of the ovulatory pathway in induced ovulators. One study conducted in ovariectomized cats observed an LH surge when electrodes stimulated the medial basal and medial preoptic region of the hypothalamus, both regions known to contain concentrated GnRH neurons [116]. A similar observation was made in ferrets, where electrostimulation of similar regions in the hypothalamus induced LH secretion in estrus and anestrus ferrets [117]. Contrary to these results, rabbits that were given GnRH analogues 30 minutes prior to copulation were still capable of ovulation. Only after multiple injections of GnRH analogues prior to copulation was there a decrease in ovulation rates, however, 40% of the does still ovulated [118]. Results from these studies indicated that there may be another factor working in junction with GnRH to regulate LH release from the pituitary gland. Nevertheless, synthetic GnRH peptides have been successful in stimulating LH secretion and inducing ovulation in reflex ovulators even without confirmed *in vivo* data [96, 100, 119, 120].

## 1.7. The pituitary gland

### 1.7.1. Pituitary anatomy

"[T]he sudden removal in its entirety of the epithelial lobe (pars anterior) of the hypophysis during a presumed state of health is incompatible with a lengthened maintenance of life"[121].

The hypophysis is often referred to as the "Master Gland" in the mammalian body in response to its role in regulating normal body function [122]. The importance of this gland was first recorded by Paulesco when the complete removal of the pituitary led to death in dogs within days; examination of dogs that did survive identified remnants of the pituitary *in situ* [121]. The hypophysis or pituitary gland is generally isolated into two lobes both of which have different embryonic origin and consequently results in a difference of function. The neurohypophysis is a specialized extension from the wall of the diencephalon of the developing brain and is

subdivided into the pars nervosa (neural lobe), and the infundibulum [123, 124]. The infundibulum is further divided into the infundibular process and the median eminence which is an extension of the hypothalamus [123]. On the other hand, the adenohypophysis is of epithelial origin formed by an evagination of the ectodermal roof of the developing mouth commonly referred to as Rathke's pouch, named after its discoverer [123]. The cranial portion, the pars distalis, is the site where cell replication is faster; the caudal wall develops to become the pars intermedia. The anterolateral portion of the adenohypophysis grows on both sides of the infundibulum, forming the pars tuberalis [125]. The adenohypophysis quickly enlarges, flattens and encircles the infundibular portion of the neurohypophysis [124]. The degree to which the adenohypophysis encases the neurohypophysis depends on the species. The embryonic development results in complete neurologic connection between the neurohypophysis and hypothalamus and a lack of innervation to the adenohypophysis [124, 126].

The hypophysis lies in a depression of sphenoid bone called the hypophyseal fossa. The gland is surrounded by dura, and the roof is formed by a reflection of the dura, the diaphragm sellae [127]. The diaphragm sellae adds another layer of protection to the pituitary as it inhibits cerebrospinal fluid from coming in contact with the pituitary [127]. The pars distalis is the largest portion of the adenohypophysis and contains five populations of cells: gonadotropes, thyrotropes, lactotropes, corticotropes and somatotropes [124]. As the secretion of gonadotrope cells relate to reproduction, gonadotrope cells will be the focus of the rest of this section.

### 1.7.2. Gonadotrope cells and reproduction

Gonadotrope cells have been localized in the ventral and lateral regions of the pars distalis [15, 128]. In one study conducted in primates, investigators used immunostaining and also found populations of gonadotrope cells in the pars tuberalis with the greatest accumulation of cells lateral to the median eminence [129]. Similarly, gonadotrope cells have been found throughout the pars tuberalis in humans [130]. In pigs, gonadotrope cells were the only cells present in the pars tuberalis, and *in vitro* studies indicate that under GnRH stimulation, these cells secrete LH [131].

Gonadotrope cells primarily secrete the glycoproteins luteinizing hormone (LH) and follicle stimulating (FSH) both which are important in regulating reproductive function [15].

Each glycoprotein is composed of an α and β subunit. The α subunit has a common amino acid sequence with LH, FSH, TSH (thyroid stimulating hormone) and human-chorionic gonadotropin (hCG) glycoproteins, but the configuration of the β subunit is specific to each hormone and confers biologic specificity [132]. Protein translation and combination of the α and β subunits take place in the rough endoplasmic reticulum (RER) in gonadotrope cells. From the RER, precursor glycoprotein hormones enter the Golgi complex to undergo posttranslational modifications such as glycosylation[132]. The hormone is then transported to storage vesicles in the cytoplasm. At the time of secretion, vesicles bind to the plasma membrane and the hormone is excreted by exocytosis into the extracellular space [132].

FSH receptor mRNA has been localized to granulosa cells using cloned receptor cDNAs, while LH receptor mRNA have been found on thecal and interstitial cells, corpus luteum and depending on the stage of the estrous cycle, on granulosa cells [133]. The importance of FSH and LH on follicle growth and maturation was observed in hypophysectomised mice. The number of ovarian follicles decline significantly by 4 to 20 days post removal of the pituitary gland [134]. In addition, LH and FSH receptors found on granulosa cells, theca cells and corpora luteum disappeared in mice four days after pituitary removal [134].

Not all gonadotrope cells are the same. These cells have been divided into subtypes, Types I-III, based on their morphologies and suspected correlation with function. Type I cells are ovoid with a population of large granules (≥ 400 nm in diameter) and a population of small granules. Type II cells are angular or stellate and contain numerous secretory granules averaging 200-220 nm in diameter. Type III cells are stellate with granules arranged around the cells periphery [135].

The shape, size and storage pattern of gonadotrope cells change during the estrous cycle; hence, gonadotrope function may correlate with its morphology [15]. In one study, rats were euthanized at different stages of the estrous cycle [136]. In rats euthanized during estrus, metestrus and proestrus, periods where serum levels of LH are low, cells were often polygonal and less frequently ovoid [136, 137]. The cytoplasm was homogenous, containing a moderate number of secretion granules which either clumped or scattered throughout the cytoplasm [136]. In proestrous rats, there was an increase in the number of degranulated gonadotrope cells and cells were typically round in shape [136]. Another observation in this study was that cell size

increased from being small in diestrus to large in proestrus which suggested that the pituitary cell becomes more dense with secretory granules and larger during different stages and the amount of LH secreted is related to the amount of cells present [136, 138]. Similar observations in morphology have been observed in rats [139].

## 2.0 OBJECTIVES & HYPOTHESIS

Based on the following observations:

- Administration of llama seminal plasma intramuscularly stimulates ovulation in 93% of treated alpacas [64]
- OIF promotes longer lasting LH surge in comparison to that characterized following GnRH treatment in llamas and alpacas [64]
- OIF induces ovulation by a systemic route, rather than locally at the ovary [43]
- OIF has a luteotrophic effect evidenced by producing larger CL diameter and more progesterone [64]
- OIF has a dose dependent effect on the proportion of ovulations, CL diameter and plasma progesterone concentrations in llamas [66]
- Gonadotrope cells express OIF receptors (unpublished data)
- Camelids ovulate after copulation with a vasectomised or intact male [42]
- All male mammals have seminal plasma which is produced by male accessory glands
- 2 mL of bull seminal plasma induces ovulation in 26% of camelids [140]

I hypothesize that:

OIF is a conserved constituent of mammalian seminal plasma which elicits its ovulatory effect by triggering LH secretion by directly binding to its receptors on gonadotrope cells in the anterior pituitary.

General Objective:

The main goal of the work described in this thesis was to contribute to the hypothesis that OIF is a conserved component of mammalian seminal plasma.

The specific objectives of the first study (Chapter 4) were to:

- Determine if OIF from llama seminal plasma will induce ovulation in prepubertal mice
- Test the hypothesis that OIF is found horse and pig seminal plasma
- Test the hypothesis that OIF elicits its response by influencing LH secretion

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

- Determine if purified OIF will stimulate LH secretion from llama and bovine anterior pituitary cells
- Determine if the level of LH secretion stimulated from OIF is related to dose in llama and bovine pituitary cell cultures

## 3.0 EVIDENCE FOR THE CONSERVATION OF BIOLOGICAL ACTIVITY OF OVULATION-INDUCING FACTOR (OIF)

### 3.1 Abstract

An ovulation-inducing factor (OIF) in the seminal plasma of llamas and alpacas (induced ovulators) and cattle (spontaneous ovulators) suggests that OIF is a conserved constituent of seminal plasma among mammals. Three experiments were designed to determine the biological effects of OIF in different species. In Experiment 1, superstimulated prepubertal female CD1 mice (n = 36 per group) were given a single 0.1 mL intraperitoneal dose of 1) phosphate-buffered saline, 2) 5 μg GnRH, 3) 5 IU of hCG, or 4) llama seminal plasma. The mice were euthanized the day after treatment, the oviducts were collected, and oocytes were counted. The proportion of mice that ovulated were similar among groups treated with GnRH, hCG, and seminal plasma (31/36, 31/36, 28/36, respectively), and all were higher than the saline-treated group (9/36) ($P < 0.001$). In Experiments 2, female llamas (n = 8 or 9 per group) were treated intramuscularly with 1) 2 mL phosphate buffered saline, 2) 1 mL diluted llama seminal plasma, 3) 3 mL of equine seminal plasma, or 4) 3 mL porcine seminal plasma. Experiment 3 was the same as Experiment 2 except that the dose of equine and porcine seminal plasma was increased to 8 mL and 10 mL, respectively. In the absence of an effect of experiment for any end point, data from Experiments 2 and 3 were combined. All llamas treated with llama seminal plasma ovulated (positive control) and none treated with saline ovulated (negative control; $P<0.0001$). The proportion of llamas that ovulated in response to equine and porcine seminal plasma was intermediate. Compared to negative controls (PBS), the proportion of llamas that ovulated was higher ($P=0.03$) in the equine seminal plasma group and tended to be higher ($P=0.1$) in the porcine seminal plasma group. Treatment with seminal plasma of all species was associated with an increase ($P<0.05$) in plasma LH pulse frequency. Results support the hypothesis that OIF and its effects are conserved among species. We conclude that the mechanism for the biological response to OIF is present in prepubertal CD1 mice, and that OIF is present in equine and porcine seminal plasma.

## 3.2. Introduction

The mechanism by which ovulation is initiated has been used to classify mammals as either spontaneous- or induced-ovulators, based on the biological process that triggers the release of GnRH and initiates the ovulatory cascade. Mice, cattle, horses and pigs are considered spontaneous ovulators because ovulation occurs at regular intervals as a result of increasing systemic concentrations of estradiol from a growing dominant follicle which stimulates GnRH secretion from neurons in the hypothalamus, which in turn elicits a surge release of LH from the anterior hypophysis [88]. In contrast, ovulation in induced ovulators does not occur at regular intervals, but rather in response to a copulatory stimulus. Documented examples of induced ovulators include camelid species [141-143], domestic cats [144], rabbits [145], koalas [6], bushtail possums [146], voles [4, 147] bears [148] and ferrets [2, 96]. The line that distinguishes spontaneous and induced ovulators, however, is often blurred as copulation has been reported to influence ovarian function in some spontaneously ovulating species. In early studies, mating hastened the onset of ovulation in sows [59], and mating and/or mechanical stimulation of the vagina and cervix resulted in ovulation in rats [18]. Spontaneous ovulation has also been reported in induced ovulators. Spontaneous ovulation was detected by ultrasonography and rectal palpation in 5% of dromedary camels [10] and 4 to 8% of llamas and alpacas [44], and was detected based on elevated progesterone concentrations in 13/15 group-housed domestic cats isolated from copulatory stimuli [149].

The term “reflex” ovulator is often used synonymously with “induced” ovulator because of the perception that ovulation occurs as a response to the stimulation of sensory nerves in the vagina and cervix by the penis during copulation [42, 46]. In contrast to the concept of a direct neural stimulus, there is increasing evidence for the presence of a biochemical substance in seminal plasma that acts in an endocrine fashion to elicit pituitary LH release and ovulation [43, 62, 64, 67]. Results from one study [64] documented the existence of a potent factor in the seminal plasma of alpacas and llamas that elicited a surge in circulating concentrations of LH and induced an ovulatory and luteotropic response. To determine the effect of seminal plasma of conspecific versus hetero-specific males, the ovulation-inducing effect of seminal plasma of alpacas and cattle was compared with that of the llama using female llamas as a bioassay [140]. Ovulation was induced by seminal plasma of all three species, providing rationale for the hypothesis that OIF is a conserved constituent of seminal plasma among mammals, and has an

effect on ovarian function in females of unrelated species. As the effect of OIF in bovine seminal plasma had already been reported, pigs and horses were used to represent unrelated species of economic value with a well characterized estrous cycle [150-153].

The objectives of this study were to determine if the biological response (ovulation) to llama seminal plasma is present in mice, (Experiment 1), and if equine and porcine seminal plasma will induce ovulation in llamas (Experiments 2 and 3).

## 3.3. Materials and methods

### 3.3.1. Experiment 1

Semen was collected from male llamas (n=4) by artificial vagina [154] twice per week over a period of 4 months at the University of Saskatchewan. Raw ejaculates were diluted 1:1 (v/v) with phosphate buffered saline (PBS, Gibco, Grand Island, NY, USA) and drawn back-and-forth through a 7 mL disposable transfer pipette (VWR, Mississauga, ON, Canada) to reduce semen viscosity, and then centrifuged for 30 minutes at 1500 x g [64]. The supernatant was decanted to separate it from spermatozoa and a drop was evaluated microscopically to confirm the absence of cells. If spermatozoa were detected, the sample was recentrifuged. Seminal plasma was stored at -80ºC. Upon thawing, seminal plasma from different animals and different ejaculates was pooled and 1% penicillin/streptomycin (10,000 units/mL of penicillin and 10mg/mL streptomycin; Sigma-Aldrich, St. Louis, MO, USA) was added.

Prepubertal female CD1 mice (*n* = 144), 20 days of age and weighing 20 to 25 g, were housed at 24°C with lights on from 0500 to 1900 h and access to food and water *ad libitum*. An intraperitoneal dose of 5 IU of eCG (Pregnecol, Bioniche Animal Health, Belleville, ON, Canada) was given (Day 0) for ovarian superstimulation. On Day 2, mice were assigned randomly to four groups (*n* = 36 per group) and given a single 0.1 mL intraperitoneal dose of 1) phosphate-buffered saline (negative control), 2) 5 µg GnRH (Cystorelin$^{TM}$, Merial, Ltd., Iselin, NJ, USA), 3) 5 IU of hCG (Chorulon$^{TM}$, Intervet Canada, Ltd., Whitby, ON, Canada), or 4) llama seminal plasma. On Day 3, mice were euthanized by administering an overdose of inhaled halothane. Oviducts were collected and oocytes were counted by oviductal trans-illumination stereomicroscopy. Ovulation was defined as the observational presence of oocytes in the oviduct. Thus, mice with no oocytes were considered not to have ovulated.

### 3.3.2. Experiment 2

Semen was collected from 6 stallions [155] (4 ejaculates per stallion) by artificial vagina over a period of 2 months at the University of Saskatchewan. Immediately after collection, the semen was filtered to remove the gel fraction. Porcine semen was collected by the gloved-hand method [156] from 4 boars (4 ejaculates per boar) at the Prairie Swine Center, University of Saskatchewan. The gel fraction was separated at the time of collection using a gauze filter. Seminal plasma was decanted from spermatozoa by centrifugation in the same fashion described for llama semen (Experiment 1), but was not diluted. The seminal plasma was stored at -80˚C. Upon thawing, the seminal plasma from different animals within species was pooled and 1% penicillin/streptomycin (v/v) (10,000 units/mL of penicillin and 10 mg/mL streptomycin, Sigma-Aldrich, Oakville, ON, Canada) was added. Llama seminal plasma was that used in Experiment 1.

Experiment 2 was conducted during May to June at the University of Saskatchewan (52°N, 106°W and 500 m above sea level) using a herd of mature non-lactating female llamas ≥ 5 years of age and weighing from 90 to 120 kg (n=36). To facilitate data collection in Experiment 2, ovarian follicular development among females was synchronized by administering a single intramuscular dose of 5 mg pLH (Lutropin-V, Bioniche Animal Health, Belleville, ON, Canada) to induce ovulation. We expected 80% to 90% of the animals to ovulate in response to pLH treatment resulting in synchronous emergence of a new follicular wave 2 days after treatment, and for those that did not ovulate to be temporally near natural wave emergence [157]. Llamas were examined daily by transrectal ultrasonography (Aloka SSD900, Tokyo, Japan with a 7.5 MHz linear array probe) for three days, and those that ovulated were given Estrumate (250 µg cloprostenol im; Schering-Plough Animal Health) 8 days after pLH treatment to ensure luteolysis. At 10-12 days after pLH treatment, llamas with a follicle ≥7 mm in diameter were assigned randomly to four groups (n=8 or 9 per group) and given: 1) 2 mL of phosphate buffered saline (PBS; negative control), 2) 3 mL equine seminal plasma, 3) 3 mL porcine seminal plasma, or 4) 1 mL llama seminal plasma (positive control) by intramuscular injection. Equine and porcine seminal plasma concentrations were based on total protein concentration. Injection sites were monitored post-treatment. No inflammation or abscessation was detected.

Llamas were subsequently examined by transrectal ultrasonography daily for 3 days to detect ovulation, and again on Day 7 for CL formation (Day 0 = day of treatment). Ovulation was defined as the sudden disappearance of a dominant follicle from one day to the next and was confirmed by later detection of a CL [158]. A blood sample was collected into a heparinized tube by jugular venipuncture immediately before treatment on Day 0 and once more on Day 7 for measurement of progesterone concentration (BD Vacutainer Systems; Becton Dickinson, Franklin Lakes, NJ, USA).

A subset of three llamas per group was catheterized to determine the effects of seminal plasma treatment on LH secretion. Catheters were inserted into the jugular vein the day before treatment to minimize the effect of stress at the time of sampling [64]. Beginning immediately before treatment, samples were taken every 15 minutes for 8 hours, and then every hour until hour 12. Blood samples were collected in heparinized glass tubes (Hepalean, Organon Canada Ltd., Toronto, ON) and centrifuged within 1 hour of collection at 1700 x g for 20 minutes, and the plasma was stored at -20ºC until the time of assay. Due to high individual variation, LH concentrations were expressed as the proportion of change in LH levels in relation to mean basal concentrations. The mean basal concentration was calculated by taking the mean of the first three values (time 0, 15 and 30 min), where the difference among concentrations are minimal.

### 3.3.3. Experiment 3

Experiment 3 was conducted during July to August at the University of Saskatchewan using the same herd of female llamas and experimental design as that of Experiment 2 except that the dose of equine and porcine seminal plasma was increased to 8 mL and 10 mL, respectively. The llamas were randomly reassigned to groups to ensure that they were not given the same treatment as in the previous experiment. The llamas catheterized for frequent blood sampling (n=3 per group) were not the same as those used in Experiment 2.

### 3.3.4. Hormone assay

Plasma progesterone concentrations were determined using a commercial, double antibody radioimmunoassay kit (Coat-a-Count progesterone, Siemens Medical Solutions Diagnostics, Los Angeles, CA). All samples were analyzed in a single assay. The intra-assay coefficients of variation for the low, medium and high reference plasma progesterone

concentrations (1.78 ng/mL, 3.59 ng/mL and 14.77 ng/mL) were 7.56%, 6.1% and 3.57%, respectively.

Plasma LH concentrations were measured using a double-antibody radioimmunoassay [159]. Concentrations of LH are expressed in terms of NIAMDD-bLH-24. The minimum detectable limit of the assay was 0.063 ng. The range of the standard curve was from 0.063 ng (80% ligand labelled LH) to 8.0 ng (20% ligand labelled LH). The intra- and interassay coefficients of variation were 9.4% and 10.6%, respectively for the high reference plasma LH concentration (2.73 ng/mL). The intra- and inter-assay coefficients of variation were 13.3% and 12.3%, respectively for the low reference plasma LH concentration (1.20 ng/mL). The PC-Pulsar program was used to assess mean and basal plasma LH concentrations as well as LH pulse frequency and amplitude in blood samples collected every 15 minutes for 8 hours [160].

To minimize individual variation, LH profiles were expressed as a proportion of LH secreted from the reference mean. For each animal, the mean of the first three LH concentrations (times 0, 15 and 30 minutes post treatment) were used as basal LH concentrations and subsequent values were expressed in relation to the calculated basal mean.

Values that exceeded more than three standard deviations from the mean were considered outliers and removed from further analysis.

### 3.3.5. Statistical Analyses

Statistical analyses were done using SAS statistical software (Statistical Analysis System Inc., Cary, NC). Single point measurements (i.e., number of oocytes, follicle size at the time of treatment, LH pulse frequency (the number of LH pulses detected per hour), corpus luteum diameter and progesterone concentrations) were compared among groups by one-way analyses of variance. Serial data (i.e., LH concentrations) were compared among groups by analysis of variance for measures (Proc Mixed, SAS). In the absence of an experiment-effect, data from Experiments 2 and 3 were combined and analyzed as a total data set. When main effects or the interactions were significant (i.e., $P \leq 0.05$), means were compared using Tukey's multiple comparison as a post-hoc test. Ovulation rates were compared among groups by chi-square analysis (Experiment 1) and Fisher's exact test (Experiments 2 and 3; Proc Genmod, SAS).

## 3.4. Results

### 3.4.1. Experiment 1

A greater number of mice ovulated after treatment with GnRH, hCG or seminal plasma in comparison to those treated with saline (P<0.05; Table 3.1.). Correspondingly, the mean number of oocytes observed per treatment group was lower in saline treated animals compared to other groups (P<0.001). The proportion of mice that ovulated was similar in the GnRH, hCG and seminal plasma groups; although the mean number of oocytes per mouse was lower in the latter (P<0.05).

**Table 3. 1.** Comparative ovulation-inducing effect of saline, GnRH, hCG and llama seminal plasma in superstimulated prepubertal CD-1 mice (Experiment 1; mean ± SEM).

| End points | PBS (n = 36) | GnRH (n = 36) | hCG (n = 36) | Seminal Plasma (n = 36) |
|---|---|---|---|---|
| Ovulations (%) | 6/36[a] (16.7%) | 31/36[b] (86.1%) | 31/36[b] (86.1%) | 28/36[b] (77.7%) |
| Number of oocytes | 6.2 ± 2.1[a] (n=6) | 27.4 ± 2.7[b] (n=31) | 25.8 ± 2.9[bc] (n=31) | 19.2 ± 2.8[c] (n=31) |

[abc] Within rows, values with no common superscript are different (P<0.05)

### 3.4.2. Experiments 2 and 3

There was no difference among treatment groups in the diameter of the pre-ovulatory follicle at the time of treatment (P=0.6; Table 3.2.). All llamas treated with llama seminal plasma ovulated (positive control) and none that were treated with saline ovulated (negative control; P<0.0001). The proportion of llamas that ovulated in response to equine and porcine seminal plasma was intermediate (Table 3.2.). Compared to negative controls (PBS), the proportion of llamas that ovulated was higher (P=0.03) in the equine seminal plasma group and tended to be higher (P=0.1) in the porcine seminal plasma group. Among those that ovulated, the interval from treatment to ovulation did not differ among groups (1.7 ± 0.1, 2.0 ± 0.3, 1.7 ± 0.3 days after treatment in the llama, equine and porcine seminal plasma groups, respectively, P=0.56). Among

those that ovulated, the diameter of the CL and plasma progesterone concentrations did not differ among groups (Table 3.2.).

**Table 3. 2.** Effects of llama, equine and porcine seminal plasma on ovulation and the form and function of the CL in llamas (Experiments 2 and 3 combined; mean ± SEM; Day 0 = treatment).

| | PBS | Seminal Plasma | | |
|---|---|---|---|---|
| | | Llama | Equine | Porcine |
| Follicle diameter on Day 0 (mm)* | 10.1 ± 0.5 | 9.9 ± 1.8 | 9.6 ± 0.4 | 9.5 ± 0.4 |
| Ovulation rate | 0/16[a] | 18/18[c] | 5/17[b] | 3/17[ab] |
| (%) | (0%) | (100%) | (29%) | (18%) |
| CL diameter on Day 7 (mm) | -- | 10.9 ± 0.6[a] | 11.0 ± 1.2[a] | 10.0 ± 0.0[a] |
| Progesterone concentration on Day 7 (ng/mL) | 0.3 ± 0.05[a] (n=16) | 3.7 ±0.05[b] (n=18) | 3. 2 ± 0.9[b] (n=5) | 3.4± 0.5[b] (n=3) |

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

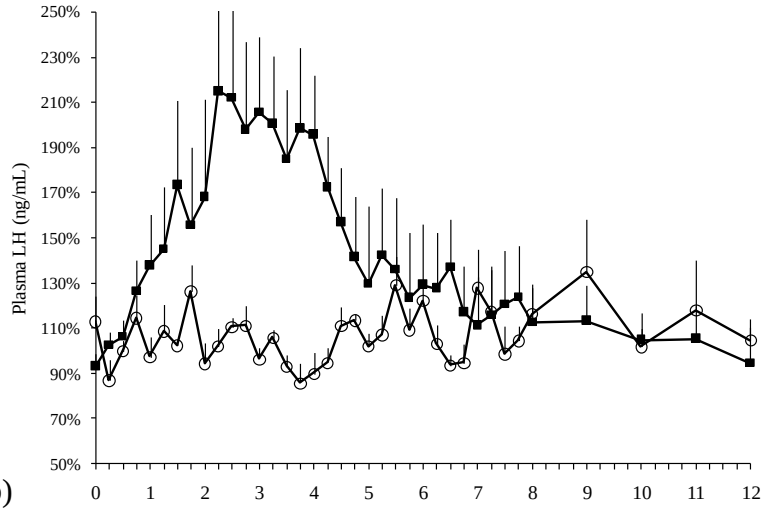
*No significant difference among groups

Ovulations were observed in all groups treated with seminal plasma when the dose of equine and porcine seminal plasma increased to 8 mL and 10 mL, respectively (Experiment 2 vs. 3). The proportion of llamas that ovulated in response to treatment with a low dose of equine seminal plasma (3 ml) vs. high dose (8 ml) did not differ (3/8 vs. 2/9; P=0.45). However, the proportion of llamas that ovulated in response to treatment with a low dose of porcine seminal plasma (3 ml) vs. high dose (10 ml) tended to differ (0/8 vs. 3/9; P=0.1).

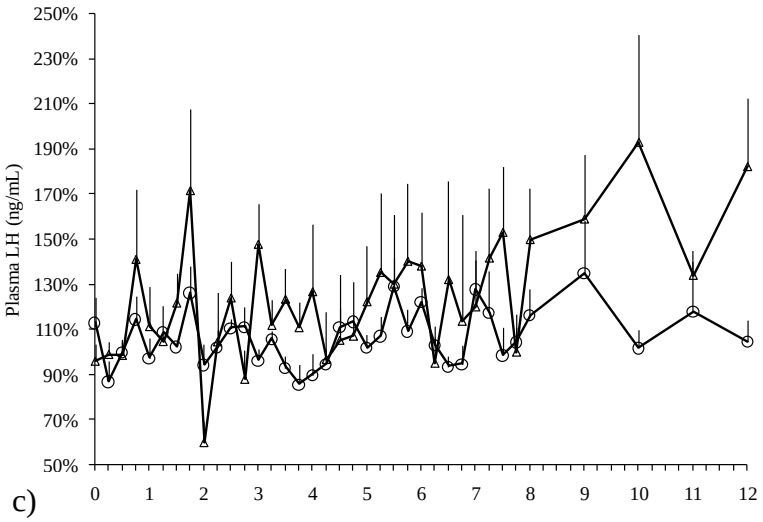
Due to complications in catheter placement, one of the catheterized llamas in the saline-treated group was removed from the experiment after 2-hours of sampling and her data were not included in LH analyses. Of the frequently sampled animals, only those treated with llama seminal plasma ovulated. Mean LH concentrations after llama seminal plasma treatment were higher than in the porcine seminal plasma and saline treated groups, while animals treated with

equine seminal plasma were intermediate (treatment effect, P<0.01; time effect, P=0.21; treatment-by-time effect, P=0.09; Figure 3.1) The treatment-by-time interaction in the LH profile (Figure 3.1a) was attributed to an LH surge in the llama seminal plasma-treated group and not in the others (Figure 3.1b. and 3.1c.). Mean plasma LH concentrations began to increase within 15 minutes of treatment with llama seminal plasma, peaked after 2 hours, and declined to basal concentrations by 7 hours after treatment (Figure 3.1a.). By excluding the positive control group from analysis, a treatment effect was still observed as equine seminal plasma stimulated higher levels of LH secretion, while mean LH concentrations after porcine seminal plasma were intermediate (treatment effect, P=0.02; time effect, P=0.18; treatment-by-time effect P=0.35). Compared to the PBS- treated llamas, LH pulse frequency was higher in the llama and porcine seminal plasma groups (P=0.04), while LH pulse frequency in the equine seminal plasma group was intermediate (Figure 3.2.).

a)


250%
230%
210%
190%
170%
150%
130%
110%
90%
70%
50%
Plasma LH (ng/mL)
0 1 2 3 4 5 6 7 8 9 10 11 12

b)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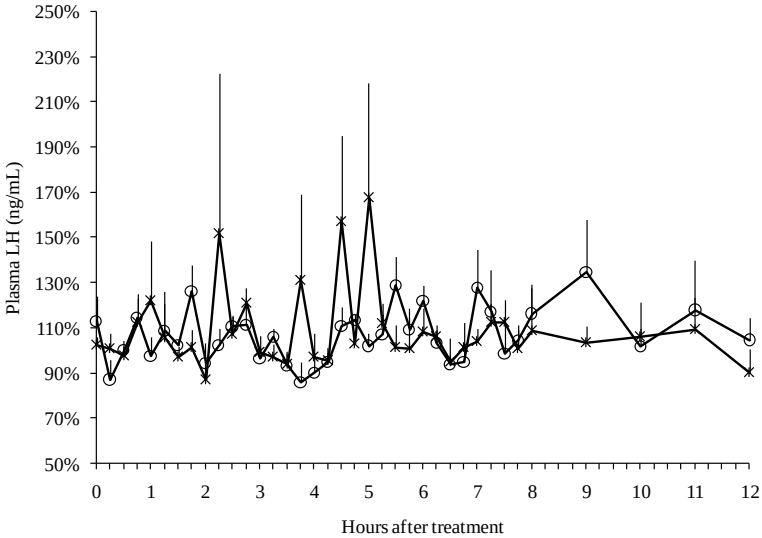
Plasma LH (ng/mL)
0 1 2 3 4 5 6 7 8 9 10 11 12

c)


250%
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210%
190%
170%
150%
130%
110%
90%
70%
50%
Plasma LH (ng/mL)
0 1 2 3 4 5 6 7 8 9 10 11 12
Hours after treatment

**Figure 3. 1.** Plasma LH concentrations (mean ± SEM) in female llamas after intramuscular treatment with a) llama (■; n=6), b) equine (▲; n=6), or c) porcine seminal plasma (∗; n=6), all in comparison to llamas treated with phosphate buffered saline (PBS,○; n=5). Values are expressed as the proportion of LH secreted from the mean values of the first three time points (P<0.05).

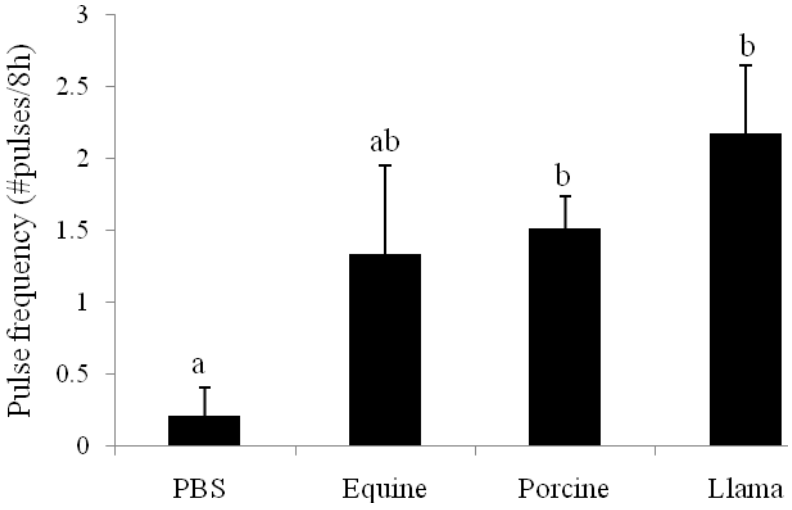



**Figure 3. 2.** Plasma LH pulse frequency (mean ± SEM) determined from samples collected at 15-minute intervals for 8 hour starting from the time of treatment with phosphate buffered saline (PBS, n=5 llamas), or equine, porcine or llama seminal plasma (n=6 llamas per group). [ab]Values with no common superscript are different (P=0.04).

### 3.5. Discussion

Our results indicate that a similar biological response (ovulation) to a single application of OIF observed in llamas and alpacas is conserved in prepubertal mice. In the first experiment the proportion of mice that ovulated was similar among GnRH, hCG and llama seminal plasma groups. However, the mean number of oocytes observed in the oviduct was lower in seminal plasma treated mice in comparison to mice treated with GnRH or hCG. The simplest explanation is that seminal plasma is not as effective in stimulating multiple ovulations in mice in comparison to GnRH or hCG. However, as the structure and mechanism of action of OIF is not clear, perhaps this discrepancy may be the result of an incompatibility between mouse OIF receptor to llama OIF ligand, or this observation may indicate the use of an unsuitable dose.

These results warrant a titration experiment to study the effect of different doses on ovulation rate.

In Experiment 1, hCG was used as a control for GnRH (positive control) in the event that the hypothalamo-hypophyseal axis was not fully developed in the prepubertal mice, and no response to GnRH would have been observed. Since similar proportions of mice ovulated in GnRH and hCG groups, it was assumed that the hypothalamo-hypophyseal axes were functional and that the ovaries were capable of responding at a local or systemic level. It appears that OIF elicits its ovulatory effect by stimulating LH secretion from the pituitary based on results generated from camelid studies and in vitro studies pituitary explants culture [43]. These findings suggests the conservation of not only the OIF ligand, but also its receptor and other factors which contribute to the physiological pathway in which OIF elicits its effect.

Experiments 2 and 3 were conducted using a female llama bioassay approach to determine the presence of ovulation-inducing factor in equine or porcine seminal plasma. Results were similar to that obtained using bull seminal plasma (5/19 llamas ovulated) demonstrating the presence of an ovulation-inducing factor in these species.

To determine a dose effect, llamas were treated with two different volumes of equine and porcine seminal plasma, while the dose of the controls remained constant. The preliminary dose of equine or porcine seminal plasma was based on total protein concentration using a spectrophotometer as a way to account for differences in ejaculatory volume: the average volume of one stallion ejaculate (70 mL [51]) varies from that of a boar (250 mL [51]). Similar ovulation rates were detected in llamas treated with 3 mL or 8 mL of equine seminal plasma, while ovulations did not occur in llamas treated with porcine seminal plasma until the dose increased to 10 mL. There are different hypotheses which arose from these findings. When further scrutinizing results in the context of a study conducted to determine the dose response to purified llama OIF, an increase in the number of llamas that ovulated (3/10, 7/10, 9/10) with increasing fractions of OIF (60 µg, 125 µg, 250 µg, respectively) was observed [66]. Similarly, llamas treated with porcine seminal tended to ovulate when the dose of seminal plasma increased. In the same experiment, no difference in ovulation rates (9/10) were noticed when llamas were treated with 250µg or 500 µg of purified OIF which indicated that the minimum dose needed to achieve maximum number of ovulations was 250µg. In retrospect, it may not have been accurate to base our initial dose on total protein. On average, llamas produce a 3 mL ejaculatory volume. As a

positive control 1 mL of diluted llama seminal plasma was used, representing 1/6$^{th}$ of an ejaculate. The 3 mL volume of equine seminal plasma represents 1/23$^{rd}$of an ejaculate, and yet ovulation occurred in 3/8 llamas. The 3 mL dose of porcine seminal plasma represents 1/80$^{th}$ of a boar ejaculate. In this context, it is not surprising that no ovulations occurred. The 60 µg dose of purified OIF used in a previous study [66] in which only 3/10 test llamas ovulated, would represent approximately 1/50$^{th}$ of a llama ejaculate. When the dose of porcine seminal plasma was increased to represent 1/25$^{th}$ of an ejaculate, ovulations occurred in 3/9 test llamas, which is similar to the proportion observed in llamas treated with 3 mL of equine seminal plasma. In light of these findings, it is unreasonable to assume that the quantity of OIF in porcine seminal plasma is lower than in equine, bull or llama seminal plasma. Instead, a more likely explanation is that inappropriate doses for side-by-side comparisons were used.

In most if not all mammals, a surge in circulating levels of LH precedes ovulation. In llamas and alpacas this rise occurs within 15 minutes of mating or seminal plasma treatment, and peaks after 2 hours before returning to basal levels by 7 hours after treatment [64]. Similar observations have been documented in other animals in which coitus is necessary for ovulation. In rabbits, an LH surge occurred after 3 minutes and peaked at 15 minutes after copulation [101]. In ferrets, the first significant increase in LH concentration occurred within 1 hour, peaked between 6 and 8 hours and reached basal concentrations by 14 hours after copulation [96]. In cats multiple copulations were needed to achieve ovulation, and with each copulation, the concentration of LH increased [97]. Collectively, these studies document an increase in LH secretion and ultimately ovulation following copulation.

In this study a subset of llamas was catheterized to measure the effects of each treatment on LH secretion into the peripheral circulation (Figure 1). Catheterized llamas treated with equine or porcine seminal plasma did not ovulate. Thus, the LH profile of an ovulating llama following treatment with equine or porcine seminal plasma remains unknown. However, there was an obvious increase in LH pulse frequency in animals treated with porcine or llama seminal plasma compared to saline treated controls, even though ovulation did not occur. These results support the hypothesis that OIF elicits its effects systemically on either the hypothalamus or directly on the pituitary rather than at the level of the gonads.

In summary, OIF from llama seminal plasma induced ovulation in prepubertal mice. Mice are conventionally classified as a spontaneous ovulator. The interpretation of these results documents the presence of an ovulation-inducing factor in the seminal plasma of horses and pigs. It appears that both the OIF ligand and receptor are conserved among mammals, and the prepubertal mouse may be a useful model for receptor/ligand studies. The purpose of OIF in spontaneous ovulators remains unclear, but its conservation among species indicates of an important functional role.

### 3.6. Acknowledgements

This research was supported by grants from the Alpaca Research Foundation and the Natural Science and Engineering Research Council of Canada.

# 4.0. THE EFFECT OF OVULATION-INDUCING FACTOR ON THE SECRETION OF LH FROM PITUITARY CELLS

## 4.1. Abstract

A substance in the seminal plasma of llamas and alpacas has been discovered that induces ovulation and growth of the corpus luteum (CL) in the female of the same species. The ovarian effects of the ovulation-inducing factor (OIF) are associated with a surge release of LH into circulation. From these results we hypothesize that OIF directly stimulates LH release from gonadotrope cells in the anterior pituitary gland. In this study, four experiments were conducted to determine whether purified OIF isolated from llama seminal plasma would stimulate LH secretion in a cell culture using pituitaries from an induced ovulator (llama) and spontaneous ovulator (cattle). Anterior pituitary cells ($2 \times 10^6$ cells/well) were cultured for 2 days and on the $3^{rd}$ day wells were incubated for 2 hours with either media containing no treatment (control), GnRH or OIF. LH concentration was detected using radioimmunoassay and compared among groups using 2-way analysis of variance. In all experiments, GnRH and OIF induced pituitary LH secretion higher than control groups ($P<0.05$). An effect of dose was evident in the llama pituitary cell culture, with mean LH secretions greater in wells treated with a higher dose of OIF ($5.41 \pm 0.28$ ng/mL) compared to wells treated with a lower dose ($2.70 \pm 0.50$ ng/mL), both of which were higher than in wells with no treatment ($0.87 \pm 0.18$ ng/mL; $P< 0.05$). Although OIF stimulated LH release in bovine cell cultures, an apparent dose response was not detected. We conclude from these observations that OIF stimulates LH secretion directly at the level of the pituitary.

## 4.2. Introduction

The precise stimulus responsible for triggering the cascade of events leading to ovulation among induced ovulators is unclear. In one study involving ovariectomized ferrets, mechanical stimulation of the vagina influenced LHRH and LH secretion [46], while in another study physical stimuli alone, such as neck gripping, mounting and thrusting, were not effective; ferrets ovulated only when intromission occurred [96]. In the vole, ovulation occurred in copulated females but not in those exposed only to mechanical stimulation [161]. In early studies, electrical stimulation of the central nervous system induced ovulation in rabbits [162] and physical stimulation of the vagina with a glass rod induced ovulation in cats [45]. In alpacas, the

proportion of females that ovulated in response to mounting alone (i.e., without intromission) was similar to the negative control group and was lower than in alpacas where intromission occurred [42]. The interpretation was that physical stimulation of the vagina and cervix during copulation was the primary factor for eliciting ovulation and not necessarily the product of copulation (semen). In a more recent study physical stimulation alone was insufficient for inducing ovulation in llamas and alpacas [43].

A surge in circulating concentrations of LH preceding ovulation has been reported in reflex ovulators including rabbits [163, 164], cats [97, 144], ferrets [96, 165], voles [102] and camelids [64, 143]. As in spontaneous ovulators [137, 166], the preovulatory surge in LH is presumably the result of a surge release of GnRH from the hypothalamus. Based on early studies, the pathway in reflex ovulators was thought to involve activation of somatosensory neurons in the vagina and cervix by penile intromission which in turn synapse with neurons responsible for GnRH release [2]. However, results of more recent studies in camelids document the presence of a substance in seminal plasma, an ovulation-inducing factor (OIF), that is responsible for eliciting ovulation in these species [62, 64, 67]. Further, this factor appears to be conserved among both spontaneous and reflex ovulators. Intramuscular administration of camelid [64], bull [140], horse and pig seminal plasma [167] induced ovulations in female llamas. GnRH and its analogues have been used effectively to stimulate LH secretion and ovulation although changes in GnRH release during pre- and post-copulatory stages in camelids have not been reported, [100]. The mechanism of action has yet to be elucidated, but the preovulatory LH surge caused by OIF [64] appears to be mediated via an endocrine route [43]. Preliminary data suggests that alpaca seminal plasma stimulates LH secretion from rat anterior pituitary cells *in vitro* [168]. We hypothesize that OIF is the biological substance responsible for stimulating LH secretion from gonadotrope cells within the anterior pituitary gland.

In this study, we sought to determine if purified OIF acts directly on the anterior pituitary to stimulate LH secretion from gonadotrope cells and if the response is conserved among species. The specific objectives were to determine if purified OIF isolated from llama seminal plasma will stimulate LH secretion in a primary culture of llama or bovine anterior pituitary cells, and whether the response is related to dose.

## 4.3. Materials and Methods

### 4.3.1. OIF purified from llama seminal plasma

Ovulation inducing factor was purified from a pool of llama seminal plasma (12 mL; n=4 llamas). Ejaculates were collected by artificial vagina [38], and seminal plasma was harvested after centrifugation, diluted, and stored frozen, as previously described [64]. After thawing, pooled seminal plasma was eluted by hydroxyl apatite column chromatography to obtain a partially purified fraction FPLC was used to obtain the pure biological fraction of seminal plasma responsible for ovulation (confidential information, Ratto and Adams, 2009). Purified OIF was confirmed *in vivo* in llamas. Seminal plasma yielded approximately 6 mg of purified OIF which was diluted in phosphate buffered saline (pH 7.4) to a stock solution of 1.6 mg/mL [169]. Purified OIF was stored in 100 µL aliquots and stored at -20ºC.

### 4.3.2. Culture media

Media preparation was adapted from previous published reports [170]. Collection medium used to immerse pituitaries in immediately after collection, consisted of 137 mM NaCl, 5 mM KCl, 10 mM D-glucose, 25 mM Hepes buffer (Sigma-Aldrich Inc., St. Louis, MO, USA; pH 7.2) and 1% v/v of a mixture of penicillin (10,000 units/mL) and streptomycin (10 mg/mL; Sigma-Aldrich Inc., St. Louis, MO, USA) dissolved in ultra-pure water. The medium was filtered using a 0.2 µm filter unit (Nalge Nunc International Corp., Rochester, NY, USA) and refrigerated at $4^{o}$C.

Dissociation medium used to dissociate pituitary cells from the tissue sample (15 mL per pituitary), consisted of collection medium plus an enzymatic cocktail of 1.0 mg/mL collagenase (type II from *Clostridium histolyticum*; Sigma-Aldrich Inc., St. Louis, MO, USA), 1.0 mg/mL hyaluronidase (type IV-S from bovine testes; Sigma-Aldrich Inc., St. Louis, MO, USA), and 0.02 mg/mL deoxyribonuclease (type I from bovine pancreas; Sigma-Aldrich Inc., St. Louis, MO, USA). Enzymes were prepared immediately before use and filtered though a 0.22 µm filter (Millipore, Billerica, MA, USA).

Culture medium, used to maintain cell viability and growth, consisted of phenol red-free Dulbecco's modified eagle medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% charcoal/dextran-treated fetal bovine serum (HyClone, Logan, UT, USA), 4 mM L-

glutamine (Gibco, Grand Island, NY, USA), 1% non-essential amino acids (Sigma-Aldrich Inc., St. Louis, MO, USA) and 1% v/v of a mixture of penicillin (10,000 units/mL) and streptomycin (10 mg/mL; Sigma-Aldrich Inc., St. Louis, MO, USA). Medium used for the treatment period consisted of culture medium without fetal bovine serum (to minimize the potential effects of calf serum on gonadotroph function) and the addition of GnRH (positive control), OIF, or nothing (negative control).

### 4.3.3. Pituitary collection and cell culture

Mature non-lactating female llamas (n=4; >5 years in age) were euthanized using the captive bolt method [171], and pituitaries were collected (Figure 4.1) within 20 minutes of euthanasia (Experiments 1 and 2; n=2 pituitaries per experiment). The heads of mature cows (n=4) were collected from a local abattoir and pituitaries were extracted within two hours of slaughter (Experiments 3 and 4; n = 2 pituitaries per experiment).

Immediately after collection, pituitaries were plunged into ice-cold collection medium. The neurohypophysis was dissected from the adenohypophysis and discarded (Figures 4.2 and 4.3). The adenohypophysis was cut into approximately 1 mm x 1 mm pieces using a pair of iris scissors, and transferred into a 50 mL falcon tube (Becton Dickinson, Franklin Lakes, NJ, USA). Pituitaries were washed by adding fresh collection medium and manually agitating the tube for 15 seconds. Once the tissue pieces had settled to the bottom of the tube, the supernatant was aspirated and discarded. The washing process was repeated five times. Tissue pieces were then immersed in dissociation medium and placed in an incubated shaker at 37ºC and 300 rpm. The cell solution was removed from the shaker after 30 minutes and was drawn back-and-forth through a 7 mL transfer pipette for one minute and again placed in the incubated shaker. The process was repeated every 15 minutes for 60 minutes or until the tissue was completely dissociated (total dissociation time approximately 90 minutes). The dissociated cells were passed through a 40 µm nylon cell strainer to remove undigested tissue (BD Bioscience, Bedford, MA, USA). The cell suspension was centrifuged at 400 g for 4 minutes and the supernatant was discarded. The cells were then washed 4 times by re-suspending in 15 mL of collection medium followed by centrifugation at 400 g for 4 minutes and discard of the supernatant. After the last centrifugation, the cells were resuspended in culture medium. The percentage of viable cells was estimated using a trypan blue test [172] (Sigma-Aldrich, St. Louis, MO, USA). Cells were plated

in 6-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ, USA) of a concentration of 2 x $10^6$ viable cells per well and incubated at 37ºC in an atmosphere of 5% $CO_2$ in ambient air.

The culture medium was changed to culture medium without fetal bovine serum. After an additional day of incubation, the serum-free culture medium was removed and cells were washed once by adding and immediately removing 2 mL fresh serum-free culture medium, and were then resuspended in 2 mL serum-free culture medium containing the respective treatments.

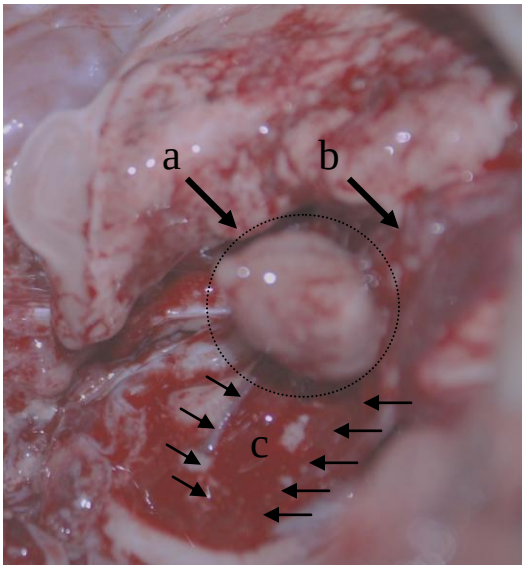



**Figure 4. 1.** Dorsal view of a llama pituitary gland (a) attached to the pituitary stock (b). Arrows delineate the hypophyseal fossa (c).

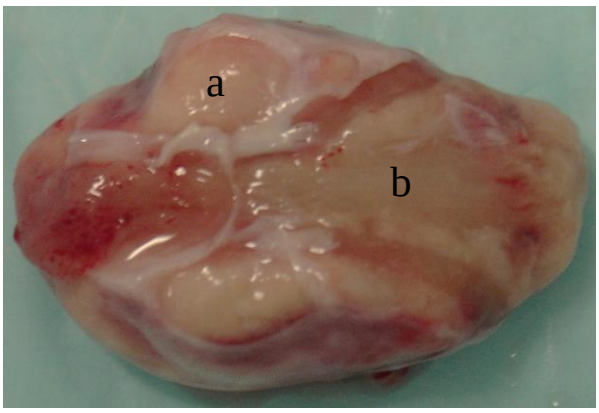



**Figure 4. 2.** Ventral view of bovine pituitary gland with anterior (a) and posterior (b) regions attached.

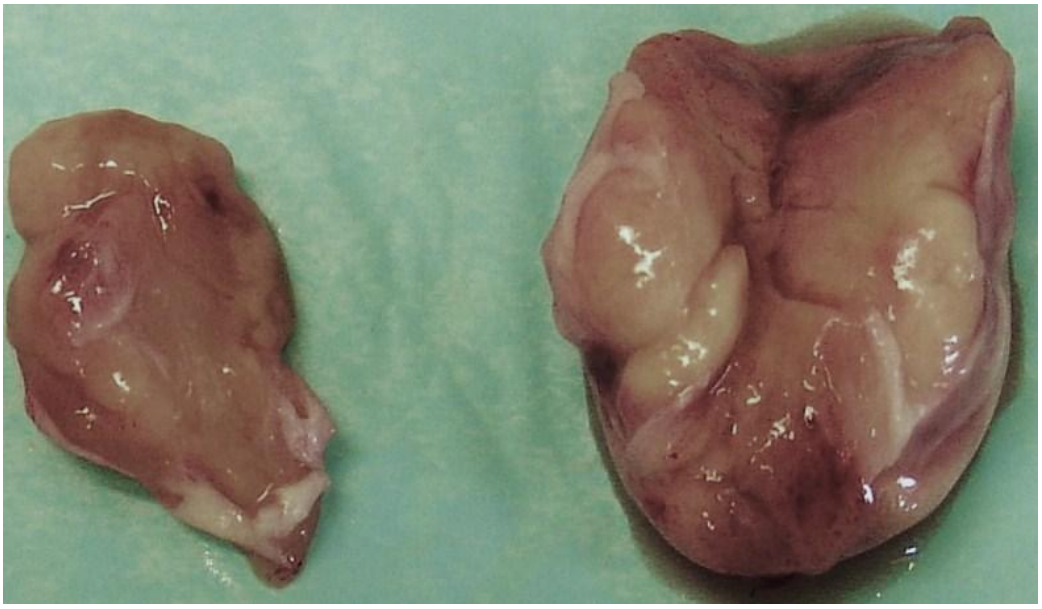


**Figure 4. 3**. Ventral view of a bovine pituitary gland with the posterior pituitary gland (left) removed from the anterior pituitary gland (right).

### 4.3.4. Experiment 1 – llama pituitary cells

Two initial concentrations of OIF were chosen (100 ng/mL and 500 ng/mL) based on results of a previous study done *in vivo* [173]. For comparison, a low and a high dose of GnRH (10 nM and 1000 nM); Buserelin, Sigma-Aldrich, St. Louis, Missouri, USA) was used as a positive control and non-treated medium was used as a negative control. After 2 hours of incubation at 37ºC in an atmosphere of 5% $CO_2$ in ambient air, media collected individually from each well (n=6 per treatment group) were centrifuged (400 x g for 5 minutes) to remove cells and the supernatant was stored at -20°C until LH assay. The doses of GnRH were chosen based on previous reports involving LH stimulation of gonadotropes [168, 174, 175]. In all experiments, values of LH concentration that were >3 standard deviations of the mean for the specific group were considered outliers and were removed from further analyses.

### 4.3.5. Experiment 2 – dose response of llama pituitary cells

To determine if llama pituitary cells would respond to OIF in a dose-dependent manner, Experiment 2 was designed in a similar manner as Experiment 1 except that three doses of OIF

(100, 200, and 500 ng/ml) and GnRH (10 nM, 100 nM, 1000 nM) were used to treat cells (n=6 cell cultures per group).

### 4.3.6. Experiment 3 – bovine pituitary cells

To determine if OIF would elicit LH release from bovine pituitary cells *in vitro,* cells pooled from 2 bovine adenohypophyses were treated, as described in Experiment 1, with either 200 ng/mL OIF, 100 nM GnRH (positive control), or no treatment (negative control; n=6 cell cultures per group). The dose of purified OIF represented the estimated concentration of OIF that reached the pituitary in a previous study done in vivo in cattle [173].

### 4.3.7. Experiment 4 – dose response of bovine pituitary cells

To determine if bovine pituitary cells would respond to OIF in a dose-dependent manner, three doses of OIF (100, 200, and 500 ng/ml) and GnRH (10 nM, 100 nM, and 1000 nM) were used to treat cells pooled from 2 bovine adenohypophyses, as described in Experiment 2 (n=6 cell cultures per group).

### 4.3.8. LH assay

Concentrations of LH in media were measured in duplicate using a double-antibody radioimmunoassay [159]. The standard curve was made using known concentrations of bovine LH in serum-free culture medium. Serum from steers of known low and high LH concentration was used as references. The primary antibody was raised in rabbits against bovine LH and concentrations of LH are expressed in terms of NIAMDD-bLH-24. The range of the standard curve was from 0.063 ng (80% ligand labelled LH) to 8.0 ng (20% ligand labelled LH) with a minimum detectable limit of 0.09 ng/mL. The intra-assay coefficient of variation for the llama pituitary cell culture was 6.1% for the low (0.97 ng/mL) and 3.4 % for the high (3.1 ng/mL) LH reference. The intra-assay coefficient of variation for the bovine pituitary cell culture was 4.3% for the low (1.06 ng/mL) and 4.5 % for the high (2.41 ng/mL) LH reference. The inter-assay coefficients of variation were 5.3% and 16.4%, respectively for the low (1.01 ng/mL) and high (2.78 ng/mL) reference concentration. To verify that OIF did not cross-react in the LH assay, concentrations of purified OIF (0, 10, 25, 50, 75, 100, 250, 500 and 640 ng/mL) were prepared in culture medium and assayed in duplicate. No relationship between OIF concentration and LH

measurement was detected using regression analysis (LH=0.0362OIF + 0.3859, $R^2$=0.2989; P=0.13), and all measurements were between 0.3 and 0.8 ng/ml.

### 4.4. Statistical Analyses

Statistical analyses were made using SAS statistical software (Statistical Analysis System Inc., Cary, NC). Mean LH concentrations were compared by 2-way analysis of variance to determine the effects of treatment and dose. In the absence of a dose effect, data from different doses within the same treatment group were combined to increase statistical power. Tukey's multiple comparison was used as a post-hoc test when a main effect of treatment or a treatment-by-dose interaction was detected. Data are presented as mean ± SEM throughout.

### 4.5. Results

The dissociation procedure yielded 50-70 x $10^6$ cells per pituitary (n=8 pituitaries) with >80% cell viability in all experiments.

#### 4.5.1. Experiment 1 – llama pituitary cells

Mean LH concentrations in media were numerically higher in wells treated with the high versus low dose of GnRH (3.48 ± ng/mL vs. 3.33 ± 0.27 ng/mL) and OIF (4.29 ± 0.43 ng/mL vs. 3.72 ± 0.37 ng/mL), but differences were not statistically significant (treatment effect P=0.11, dose effect, P=0.35; treatment-by-dose interaction: P=0.54). Hence, LH data were combined within treatment groups for subsequent comparison with negative controls (Figure 4.1). Concentrations of LH in media samples from OIF-treated cells were higher than in negative controls (P<0.0001) and tended to be higher than in positive controls (GnRH, P=0.10).

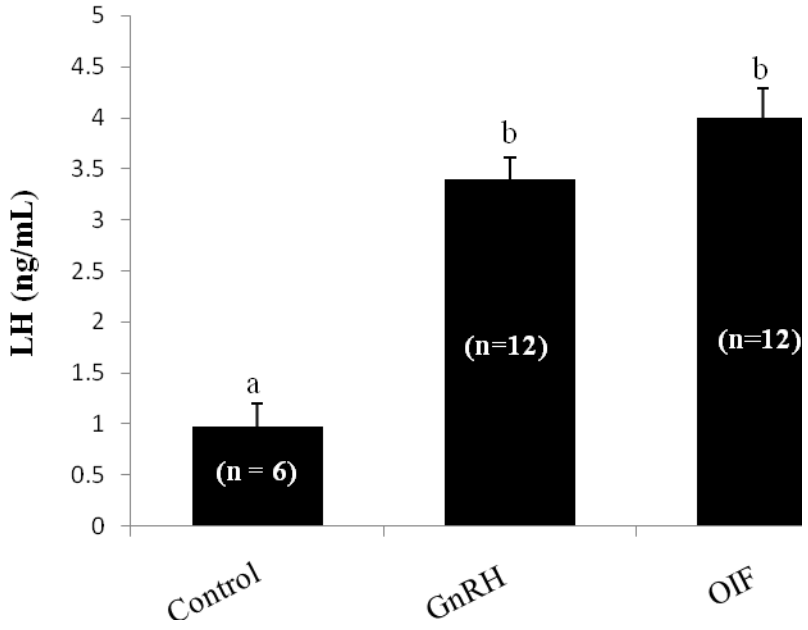



**Figure 4. 4.** LH response (mean ± SEM) of llama pituitary cells (2 x $10^6$ cells/well) incubated *in vitro* for two hours with untreated culture medium (negative control), or culture medium treated with GnRH or OIF. The number in parentheses indicates the number of wells sampled for each treatment. [ab]Values with different superscripts are different (P<0.0001; Experiment 1).

### 4.5.2. Experiment 2 – dose response of llama pituitary cells

Mean LH concentrations were not different between wells treated with intermediate and high doses of GnRH (P=0.67) or between wells treated with intermediate and high doses of OIF (P=0.32); hence, data from the two upper dose groups within treatments were combined to represent a high dose (Figure 2). Mean LH concentrations were higher in both low and high dose groups of GnRH and OIF treatment compared to control media (P<0.0001). Mean LH concentrations were higher in cells treated with a high dose of GnRH and OIF than in the corresponding low-dose groups (P<0.0001).

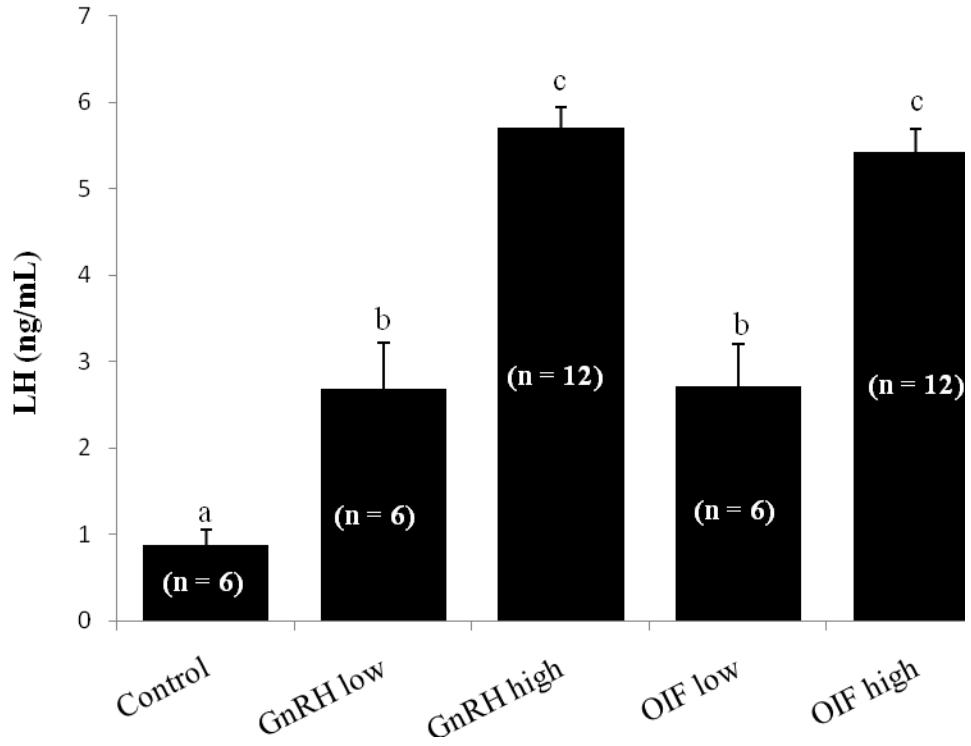



**Figure 4. 5.** LH response of llama pituitary cells (2 x $10^6$ cells/well) incubated *in vitro* for two hours with untreated culture medium (negative control), or culture medium treated with low or high doses of GnRH or OIF. The number in parentheses indicates the number of wells sampled for each treatment. [abc]Values (mean ± SEM) with different superscripts are different (P<0.0001; Experiment 2).

### 4.5.3. Experiment 3 – bovine pituitary cells

Similar concentrations of LH were measured when bovine pituitary cells were incubated with GnRH (positive control) or OIF (P=0.39). LH concentrations from both GnRH and OIF-treated cells were elevated compared to the negative control group (P=0.0007; Figure 3).

### 4.5.4. Experiment 4 – dose response of bovine pituitary cells

Treatment of bovine pituitary cells with either GnRH or OIF resulted in an increase in LH concentration in the culture media (P<0.001; Fig. 4). In GnRH- and OIF-treated groups, LH concentrations followed a dose-related pattern (treatment effect P=0.73, dose effect P<0.01, treatment-by-dose interaction P=0.08).

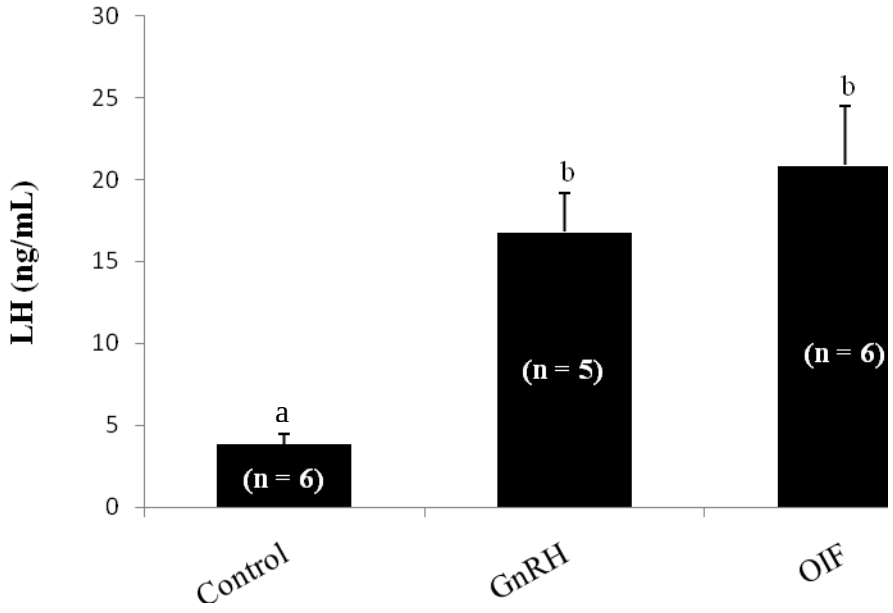



**Figure 4. 6.** LH response (mean ± SEM) of bovine pituitary cells (2 x $10^6$ cells/well) incubated *in vitro* for two hours with untreated culture medium (negative control), or culture medium treated with GnRH or OIF. The number in parentheses indicates the number of wells sampled for each treatment. [ab]Values with different superscripts differ (P<0.0001; Experiment 3).

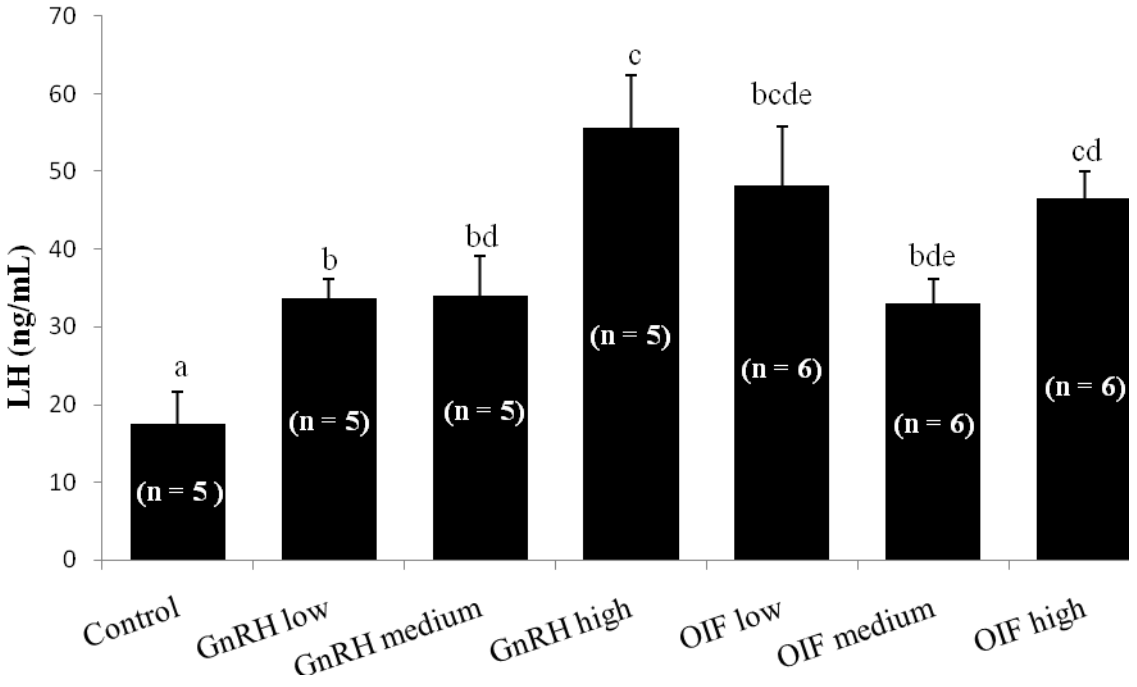



**Figure 4. 7.** LH response of bovine pituitary cells (2 x $10^6$ cells/well) incubated *in vitro* for two hours with untreated culture medium (negative control), or culture medium treated with low, medium or high doses of GnRH or OIF. The number in parentheses indicates the number of wells sampled for each treatment. $^{abcde}$Values (mean ± SEM) with no common superscripts are different ($P<0.05$; Experiment 4).

### 4.6. Discussion

The results provide compelling evidence for a direct effect of OIF on gonadotropin secretion within the anterior portion of the pituitary gland. Moreover, we interpreted our results to mean that the response to OIF is conserved at the pituitary level regardless of the type of reproductive strategy (i.e., induced or spontaneous ovulator). The OIF used in this experiment was purified from llama seminal plasma. The relative degree of response (i.e., difference in LH concentration between untreated and OIF-treated wells) was similar between llama and bovine pituitary cells cultures. In Experiments 1 and 2, OIF stimulated LH secretion to levels up to four times greater than in negative controls which corresponds to the response observed in *in vivo* studies when llamas were given an intramuscular treatment of seminal plasma [64], Up to six times more LH was stimulated in OIF-treated wells than in the negative control in bovine cell cultures. Collectively, these observations suggest that the OIF receptor required to conduct the response is conserved among mammals. OIF did alter circulating concentrations of FSH and

follicular wave emergence in prepubertal heifers, although OIF treatment did not induce ovulation or LH release [66]. However, more studies are needed before conclusions are made on the effect of OIF on FSH secretion *in vitro*. OIF has a direct effect on LH release at the level of the pituitary. Further analysis is needed to determine if OIF has an effect at the level of the GnRH nuclei of the hypothalamus.

Three doses of OIF were chosen to illustrate the dose-response of pituitary cells to OIF. We estimated a circulating plasma volume of 5.3 L in an average size (120 kg) llama based on a blood volume of 6.35% BW [176] and a packed-cell volume of 30% [177], Assuming that 1/3 of OIF from a single ejaculate (~800 µg [169]) is absorbed into circulation, the dose of 100 ng/mL was use to represent the physiologic concentration of OIF that reaches the pituitary after copulation. The second dose was double the estimated physiologic dose (200 ng/mL) and corresponded to the amount of OIF used in an in vivo study conducted in cattle [173]. The dose of 500 ng/mL was chosen arbitrarily as a high dose. The term "low dose" only applies in the context of this experiment as 100 ng/mL is the physiological relevant dose. Further studies using lower concentrations of OIF are warranted to better assess the influence of dose. Results supported the hypothesis of a dose-response relationship between the dose of OIF and LH secretion by llama pituitary cells in culture. More LH was detected in llama cells treated with a higher dose of OIF in both Experiments 1 and 2, although low and high values in Experiment 1 were not different statistically.

The dose of 200 ng/mL was used to represent the concentration of purified OIF used in a study to determine its effect in cattle [173]. where OIF did not influence LH secretion. In the present study, an interesting observation involving dose appeared when 200 ng/mL of OIF stimulated less LH release than in wells treated with 100 ng/mL or 500 ng/mL of OIF. More replicates are needed to determine if this observation is unique to this experiment or if a dose of 200 ng/mL of OIF consistently stimulates less LH than lower and higher doses of OIF. In the event of the latter, the notion of an unsuitable dose of OIF in the cattle study may explain why an ovulatory effect was not observed [178].

The roughly 10-fold higher concentration of LH detected in the bovine versus llama pituitary cell cultures treated with GnRH and OIF in the present study was unexpected. As the same references were used in all assays and the coefficients of variation were within accepted

ranges, the discrepancy may be attributed to assay specificity. The primary antibody used for LH detection was specific against bovine LH and higher concentrations in bovine samples may be a reflection of greater displacement of iodinated LH with bovine than with llama LH. Alternatively, the bovine gonadotrophs may simply have a greater capacity to secrete LH in response to GnRH and OIF than llama gonadotrophs.

In this study, we tried to minimize variation by pooling two pituitaries per experiment. Pooled pituitaries were used to increase the number of observations per treatment and reduce individual variation which may arise from physical and nutritional status, stage of ovarian cycle, and overall health of the animal. Post mortem analysis of llama ovaries in Experiment 1 showed a fully developed corpus luteum approximately 13 mm in diameter, while only follicles were present in the other llama. Thus, two llamas which were housed in similar environments were in two separate stages: follicular and luteal. Different ovarian phases influence the number of secretory granules in gonadotrope cells which reflects in the abundance of LH secreted when treated with GnRH and OIF [138]. Ovarian assessment could clarify why an effect of dose was not evident in Experiment 1. One limitation of this study was that the bovine pituitaries were collected from a local slaughterhouse and nothing was known about the stage of the estrous cycle. An important difference between llamas and cattle is that cattle have a clearly defined estrous cycle alternating between a long luteal phase and a short follicular phase, while non-mated llamas remain solely in a follicular phase. In a study of antigen labelling in rats, changes in the population of gonadotrope cells were detected in relation to different stages of the estrous cycle [15]. Pituitaries from rats in the preovulatory period showed more gonadotrope cells than in other stages of the estrous cycle.

In conclusion, our results indicate that OIF alone is capable of stimulating LH secretion directly at the level of the pituitary in llamas and cattle and support the hypothesis that OIF elicits a specific dose-related response from the adenohypophysis. The exact mechanism by which OIF regulates LH secretion remains to be elucidated. Results support the hypothesis that OIF acts directly at the level of the pituitary to elicit a pre-ovulatory surge in LH.

## 4.7. Acknowledgements

This research was supported by a grant from the Natural Science and Engineering Research Council of Canada.

## 5.0. GENERAL DISCUSSION

The female egg must be released from its housed follicle in order for fertilization to occur in mammals, a process known as ovulation. Reproductive physiologists have struggled for more than a century to answer the following question: what is the stimulus necessary for ovulation in induced ovulators? During copulation, a female is exposed to an assortment of stimuli which on their own, or in combination, have been shown to initiate the ovulatory process. Currently, physical stimulation of the vagina and cervix during intromission is the predominant stimuli of ovulation in reflex ovulators. The results generated in our lab and during this thesis do not support this claim. Instead, we postulate that the ovulation-inducing factor (OIF) within seminal plasma is the primary stimulus needed for ovulation to occur in camelids and potentially other induced ovulators.

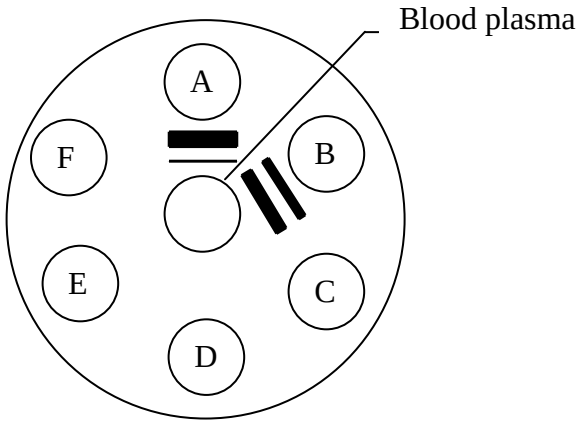
The notion that seminal plasma contains factor(s) that may influence female reproduction and may play a role in male fertility is not a novel concept [56]. Studies in mice showed that the removal of the prostate gland and seminal vesicles impaired male fertility and consequently pregnancy rates [179] and in some cases reduced sperm motility in the uterus [53]. Similar observations involving different seminal proteins and male fertility have been made in horses [57] and cattle [49]. Collectively, these observations support the notion that seminal plasma is not only a vehicle for sperm transport but it also carries other factors that play a crucial role in events required for conception.

However, not all seminal plasma is the same. The variation in seminal plasma among species is attributed to the diverse arrangement of accessory glands. Regardless of the assembly of glands or ovulation classification of the corresponding male, OIF appears to be a component that is consistently present. Thus far, OIF has been documented in the seminal plasma of camels, llamas, alpacas, rabbits (*in vitro* only; unpublished data), bulls, horses (Chapter 3) and pigs (Chapter 3). Studies to localize the accessory gland responsible for producing OIF need further investigation. The bulk of the seminal plasma in bulls, horses and pigs is anatomically related to the secretions of large vesicular glands. However, it is worth noting that vesicular glands are not present in camelids.

With the discovery of OIF in camelid seminal plasma [62, 64], it is no longer accepted that ovulation occurs via physical stimulation of the vagina and cervix during copulation in camelids. This notion is reinforced as seminal plasma from unrelated species is capable of inducing ovulation in camelids. The response (ovulation) to seminal plasma from bulls, horses and pigs, all animals whose females are considered spontaneous ovulators, was higher than llamas treated with saline. However, the ovulation rates decreased when non-camelid seminal plasma was given in comparison to those treated with camelid seminal plasma [62, 64, 140] (Chapter 3). There are several reasons for this weakened response. The most obvious is that OIF is present at a lower concentration in the seminal plasma of animals whose females are considered spontaneous ovulators. It has been proposed that some spontaneous ovulators have the necessary components to facilitate both spontaneous and induced ovulation and have been deemed "*facultative* ovulators" [12]. Thus, it may be appropriate to consider that the lack of OIF present may be the evolutionary consequence of a subordinate function since coitus is not the primary ovulatory stimulus in these animals.

Increasing the dose of either equine or porcine seminal plasma should increase the ovulatory response presuming that the inferior response observed after bull, horse or pig seminal plasma treatment is the result of a lower OIF concentration. Our results neither support nor dispute this claim. After an almost 3-fold increase of equine seminal plasma, no change in ovulation number was observed (Chapter 3). In contrast, increasing the porcine seminal plasma dose from 3 mL to 10 mL, tended to result in a higher ovulation rate (Chapter 3). We conducted another experiment where the doses of equine and porcine seminal plasma increased to 15 mL and 20 mL, respectively. None of the llamas ovulated in both treatment groups. Our original thought was that OIF had exceeded its maximum effect dose and desensitized its receptors, a notable consequence with excessive doses of GnRH. We organized a final study and decreased the dose of equine and porcine seminal plasma to 5 mL. No ovulations were detected in any treatment groups, including the positive controls. Retrospective analysis of each experiment identified a steady decline in our llama seminal plasma (positive control) treated animals. In both Experiments 1 and 2, 100% of llamas ovulated with llama seminal plasma, followed by 75% in Experiment 3 and finally 0% in Experiment 4. The decrease in response in the positive control group was reason to exclude data collected in Experiments 3 and 4. The llamas used were the same for all four experiments and experiments were designed to ensure that one animal would

not receive the same treatment twice. Hence, by the end of the fourth experiment, every animal was given each treatment (PBS, llama or equine or porcine seminal plasma) only once. The hypothesized reason for the successive decline in response is that the llamas were developing antibodies against seminal plasma. However, this hypothesis was not supported using the immunodiffusion technique [180]. Instead, we observed the pre-existence of antibodies in llama blood plasma against porcine seminal plasma. These results were generated using blood plasma from eight llamas that was taken before and one month after treatment and exposed samples to each of the four treatment groups (saline, llama, equine or porcine seminal plasma). Clear and multiple precipitate bands formed between the blood plasma and porcine seminal plasma at the before and after treatment stages, while no bands were observed in other treatment groups, demonstrating the presence of antibodies specifically against porcine seminal plasma (Figure 5.1). Perhaps the presence of these antibodies explains why a greater dose of porcine seminal plasma was needed to stimulate ovulation in llamas.




Llama 1

Figure 5.1. Immunodiffusion plate from llama 1. Letters within wells represent different treatment groups: A. Porcine seminal plasma sample #1, B. Porcine seminal plasma sample #2, C. Equine seminal plasma sample #1, D. Equine seminal plasma sample # 2, E. Llama seminal plasma, F. Phosphate buffered saline. Black lines depict precipitation lines which occured between llama blood plasma and porcine seminal plasma.

In junction with the theory that OIF is present in a lower concentration in spontaneous ovulators, we suspect another likely reason for this deficiency in ovulation rates involves

structural changes in the OIF protein or OIF receptor. Western blot analysis of bovine seminal plasma describes OIF at a higher molecular mass (approximately 60 kDa; unpublished data) than OIF identified in llamas or rabbits (26 kDa; unpublished data). The higher molecular mass OIF in cattle may be a prohormone form which has not yet been cleaved into its more biologically active form (Adams and Ratto, 2009; unpublished data).

A plausible explanation for the variation in response and molecular weight involves protein glycosylation. Glycosylation is a common process that newly synthesized proteins undergo in which monosaccharides combine with each other in a variety of ways that differ not only in sequence and chain length, but also in the position of linkages and branching points [181]. Glycosylation produces different glycoforms of a single protein which share the same backbone but differ in structure resulting in different physical and biochemical properties which in turn may lead to functional diversity [181]. This modification may explain the impaired and varied response when OIF from different species are introduced into a camelid. These changes may be observed in the transcribed protein, or in the second gene product, the carbohydrates. We suspect a combination of both scenarios is responsible for lower ovulation rates and possibly explains why the function of purified OIF was altered in cattle *in vivo* studies [173].

The ability for llama seminal plasma to stimulate ovulation in prepubertal mice signifies that the OIF receptor may also be conserved amongst mammals (Chapter 3). The OIF receptor has been detected and sequenced in an immortalized cell culture of mouse gonadotrope cells (unpublished data). Along with possible modifications to the OIF ligand, it is just as likely that the receptor may have tolerated its own alterations. Similar to the cross-species effect observed after seminal plasma from different species is given to llamas, the response to OIF derived from llama seminal plasma is decreased or altered when the host animal is not a camelid. So far, our lab has tested the biological effects of OIF in two species other than camelids: mice and cattle. The ovulation rates obtained in mice after llama seminal plasma treatment were comparable to positive controls and rates observed in camelids (Chapter 3). However, the number of oocytes observed was lower in mice treated with llama seminal plasma than GnRH-treated mice, demonstrating a moderately weaker response (Chapter 3). An ovulatory effect was not observed in cattle. Instead OIF had an effect on FSH secretion 24 hours after treatment and hastened the onset of a new follicular wave [173]. Whether this FSH-response is restricted to cattle is unclear

as the effect of OIF on FSH secretion has only been reported in the Bactrian camel 6 hours after treatment, where investigators reported no effect [182].

A preovulatory LH surge is observed in llamas treated with llama seminal plasma or purified OIF and FSH secretion is increased following OIF treatment in cattle. From these observations, we hypothesized that OIF directly stimulates gonadotrope cells in the anterior pituitary gland (Chapter 4). In primary cell cultures, we observed that OIF influences LH secretion by stimulating LH release directly from the anterior pituitary gland (Chapter 4). In the work presented in this thesis, none of the catheterized llamas treated with either equine or porcine seminal plasma ovulated and the LH profile of an animal treated with non-camelid seminal plasma remains unknown. Nevertheless, equine and porcine seminal plasma still influenced LH secretion observed by an increase in LH pulsatility (Chapter 3). Although the LH frequency in llamas treated with equine seminal plasma did not differ from negative controls, irregular spikes observed in individual animals reflected a change in LH secretion.

The first study in this thesis was designed to illustrate the conservation of OIF across species. The second study was designed to provide insight of response-site in which OIF produces its effect. Contradicting cattle *in vivo* studies, purified OIF from llama seminal plasma stimulated LH secretion when applied directly to an anterior pituitary cell culture from llama or cattle (Chapter 4). These results are the first of its kind which demonstrate a possible binding site of OIF. In the absence of the gonads, hypothalamus and posterior pituitary, OIF is still capable of stimulating LH secretion to levels that were significantly higher than untreated groups. This begs the question, why did OIF not have an effect in cattle *in vivo*? To determine if the results observed in cattle was related to OIF ligand/receptor incompatibility, a study observing the effect of purified cattle OIF is needed.

In light of the data presented during this thesis, the prospected mechanism of action of OIF is as follows: during intromission, the endometrium is abraded exposing seminal fluids to the circulatory system. OIF, produced by the accessory glands is ejaculated into the female reproductive tract. OIF enters circulation, binding to receptors on gonadotrope cells. Ligand-receptor complex stimulates excretion of LH granules, releasing LH into circulation, and consequently ovulation approximately 30 hours later.

## 6.0. GENERAL CONCLUSIONS

- OIF ligand is conserved in species other than camelids
- Evidence for the conservation of the OIF receptor
- OIF receptors are present on gonadotrope cells
- OIF induces ovulation by stimulating pituitary LH secretion

## 6.1. Specific Conclusions: Ovulation induction in studies

- Llama seminal plasma induced ovulations in prepubertal mice to rates to comparable to mice treated with GnRH and hCG
- Llama seminal plasma treatment produces a lower number of follicles to ovulate in comparison to GnRH treated mice
- A fraction ($1/6^{th}$) of a llama ejaculate is sufficient to stimulate ovulation in 100% of treated llamas
- A fraction ($1/23^{rd}$) of a horse ejaculate is sufficient to stimulate ovulation in 38% of treated llamas
- A fraction ($1/25^{th}$) of a pig ejaculate is sufficient to stimulate ovulation in 33% of treated llamas
- Llama seminal plasma triggered a pre-ovulatory LH surge in llamas
- Anovulated llamas treated with porcine seminal plasma triggered an increase in LH pulse frequency
- Anovulated llamas treated with equine seminal plasma triggered an increase in LH pulse frequency but was not significantly different from negative controls

## 6.2. Specific conclusions: Pituitary LH secretion studies

- OIF directly stimulates LH secretion from a primary cell culture of llama or cattle anterior pituitary cells
- The level of LH secretion is dependent on the concentration of OIF in a llama anterior pituitary cell culture

## 7.0 FUTURE STUDIES

These results inspired new questions and new hypotheses. The most critical is determining the role of GnRH in the preovulatory surge in camelids. We observe a preovulatory LH surge after intramuscularly administering seminal plasma or purified OIF. Based on *in vitro* data, we see that OIF may bypass the hypothalamus, and induce gonadotropin secretion at the level of the pituitary gland. To confirm these results we need to determine all OIF binding sites. Although one possible site of action is the pituitary gland, we do not have sufficient evidence to exclude the possibility of other OIF binding sites which may exist in the hypothalamic or even uterine and ovarian levels. OIF binding sites can be established by administering radioactively-labelled OIF and/or by using immunohistochemsitry to visualize binding sites.

However, whether or not we find an OIF target region in the hypothalamus does not answer one fundamental question: Can OIF alone induce a preovulatory LH surge? To address this question, we need to remove the influence of GnRH. The GnRH response could be diminished by either recession of the pituitary stalk to remove the interaction between the hypothalamus and pituitary gland, or immunization of llamas against GnRH and determine whether there is a change in the pattern of LH secretion and ovulation.

So far, our attention has been focused on determining how OIF effects the female and providing evidence that this substance exists within seminal plasma of unrelated species. The presence of an ovulation-inducing factor in the seminal plasma of bulls, horses, and pigs, all males whose corresponding female is a spontaneous ovulator motivates the question: why is OIF conserved in mammalian seminal plasma? Is OIF present at equal concentrations among all male camelids or is there a bias towards males that are more reproductively competent? Immunoassays, such as immunoblots and ELISA, are techniques that will be used in the future to provide insight to these questions.

Currently, we hypothesize that the prostate gland may be the site of OIF production as it is the only accessory gland conserved amongst mammals. One way to localize OIF production may be to conduct an *in vivo* experiment where subsequent accessory glands are excised and seminal fluids are collected. Using the llama or mouse (Chapter 3) *in vivo* bioassay, we can determine which glands are pertinent for ovulation. Tandem to this experiment, we could confirm OIF presence using an immunoblot and verify function (LH secretion) using the *in vitro* cell culture assay as outlined in Chapter 4. Perhaps OIF is not solely produced by one gland

and/or may need the influence of other proteins produced by other glands to facilitate its ovulatory effect.

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