AN ABSTRACT OF THE THESIS OF

Caitlin Elizabeth Donovan for the degree of Master of Science in Animal Sciences presented on May 3, 2013

Title: Applications of GnRH Immunization in Domestic Dogs

Abstract approved:

______________________________

Michelle Anne Kutzler

Reproductive function in the dog is controlled by feedback mechanisms that involve the hypothalamus, the anterior pituitary gland, and the gonads. Surgical gonad removal, a common procedure performed in dogs for the purposes of sterilization, disrupts the hypothalamic-pituitary-gonadal (HPG) axis and results in permanently elevated concentrations of gonadotropins. Manipulation of the HPG axis via gonadotropin releasing hormone (GnRH) immunization results in the synthesis of GnRH neutralizing antibodies, which bind to (neutralize) GnRH and prevent it from binding to its receptors. The end result of GnRH immunization is the cessation of pituitary gonadotropin secretion, namely luteinizing hormone (LH).
In 2004, a commercial GnRH vaccine was launched in the United States (Canine Gonadotropin Releasing Factor Immunotherapeutic®; Pfizer Animal Health USA), labeled for the treatment of benign prostatic hyperplasia in intact male dogs. This research investigated two novel clinical applications of this vaccine in dogs.

In the first study, physiologic responses to GnRH immunization in intact male dogs were observed. Four intact males were vaccinated with the GnRH vaccine twice at four week intervals. Blood samples were collected prior to each injection (at weeks 0 and 4) and at weeks 12 and 20 following initial vaccination. Scrotal measurements were also made at the time of each blood sample collection to calculate testicular volume. All four dogs developed a GnRH antibody titer and experienced a significant decrease in testosterone concentrations. Testicular volume also significantly decreased, and this effect was reversed by the end of the study. LH concentrations remained at basal levels. These results are indicative of temporary humoral response to the GnRH vaccine, and future studies should investigate prolonging these effects to potentiate GnRH immunization as a method of population control in dogs.

Beyond use as a temporary immunosterilant, GnRH immunization has other promising clinical applications. Elevated gonadotropin concentrations as a result of gonad removal in female dogs decreases urethral pressure, and in some bitches, this results in the development of urethral sphincter mechanism incompetence (USMI). Therefore, lowering gonadotropin concentrations in incontinent ovariectomized bitches through GnRH immunization may restore continence. In the second study, sixteen incontinent dogs that were using phenylpropanolamine (PPA) to control incontinence
were recruited. Eleven dogs were immunized against GnRH at week 0, and nine dogs were vaccinated again four weeks later. Five control dogs were vaccinated with a placebo twice at four week intervals. Vaccinated dogs discontinued PPA two weeks after re-vaccination, and control dogs remained on PPA for the duration of the study. Blood samples were collected before each injection and at 6, 8, 10, 12, 16, 20, and 24 weeks, and owners recorded episodes of incontinence throughout the study. Of the nine dogs that completed the vaccination series, four dogs remained continent after PPA was discontinued. For these four dogs, there was no difference in the episodes of incontinence when using PPA versus treatment with the vaccine. All nine vaccinated dogs developed a GnRH antibody titer, and LH concentrations decreased significantly in vaccinated dogs compared to controls. These results indicate that decreasing LH concentrations through GnRH immunization restores continence to some, but not all incontinent ovariectomized bitches. Because the development of USMI results from decreased urethral pressure that happens after ovariectomy, future studies should focus on preventing this decrease in urethral pressure to prevent USMI from occurring.
Applications of GnRH Immunization in Domestic Dogs

by

Caitlin Elizabeth Donovan

A THESIS

submitted to

Oregon State University

in partial fulfillment of

the requirements for the

degree of

Master of Science

Presented May 3, 2013

Commencement June 2013
ACKNOWLEDGEMENTS

I am so grateful to my advisor, Dr. Michelle Kutzler, for her unwavering support and guidance throughout my graduate career. Her incredible knowledge base, experience, and ability to provoke me to think outside of the box has truly helped me become the researcher I am today, and her drive has pushed me to accomplish very much in a short amount of time. I am so thankful that I had the opportunity to learn from her.

I also want to thank Dr. Jana Gordon for all her help with the urinary incontinence study and Dr. Tim Hazzard for teaching me how to be a conscientious researcher. Both individuals provided excellent insight for the project, and I am immensely appreciative of their ideas, guidance, and support. I am also hugely indebted for the support and friendship of the Theriogenology Laboratory: Laura Sahlfeld, Libby Fellows, and Justine Gullab.

Various people at Oregon State University and the surrounding community have been an immense help to me. Thank you to Dr. Gerd Bobe for numerous lessons in statistics, Meredith Hanson and Jennifer Gartner for always being available to help me out with the study dogs, Reni Stewart for her incredible ability to recruit dogs at the drop of a hat, and the owners and their dogs that participated in the study. Without these people, this study would not have been possible.

Thank you so much to the Department of Animal and Rangeland Sciences for granting me an assistantship, the Collie Health Foundation for funding the urinary incontinence study, and Pfizer Animal Health for product donation. Also, a huge thank you to my other fellow co-authors I have worked with over the last two years on various projects; Marty Greer, Katherine Peed, Jennifer Grossman, Kristin Patton, Steve Lamb, Tessa Fiamengo, Andy Schmidt, JoAnne Lemieux, and Frances Hathaway.
My family has always done everything in their power to be there for me, and I cannot thank them enough for helping me to always be the best I can be. Moving across the country to come to Oregon State was a big change, but their unwavering encouragement to follow my dreams has allowed me to be successful. I truly have the best family anyone could ask for.

Finally, I would not be here today without the support of my boyfriend Arthur. He has been my foundation through this chapter of my life, and I look forward to the beginning of a new chapter as we head on to Davis, California so I can pursue my doctorate.
CONTRIBUTIONS OF AUTHORS

Dr. Michelle Kutzler conceptualized, provided oversight, and edited the manuscripts for the male dog and incontinence study. Dr. Marty Greer assisted with male dog sample collection and edited the resulting manuscript. Dr. Jana Gordon helped conceptualize the incontinence study, assisted with incontinent dog sample collection, and edited the resulting manuscript. Katherine Peed assisted with research initiation of the male dog study, and Dr. Tim Hazzard assisted with assay preparation in the male dog study.
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CHAPTER 1
INTRODUCTION

1.1 THE HYPOTHALAMIC-PITUITARY-GONADAL AXIS IN THE BITCH

1.1.1 Summary

Gonadotropin and steroid hormone secretion in the dog is primarily controlled by feedback mechanisms that involve the hypothalamus, the anterior pituitary gland, and the gonads (Fink, 1988). Gonadectomy, a surgical technique performed frequently in veterinary practice as a reliable means of population control, disrupts the hypothalamic-pituitary-gonadal (HPG) axis (Stockner, 1991). For the purposes of this review, only the implications of gonad removal in the female dog (ovariectomy) will be discussed.

1.1.2 Gonadotropin-Releasing Hormone

Gonadotropin-releasing hormone (GnRH) is a decapeptide (pGlu\(^1\)-His\(^2\)-Trp\(^3\)-Ser\(^4\)-Tyr\(^5\)-Gly\(^6\)-Leu\(^7\)-Arg\(^8\)-Pro\(^9\)-Gly\(^10\)-NH\(_2\)) synthesized and stored in GnRH neurons located in the medial basal and preoptic area of the hypothalamus (Jeong and Kaiser, 2006). There have been three isoforms of GnRH recognized in animals; mammalian GnRH-I, chicken GnRH-II, and lamprey GnRH-III (Khan et al., 2007). However, GnRH-I is accepted as the main fertility-regulating peptide (Khan et al., 2007) and will be the only
isof orm discussed for the purposes of this review. Release of GnRH is controlled by steroidal hormone feedback as well as non-steroidal hormones, such as melatonin, catecholamines, and opioids (Fink, 1988). Under neuronal stimulation, GnRH is released in a pulsatile manner and moves through nerve terminals in the median eminence (Jeong and Kaiser, 2006). These terminals are in close proximity to the primary capillary bed of the hypophyseal portal system (Clarke and Pompolo, 2005), allowing GnRH to enter circulation and access its receptors on gonadotrophs in the anterior pituitary. GnRH has a high specificity to its receptor (GnRHR) in the anterior pituitary (Clarke and Pompolo, 2005), and GnRHR regulation is dependent on GnRH itself (Katt et al., 1985).

1.1.3 Gonadotropins

The gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH) are located in the pars distalis region of the anterior pituitary. Both gonadotropins are glycoproteins composed of an α and β subunit (Childs, 2006). The α subunit is common to both LH and FSH, while the β subunit is distinct and determines hormonal specificity (Jeong and Kaiser, 2006).

In the female, LH stimulates androgen production in ovarian theca cells, which is converted into estradiol in the ovarian granulosa cells with the assistance of FSH (Palermo, 2007). FSH is responsible for promoting initial ovarian follicle development from primary to antral follicles; only FSH receptors are expressed in the granulosa cells at these stages (Palermo, 2007). FSH then induces the expression of LH receptors in the
granulosa cells of mature follicles, allowing the follicle to become increasingly responsive to LH (Palermo, 2007). As a result, LH is the gonadotropin responsible for dominant follicle selection, subsequent ovulation, and luteinization of the dominant follicle (Childs, 2006; Palermo, 2007).

LH and FSH are both secreted by the same pituitary cell type, the gonadotrophs, but they are both differentially regulated (Crawford and McNeilly, 2002; de Gier et al., 2006). Gonadotropin secretion is modulated by hypothalamic feedback; GnRHR-ligand binding causes the rapid mobilization of intracellular calcium (Ca$^{2+}$), and this triggers the exocytosis of LH and FSH (Farnworth, 1995; Jeong and Kaiser, 2006). Frequent GnRH pulses with low amplitude support LH synthesis and secretion, while less frequent GnRH pulses with high amplitude support FSH synthesis and secretion (Palermo, 2007). Gonadotropin secretion is modulated by gonadal feedback as well. It is thought that this mechanism is also partially responsible for the differential secretion of LH and FSH because gonadectomy results in a concurrent rise in both FSH and LH, thereby reducing differential secretion (Shupnik, 1996).

1.1.4 Gonadal Feedback

The two reproductive steroid hormones produced by the ovary, estrogen and progesterone, also contribute to HPG axis modulation. Progesterone, produced by the luteal cells of the corpus luteum, is the steroid hormone that dominates the luteal phase (Messinis, 2006). When circulating concentrations are elevated, progesterone negatively
feeds back directly on the median basal hypothalamus, decreasing GnRH pulse frequency and consequently LH secretion. Progesterone also acts on gonadotrophs in the anterior pituitary to decrease LH secretion directly, but this mechanism is estrogen-dependent (Girmus and Wise, 1992). By decreasing GnRH pulse frequency and LH secretion, progesterone allows for the maintenance of the corpus luteum.

Estradiol, the estrogen that dominates during the nonpregnant reproductive cycle, has both a negative and positive feedback role. During follicular development, estradiol concentrations increase as the follicle grows and negatively feeds back on both the hypothalamus and anterior pituitary to reduce the amplitude of GnRH and LH secretion, allowing LH to build up in the gonadotrophs (Palermo, 2007). During the late follicular phase, estradiol concentrations are postulated to reach a threshold, which signals a change that results in estradiol positively feeding back on the hypothalamus and pituitary (Clarke, 1995). This increases GnRH secretion, which causes an LH surge. The LH surge, coupled with an increased responsiveness of the dominant follicle to LH, results in ovulation.

In addition to reproductive steroid hormones, the ovary also secretes the peptides inhibin, activin, and follistatin, whose biological effects act specifically upon FSH (Shupnik, 1996). Inhibin is produced by the granulosa cells of antral follicles and suppresses FSH release (Shupnik, 1996). Increased inhibin concentrations occur as the dominant follicle emerges and therefore results in a decline in FSH (Findlay, 1993). Another gonadal peptide, activin, is also produced by granulosa cells, and it stimulates FSH production (Findlay, 1993; Shupnik, 1996). It is mainly secreted by preantral
follicles, which contributes to their responsiveness to FSH (Findlay, 1993). However, precisely how activin signaling integrates into the network of other hormonal signaling pathways has yet to be elucidated (Coss et al., 2010). Finally, the granulosa cells are also the major site of production for the gonadal peptide follistatin (Findlay, 1993). While follistatin does not directly affect FSH, it does bind to and therefore inhibit activin action, therefore indirectly affecting FSH production (Shupnik, 1996). Given that gonadal peptide action is exerted on FSH only and not LH, these peptides are postulated to contribute to the differential secretion of the gonadotropins (Shupnik, 1996).

1.1.5 HPG Axis in the Intact Bitch

The canine estrous cycle is comprised of anestrus, proestrus, estrus, and diestrus. Late anestrus is characterized by the initiation of folliculogenesis, which results from an increase in LH and FSH pulsatility (Kooistra et al., 1999; Olson et al., 1982). During proestrus, follicles continue to develop in response to FSH and produce increasing levels of estradiol, which stimulates increased pulse frequency of GnRH. This accumulates in a preovulatory gonadotropin surge, resulting in a 1-3 day elevation in LH and a 1-4 day elevation of FSH (Concannon, 2011). The LH surge occurs near the onset of estrus, which lasts 1-3 days in the bitch (Concannon, 2011). Ovulation then occurs 48-60 hours following the onset of the LH surge (Concannon, 2011). Progesterone, originating from the developing corpora lutea, steadily increases during estrus and the first half of diestrus, then plateaus and declines during the remainder of diestrus. Elevated progesterone
negatively feeds back on LH, and concentrations remain low yet dynamic during diestrus, indicative of pulsatile secretion (Kooistra et al., 1999).

1.1.6 HPG Axis in the Ovariectomized Bitch

Ovary removal results in the inability for gonadal steroid hormones to feed back upon the hypothalamus and anterior pituitary, and this results in a rapid increase in LH and FSH concentrations. This effect is seen across mammalian species, including women (Yen and Tsai, 1971). In the bitch, LH and FSH concentrations stabilize approximately one year post-surgery at 7 and 14-fold increases, respectively (Reichler et al., 2004). Gonadotropin elevations remain permanently elevated; however, concentrations remain somewhat dynamic, reflective of pulsatility still occurring (Concannon, 1993).

There are potential negative effects of ovariectomy that vary between species. For example, the dog is predisposed to urinary incontinence (Reichler, 2009); the ferret is predisposed to hyperadrenocorticism (Schoemaker et al., 2002); the rodent and rabbit are at increased risk for osteoporosis (Rosen et al., 2000; Sevil and Kara, 2010; Yamazaki and Yamaguchi, 1989); and the woman is at an increased risk for cardiovascular problems, cognitive impairment, and osteoporosis (Shuster et al., 2008). These effects could be due to gonadotropin elevation, which may be impacting specific tissues through extragonadal receptor-ligand binding; extragondal gonadotropin receptors have been identified in various locations in different species.
1.2 NONCLASSICAL ACTIONS OF GNRH, LH, AND FSH IN THE BITCH

1.2.1 Summary

The expression of GnRH and gonadotropin receptors have been identified in numerous extragonadal tissues that vary among species, indicating that these hormones may exhibit actions beyond the HPG axis. Extragonadal receptors for GnRH and LH have been identified most extensively in the rodent and human (Pakarainen et al., 2007; Skinner et al., 2009), but there is less documentation available on extragonadal FSH receptors (Mizrachi and Shemesh, 1999). Extragonadal GnRH, LH, and FSH receptors identified in various female species are summarized in Table 1.1. In the dog, GnRH and gonadotropin receptors have been identified in the skin (Welle et al., 2006), urinary bladder (Coit et al., 2009; Ponglowhapan et al., 2007; Reichler et al., 2007), and urethra (Ponglowhapan et al., 2007; Reichler et al., 2007). While the presence of these receptors has been identified, the role of GnRH and gonadotropins in extragonadal tissue is still unclear.

1.2.2 Action on the Skin

Immunohistochemical studies have shown that GnRH, FSH, and LH receptors are present in canine skin, specifically the epidermis, infundibulum, isthmus, sebaceous glands, sweat glands, and the arrector pili muscles (Welle et al., 2006). Some bitches
Table 1.1. Extragonadal GnRH, LH, and FSH receptor expression in various species.

<table>
<thead>
<tr>
<th>Female Species</th>
<th>Extragonadal GnRHR</th>
<th>Extragonadal LHR</th>
<th>Extragonadal FSHR</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog</td>
<td>Bladder</td>
<td>Bladder</td>
<td>Bladder</td>
<td>(Coit et al., 2009; Ponglowhapan et al., 2007; Reichler et al., 2007; Welle et al., 2006)</td>
</tr>
<tr>
<td></td>
<td>Skin</td>
<td>Skin</td>
<td>Skin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Urethra</td>
<td>Urethra</td>
<td>Urethra</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>Adrenal gland</td>
<td>Adrenal gland</td>
<td>Not reported</td>
<td>(Pakarainen et al., 2007; Skinner et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>Bladder</td>
<td>Bladder</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Heart</td>
<td>Uterine blood vessels</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>Cervix</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>Oviduct</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Skeletal muscle</td>
<td>Skin</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Uterus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>Adrenal gland</td>
<td>Adrenal gland</td>
<td>Not reported</td>
<td>(Fields and Shemesh, 2004; Pakarainen et al., 2007; Skinner et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>Brain</td>
<td>Brain</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Eye</td>
<td>Kidney</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>Mammary gland</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>Spinal cord</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Skeletal muscle</td>
<td>Uterus</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Skin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>Bladder</td>
<td>Oviduct</td>
<td>Not reported</td>
<td>(Pakarainen et al., 2007; Skinner et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>Brain</td>
<td>Uterus</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Eye</td>
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<td></td>
<td>Heart</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Kidney</td>
<td></td>
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<tr>
<td></td>
<td>Liver</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Skin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sheep</td>
<td>Brain</td>
<td>Uterus</td>
<td>Not reported</td>
<td>(Fields and Shemesh, 2004; Skinner et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>Eye</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Spinal cord</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cow</td>
<td>Adrenal gland</td>
<td>Oviduct</td>
<td>Cervix</td>
<td>(Fields and Shemesh, 2004; Mizrachi and Shemesh, 1999; Skinner et al., 2009)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Uterus</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Uterine blood vessels</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
display a change in coat quality after ovariectomy, collectively referred to as ‘puppy coat syndrome’ (Fontaine and Fontbonne, 2011). Affected dogs have an increase in wool hair and a decrease in color intensity, and this is undesirable to dog owners (Reichler et al., 2008). This coat difference can be attributed to a significant increase in the anagen:telogen ratio, or the hair follicle growth:resting ratio, in dogs one year after ovariectomy (Reichler et al., 2008). However, the incidence of this coat change post-ovariectomy is approximately 20%, so the pathophysiological mechanism underlying the change between elevated gonadotropins and change in coat quality still needs to be elucidated (Reichler et al., 2008).

1.2.3 Action on the Urinary Bladder and Urethra

Receptors for GnRH (Reichler et al., 2007), LH (Ponglowhapan et al., 2007; Reichler et al., 2007), and FSH (Ponglowhapan et al., 2007) are present in the canine bladder and urethra. Urinary incontinence as a consequence of ovariectomy is a common problem in bitches, and the incidence of post-spay incontinence is reported to be between 5.7-20% while the incidence of incontinence in intact females is between 0-1% (Arnold et al., 1992; Thrusfield et al., 1998). Urinary incontinence in both intact and neutered male dogs is also uncommon (Aaron et al., 1996). Therefore, there may be an effect of reproductive status and gender on the expression of LH and FSH receptors. Ponglowhapan and co-workers (2008) found significantly less receptor expression in ovariectomized females compared to intact females, but there was no difference between
intact and neutered males. This is in contrast to Reichler and colleagues (2007), who found no overall difference in LH or GnRH receptor expression between intact and ovariectomized females. It is important to mention though that the dogs used in the Ponglowhapan study were all in anestrus, while the dogs used in the Reichler study were at different stages of the cycle. The stage of the estrous cycle could affect gonadotropin receptor expression and therefore result in variability between individuals; it has been demonstrated in the human female that maximum LH receptor expression in the uterus, an extragonadal tissue, occurs during the luteal phase (Shemesh, 2001).

In contrast to both these studies, Coit and coworkers (2009) found a significant increase in LH and GnRH receptor expression in gonadectomized female and male dogs compared to intact. Receptor expression for LH was also found to be significantly greater in intact females compared to intact males. The authors attributed these conflicting findings to the population of dogs observed. For example, Ponglowhapan and colleagues studied dogs that had been gonadectomized less than one year prior, and it is possible that changes in receptor expression continue longer than one year following gonadectomy (Coit et al., 2009), especially considering LH and FSH concentrations in gonadectomized dogs do not stabilize until approximately 42 weeks post-gonadectomy (Reichler et al., 2004).

1.2.4 Conclusions
It is accepted that in the dog, LH, FSH, and GnRH receptors are present in the extragonadal tissues of the skin, bladder, and urethra. However, additional research is needed to determine other extragonadal tissues that may be responsive to these hormones and what the conclusive effects of gonadectomy are on LH, FSH, and GnRH receptor expression.

1.3 APPLICATIONS OF GNRH ANALOGUES AND IMMUNIZATION

1.3.1 Summary

Given that the GnRH sequence pGlu\(^1\)-His\(^2\)-Trp\(^3\)-Ser\(^4\)-Tyr\(^5\)-Gly\(^6\)-Leu\(^7\)-Arg\(^8\)-Pro\(^9\)-Gly\(^10\)-NH\(_2\) is conserved across all mammals, developing GnRH analogues with the goal of manipulating fertility in various species has been an ongoing area of research. GnRH analogues (agonists and antagonists) and immunization have all been utilized in the dog because of the strong need for antifertility agents (Junaidi et al., 2003). Future work with these compounds includes increasing the duration of effect for long-term methods of nonsurgical sterilization.

1.3.2 GnRH Agonists

Development of agonists has focused on producing sequences with a high affinity for GnRH receptors and increased resistance to degradation or elimination, allowing for a
product more potent than GnRH itself (Padula, 2005). Areas of manipulation of the GnRH sequence for agonist development primarily target the end of the amino acid chain (replacing Gly\textsuperscript{10}-NH\textsubscript{2}), the beginning of the chain (replacing pGlu\textsuperscript{1}), and the center of the chain (replacing Gly\textsuperscript{6}). Furthermore, replacing the L-isomer Gly\textsuperscript{6} with a D-isomer has been shown to drastically increase overall potency of the agonist (Padula, 2005).

Depending upon binding affinity, the rate of dissociation, and duration of use, GnRH agonists can either stimulate or inhibit the production and release of gonadotropins from the pituitary. Initial administration results in an immediate increase in gonadotropin concentrations, referred to as the flare-up effect (Gobello, 2007). This short-term flare-up lasts days to weeks and is followed by a desensitization and therefore inhibition of gonadotropin secretion that is reversible when the GnRH agonist is discontinued (Conn and Crowley, 1991). While gonadotropin suppression is ultimately achieved for long durations of time, the initial flare-up can be problematic if the agonist is being used in an anti-fertility application.

GnRH agonists have been widely investigated in the dog for both reproductive and non-reproductive applications. Short-term administration of GnRH agonists (during the flare-up period) results in estrous induction (Fontaine et al., 2011; Inaba et al., 1998; Volkmann et al., 2006), while long-term administration has been investigated for various clinical applications. GnRH agonists that have been investigated in the dog are summarized in Table 1.2.

To date, deslorelin is the only product that has been developed specifically for long-term fertility control in domestic animals (Herbert and Trigg, 2005). Its modified
Table 1.2. GnRH agonists and antagonists investigated in the dog.

<table>
<thead>
<tr>
<th>Common Name</th>
<th>Type</th>
<th>Clinical Applications</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acyline</td>
<td>Antagonist</td>
<td>Testicular characteristic alterations</td>
<td>(García Romero et al., 2012; Valiente et al., 2007, 2009a, 2009b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Testosterone suppression</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pregnancy termination</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Estrous cycle interruption</td>
<td></td>
</tr>
<tr>
<td>Cetrorelix</td>
<td>Antagonist</td>
<td>Testosterone suppression</td>
<td>(Schwahn et al., 2000)</td>
</tr>
<tr>
<td>Deslorelin</td>
<td>Agonist</td>
<td>Delay of puberty</td>
<td>(Fontaine et al., 2011; Junaidi et al., 2003, 2009; Limmanont et al., 2011; Reichler et al., 2003, 2008; Romagnoli et al., 2009; Sirivaidyapong et al., 2012; Volkmann et al., 2006)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Induction of fertile estrus</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Estrous synchronization</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antifertility agent</td>
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<tr>
<td></td>
<td></td>
<td>Treatment for benign prostatic hyperplasia</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Treatment for urinary incontinence</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reversal of spay-induced coat changes</td>
<td></td>
</tr>
<tr>
<td>Leuprolide</td>
<td>Agonist</td>
<td>Induction of fertile estrus</td>
<td>(Inaba et al., 1996, 1998; Reichler et al., 2006a, 2006b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gonadotropin suppression</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Suppression of testicular function</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Effect on bladder and urethral function</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Treatment for urinary incontinence</td>
<td></td>
</tr>
<tr>
<td>Nafarelin</td>
<td>Agonist</td>
<td>Suppression of spermatogenesis</td>
<td>(Goericke-Pesch et al., 2009, 2010; Ludwig et al., 2009; McRae et al., 1985; Paramo et al., 1993)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Recrudescence of spermatogenesis after treatment cessation</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Suppression of estrus</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Treatment for benign prostatic hyperplasia</td>
<td></td>
</tr>
<tr>
<td>Buserelin</td>
<td>Agonist</td>
<td>Estrus induction</td>
<td>(Reichler et al., 2003; Rota et al., 2003)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Treatment for urinary incontinence</td>
<td></td>
</tr>
<tr>
<td>Goserelein</td>
<td>Agonist</td>
<td>Treatment of hormone-dependent mammary tumors</td>
<td>(Lombardi et al., 1999)</td>
</tr>
</tbody>
</table>
sequence is a D-isomer of Trp substituting for Gly\(^6\) as well as Pro\(^9\)-ethylamide substituting for Gly\(^{10}\)-NH\(_2\) (Padula, 2005). In the dog, long-term administration results in delayed puberty and acts as an anti-fertility agent in both males and females (Junaidi et al., 2003, 2009; Romagnoli et al., 2009). Deslorelin is also effective in treating benign prostatic hyperplasia by lowering testosterone concentrations (Limmanont et al., 2011). Furthermore, because elevated gonadotropin concentrations result from ovariectomy, deslorelin has been investigated as a treatment for urinary incontinence and puppy coat syndrome in ovariectomized female dogs (Reichler et al., 2003, 2008).

Leuprolide is another GnRH agonist that has been investigated in the dog (Herbert and Trigg, 2005). Its modified sequence is a D-isomer of Leu substituting for Gly\(^6\) as well as Pro\(^9\)-ethylamide substituting for Gly\(^{10}\)-NH\(_2\) (Padula, 2005). This agonist suppresses testicular function and gonadotropin concentrations with long-term applications (Inaba et al., 1996; Reichler et al., 2006a). Leuprolide was also found to restore urinary continence completely in 9 out of 22 incontinent ovariectomized bitches (Reichler et al., 2006b). While urethral closure pressure and function did not change in ovariectomized continent Beagles treated with leuprolide, bladder threshold volume significantly increased (Reichler et al., 2006a).

1.3.3 GnRH Antagonists

GnRH antagonists compete with GnRH for receptor occupancy and cause an immediate suppression of gonadotropin secretion (van Loenen et al., 2002). Development
of antagonists have focused on producing sequences with a high affinity for the GnRH receptor but no actual activation of the receptor, and this has been achieved by manipulating approximately 5-7 amino acid positions in the GnRH sequence (Herbst, 2003; Padula, 2005). However, a major limitation of GnRH antagonist application has been the difficulty in synthesizing long-term release formulations without side effects (Gobello, 2012). Depot preparations currently available require monthly administration (Agersø et al., 2003; Broqua et al., 2002; Klotz et al., 2008). While limited data is available concerning the use of GnRH antagonists in domestic carnivores (Gobello, 2012), GnRH antagonists have promising clinical reproductive applications. Antagonists investigated to date in the dog are summarized in Table 1.2.

Acyline, a third-generation antagonist, has substitutions in 7 amino acid positions with a sequence of Ac-D-2Nal\(^1\)-D-4Cpa\(^2\)-D-3Pal\(^3\)-Ser\(^4\)-4Aph(Ac)\(^5\)-D-4Aph(Ac)\(^6\)-Leu\(^7\)-ILys\(^8\)-Pro\(^9\)-D-Ala\(^10\)-NH\(_2\) (Valiente et al., 2007). It has demonstrated the ability to interrupt proestrus and block ovulation as well as safely terminate mid-pregnancy by suppressing progesterone concentrations in female dogs (Valiente et al., 2009a, 2009b). In male dogs, acyline has been investigated as an immunosterilant and decreases testosterone concentrations for approximately 14 days (García Romero et al., 2012) and furthermore impairs sperm concentration and motility for approximately six weeks (Valiente et al., 2007). Cetrorelix, another third-generation antagonist, has substitutions at 5 amino acid positions with a sequence of Ac-D-2Nal\(^1\)-D-4Cpa\(^2\)-D-3Pal\(^3\)-Ser\(^4\)-Tyr\(^5\)-D-Cit\(^6\)-Leu\(^7\)-Arg\(^8\)-Pro\(^9\)-D-Ala\(^10\)-NH\(_2\) (Padula, 2005). It suppresses testosterone
concentrations in dogs, but the effect is short-lived; dogs returned to normal testosterone levels by 48 hours after initial injection (Schwahn et al., 2000).

1.3.4 Immunization against GnRH

Another method for suppressing gonadotropin secretion is through active immunization against GnRH. Because native GnRH has a short biological half-life and low immunogenicity, effectively immunizing against GnRH requires GnRH or an analogue to be conjugated with an antigenic carrier molecule (usually an inactivated bacteria or virus) and then combined with an adjuvant to increase the immunoreactivity (Cox and Coulter, 1997; Fürst et al., 1994). When an animal is immunized with a GnRH vaccine, GnRH neutralizing antibodies are synthesized, which prevent GnRH from binding to its receptors in the anterior pituitary and consequently blocks the synthesis of gonadotropins and therefore steroid hormones (Fürst et al., 1994) (Figure 1.1).

Two commercially produced and several privately produced GnRH vaccines have been tested in dogs. GonaCon™ (United States Department of Agriculture, Fort Collins, CO) has been developed for wildlife population control, and it has been investigated in squirrels, swine, wild horses, white-tailed deer, cats, dogs, and other species (Alliance for Contraception in Cats and Dogs, 2009). While preliminary research using this vaccine as an immunosterilant in male dogs resulted in severe adverse injection site reactions (Alliance for Contraception in Cats and Dogs, 2009), reproductive inhibition did last one year in treated dogs (Miller et al., 2004). However, another study investigating the effect
Figure 1.1. Mechanism of action of gonadotropin releasing hormone (GnRH) immunization. Before immunization (left), GnRH is secreted by the hypothalamus, where it binds to its receptors on the anterior pituitary and elicits the secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH). After immunization (right), antibodies form against GnRH (GnRH-ab), preventing receptor binding and therefore halting LH and FSH secretion. Key of symbols provided at bottom.
of GonaCon™ on rabies virus antibody production demonstrated no adverse effects of Gonacon™ when administered to female dogs (Bender et al., 2009).

The other commercial GnRH vaccine, Canine Gonadotropin Releasing Factor Immunotherapeutic® (Pfizer Animal Health, Exton, PA) (package insert shown in Figure 1.2), is specifically labeled for use in dogs. This vaccine was produced for the treatment of benign prostatic hyperplasia in intact male dogs with a recommendation of revaccination every 6 months (Pfizer Animal Health). However, it is also effective in temporarily decreasing testosterone concentrations and testicular volume in healthy intact male dogs (Donovan et al., 2012) and for pregnancy termination in bitches (Chew and Purswell, 2010), suggesting the vaccine may be effective as a reversible immunosterilant.

Finally, GnRH immunization has demonstrated anti-fertility effects in various non-commercial vaccine compositions as well (Jung et al., 2005; Ladd et al., 1994; Walker et al., 2007). The different vaccine compositions and applications of GnRH immunization in the dog are summarized in Table 1.3.
Transient and self-limiting swelling at the injection site may be seen following vaccination, and should resolve within 14-28 days.

The effect of this product upon subsequent fertility in dogs is unknown.

Dispose of containers, syringes, needles and any unused contents according to local biomedical waste handling requirements.

In case of anaphylaxis, administer epinephrine or equivalent.

REFERENCES:

Technical inquiries should be directed to Pfizer Animal Health Veterinary Services, 1800-366-5238 (USA, (900) 461-0917 (Canada).

For veterinary use only

U.S. Veterinary License No. 189

Pfizer Animal Health

CAUTION: This product should be administered by or under the supervision of, a veterinarian. Care should be taken to avoid accidental self-injection or needle-stick injury when administering this product. Accidental self-injection and/or needle-stick injury may affect fertility in both men and women. As this vaccine may affect pregnancy, women of childbearing age should take particular care in its handling and administration. Seek medical advice immediately in the event of an accidental self-injection. To report an adverse event, contact Pfizer Animal Health at 1-800-366-5238.

DIRECTIONS:
General Directions: Vaccination of healthy dogs is recommended. Shake well. Asperitionally administer 1 mL subcutaneously.

Primary Vaccination: Healthy dogs should receive 2 doses administered 4-6 weeks apart. Involution of the prostate gland typically begins 10-14 days after administration of the second dose.

Revaccination: The revaccination interval for this product has not been determined. A single repeat dose administered every 6 months is recommended.

PRECAUTIONS:
Store at 2°-8°C. Prolonged exposure to higher temperatures and/or direct sunlight may adversely affect potency. Do not freeze. Use entire contents when first opened. Sterilized syringes and needles should be used to administer this vaccine.

Figure 1.2. Canine Gonadotropin Releasing Factor Immunotherapeutic® package insert. Outside cover (left) and inside leaves (right).
Table 1.3. GnRH immunization products investigated in the dog.

<table>
<thead>
<tr>
<th>Common Name</th>
<th>Vaccine Composition</th>
<th>Clinical Applications</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GonaCon™</td>
<td>GnRH peptide conjugated to keyhole limpet hemocyanin with AdjuVac™ adjuvant</td>
<td>Immunosterilization</td>
<td>(Alliance for Contraception in Cats and Dogs, 2009; Bender et al., 2009; Miller et al., 2004)</td>
</tr>
<tr>
<td>Canine Gonadotropin Releasing Factor Immunotherapeutic®</td>
<td>GnRH peptide conjugated to dipheria toxoid with a proprietary plant-based adjuvant</td>
<td>Pregnancy termination Decreasing testosterone and testicular volume Treatment for benign prostatic hyperplasia</td>
<td>(Chew and Purswell, 2010; Donovan et al., 2012; Hashimi et al., 2008; Pfizer Animal Health; Russo, 2008)</td>
</tr>
<tr>
<td>Non-commercial formulation</td>
<td>GnRH peptide conjugated to T helper cell epitope (originating from canine distemper virus and goat rotavirus) with Iscomatrix® adjuvant</td>
<td>Immunosterilization</td>
<td>(Jung et al., 2005)</td>
</tr>
<tr>
<td>Non-commercial formulation</td>
<td>GnRH peptide conjugated to tetanus toxoid with MDP A-5 adjuvant</td>
<td>Immunosterilization</td>
<td>(Ladd et al., 1994)</td>
</tr>
<tr>
<td>Non-commercial formulation</td>
<td>GnRH peptide conjugated to T helper cell epitope (originating from canine distemper virus) with Iscomatrix® adjuvant</td>
<td>Immunosterilization</td>
<td>(Walker et al., 2007)</td>
</tr>
</tbody>
</table>
1.4 ETIOPATHOPHYSIOLOGY OF URINARY INCONTINENCE IN THE BITCH

1.4.1 Summary

Urinary incontinence is the involuntary release of urine (Moore, 2001). Resting urethral closure pressure and continence largely depend on the tonic sympathetic noradrenergic activation of $\alpha$-1 adrenoreceptors on urethral smooth muscle (Brune et al., 2001). Ovariectomy causes a deterioration of urethral closure pressure (Reichler et al., 2004), and urethral sphincter mechanism incompetence (USMI) post-ovariectomy remains the most prevalent cause of canine incontinence (Holt, 1985). However, other causes of urinary incontinence exist and can be categorized into congenital anatomic abnormalities, neurologic disorders, or functional disorders.

1.4.2 Congenital Anatomic Abnormalities

Anatomic abnormalities that can result in urinary incontinence include ectopic ureters, congenital sphincter mechanism incompetence, bladder hypoplasia, and vestibulo-vaginal stenosis. Ectopic ureter is an abnormality where one or both ureters terminate distal to the neck of the bladder instead of in the bladder, allowing urine to bypass the urethral sphincter and therefore leak freely without being contained (Lautzenhiser and Bjorling, 2002). Surgical correction repositions the ureter(s) into the bladder to restore continence (Holt, 1983). Congenital sphincter mechanism incontinence
usually occurs as a result of a short and/or wide urethra, resulting in the urinary sphincter being inadequate (Holt, 1983). Bladder hypoplasia is the underdevelopment of the bladder, which results in a low capacity and incontinence. Finally, many dogs with vestibulo-vaginal stenosis, or abnormal narrowing of the caudal vagina, display incontinence because urine can pool cranial to the stenosis (where the urethra opens into the vagina) and subsequently dribble out. However, continent dogs can also have vestibulo-vaginal stenosis and therefore, it cannot be considered a cause for incontinence but rather a complicating factor (Holt, 1983).

1.4.3 Neurologic Disorders

Neurologic disorders that result in urinary incontinence can be divided into two subtypes: flaccid neurogenic bladder and spastic neurogenic bladder. Flaccid neurogenic bladder is a lower motor neuron dysfunction that results in an excessively dilated bladder and an inability to contract, resulting in a retention of urine and overflow incontinence (Ponglowhapan et al., 2012). Spastic neuropathic bladder is a upper motor neuron dysfunction where incomplete voiding of the bladder occurs (Ponglowhapan et al., 2012). A type of spastic bladder, reflex dyssynergia, is characterized by normal initiation of bladder voiding, followed by a decrease and then a sudden interruption in flow (Espiñeira et al., 1998). Treatment aims to re-coordinate bladder voiding with urethral relaxation (Espiñeira et al., 1998).
1.4.4 Functional Disorders

The most common cause of urinary incontinence is acquired urinary sphincter mechanism incompetence (USMI) as a result of ovariectomy (Holt, 1985). Gonadectomy is associated with urethral functional modifications that may contribute the development of USMI (Noël et al., 2010). In the dog, it has been demonstrated that mean urethral closure pressure is significantly reduced after ovariectomy (Arnold, 1997; Reichler et al., 2004) and is further reduced in incontinent dogs (Richter and Ling, 1985). Specifically, Arnold and colleagues (1997) reported that a urethral closure pressure of less than 7.4 cm H$_2$O allowed for the differentiation of bitches with urinary incontinence with a diagnostic accuracy of 91%. However, the definite underlying mechanism(s) of incontinence resulting from reduced urethral closure pressure has not been concretely determined (Reichler et al., 2006a). It has been hypothesized that elevated gonadotropins as a result of ovariectomy may affect urethral pressure, but when incontinent dogs were treated with GnRH agonists to reduce gonadotropin concentrations, there was no change in urethral pressure even in cases of restored continence (Reichler et al., 2006b).

The development of USMI is most likely multifactorial due to multiple predisposing factors. However, it is well evidenced that ovariectomy is the primary predisposing factor for urinary incontinence. The incidence of incontinence has been reported from 5.7-20% (Arnold et al., 1992; Holt and Thrusfield, 1993) in ovariectomized bitches whereas the incidence of incontinence in intact bitches is 0-1% (Holt and Thrusfield, 1993). Gender is also a major predisposing factor. Females are far more
affected than males; one study reported 235/244 (96.3%) of adult patients diagnosed with USMI were female (Holt, 1990a). It is also well established that medium and large dogs are also more likely to develop incontinence than small dogs (Angioletti et al., 2004; Chew and DiBartola, 2007; Holt and Thrusfield, 1993). Other potential predisposing factors include obesity; Angioletti and coworkers (2004) found that bitches that were overweight before ovariectomy had 3.5 times more risk of developing incontinence compared to non-obese bitches. Tail docking has also been suggested as a risk factor for urinary incontinence as many breeds with a high predisposition for developing incontinence (Boxers, Rottweilers, Dobermans, Old English Sheepdog) have docked tails (Holt and Thrusfield, 1993; Ponglowhapan et al., 2012). However, no studies have compared docked and undocked dogs of the same breed, so it is currently unknown if the incidence of incontinence is related to size, tail docking, or both in these breeds.

Estrogen deficiency has been hypothesized as a cause for urinary incontinence (Arnold et al., 2009) and was originally supported by the fact that approximately 65% of incontinent bitches treated with estrogens have returned to continence (Janszen et al., 1997). However, the permanent elevation of gonadotropins as a result of ovariectomy has also been hypothesized to be a cause for urinary incontinence, especially considering LH and FSH extragonadal receptors are present in the canine lower urinary tract (Coit et al., 2009; Ponglowhapan et al., 2007; Reichler et al., 2007). Estrogen therapy restores the disrupted HPG axis and therefore decreases elevated gonadotropins, so this would explain why estrogen can be an effective treatment for incontinence. Furthermore, long-acting GnRH depot agonists have been evaluated for the treatment of incontinence, and
treatment success was comparable to estrogen therapy (Reichler et al., 2003, 2006b). This is further evidence that gonadotropin excess, not estrogen deficiency, is what may cause urinary incontinence.

1.4.5 Conclusions

The major endocrinological change resulting from ovariectomy (e.g. elevated gonadotropins) has an effect on the pathophysiology of urinary incontinence. Elevated gonadotropins bombarding their receptors in the urinary tract may be what induces the decrease in urethral pressure, and this effect seems to be non-reversible as reducing gonadotropin concentrations in ovariectomized continent and incontinent dogs did not increase urethral closure pressure (Reichler et al., 2006a, 2006b). However, treating incontinent dogs with GnRH agonists to effectively decrease gonadotropin concentrations restored continence in some dogs (Reichler et al., 2003, 2006b), indicating that urinary incontinence is dependent on circulating gonadotropin levels in some cases. However, it is possible that over time, the effects of elevated gonadotropins may not be fixable by only decreasing gonadotropin concentrations. This is further evidenced incontinent dogs that are treated successfully with combinations of GnRH agonists or estrogen therapy with phenylpropanolamine when one treatment alone was not effective. Further information regarding incontinence treatment will be discussed in the next section.
1.5 CURRENT METHODS FOR THE TREATMENT OF URINARY SPHINCTER MECHANISM INCOMPETENCE (USMI) IN THE BITCH

1.5.1 Summary

Various treatment regimens have been investigated to treat acquired urinary sphincter mechanism incompetence (USMI), both surgical and non-surgical. Medical therapy is the first line of treatment for most affected dogs, but surgical treatment may be pursued if medical management has failed, side effects develop, or if owners are reluctant to medicate long term (Claeys et al., 2010; McLoughlin and Chew, 2009). Numerous treatment approaches exist for both categories.

1.5.2 Surgical Treatment Options

The overall goal of surgical treatment of USMI is to increase urethral resistance to the outflow of urine (McLoughlin and Chew, 2009). To date, surgical procedures to treat USMI in the dog have been adapted from procedures performed on women with urinary incontinence (Claeys et al., 2010; McLoughlin and Chew, 2009). Less popular surgical options for the treatment of USMI in the dog include urethral slings, artificial sphincters, and urethral lengthening. Due to increased invasiveness and/or lack of efficacy, these procedures are not commonly recommended.
Colposuspension is the most commonly performed surgical treatment for USMI in the dog (McLoughlin and Chew, 2009). Briefly, sutures are placed between the prepubic tendon and seromuscular layer of the vagina, and this relocates the bladder neck and proximal urethra to a more cranial position, exposing both structures to intra-abdominal pressure and therefore adding pressure onto the bladder neck (Claeys et al., 2010; McLoughlin and Chew, 2009; Rawlings et al., 2001). Colposuspension has been demonstrated to successfully restore continence initially in approximately 50% of cases (Holt, 1990b; Rawlings et al., 2001). However, efficacy of the procedure long-term is not as successful; Rawlings and coworkers (2001) reported that only 3 of 22 bitches that underwent colposuspension were still continent twelve months after surgery compared to 12 out of 22 bitches that were continent two months after surgery. As a result, many dogs that undergo colposuspension will need additional medical therapy to have the best urinary control possible (Rawlings, 2002).

Urethropexy is another surgical procedure to treat USMI. Briefly, sutures are placed between the body wall and the seromuscular layer of the urethra (McLoughlin and Chew, 2009). This procedure is similar to colposuspension in that the bladder neck is relocated to a more cranial position and is therefore more susceptible to intra-abdominal pressure. However, it is also speculated that additional urethral resistance is created by causing a urethral kink at the level of the urethropexy (White, 2001). The success of urethropexy to restore continence initially is similar to colposuspension. White (2001) reported approximately 56% success with a worsening of response to the procedure over time.
Urethral bulking is a minimally invasive procedure designed to increase urethral resistance. Briefly, a needle is guided endoscopically to just below the mucosal layer of the urethra, and a substance is then injected in 3-4 sites around the submucosal layer of the urethra (McLoughlin and Chew, 2009). While Teflon was the substance formerly used for this procedure, rejection of the foreign matter that resulted in a return to incontinence made the material unfavorable for use (Barth et al., 2005). Therefore, collagen is the preferred agent for urethral bulking due to a higher degree of biocompatibility (McLoughlin and Chew, 2009). Studies have demonstrated approximately 53-68% success in restoring continence using collagen implantation, but continence deteriorates over time (Arnold et al., 1996; Barth et al., 2005). Collagen flattening over time may be what leads to an eventual return to incontinence (Claeys et al., 2010).

1.5.3 Non-Surgical Treatment Options

Numerous medical therapies exist for the treatment of USMI through direct or indirect action on urethral musculature. This form of management is non-invasive and therefore the first option considered by many pet owners when treating USMI.

Phenylpropanolamine (PPA) is a synthetic adrenergic agonist that mimics the effects of endogenous catecholamines. PPA stimulates the sympathetic nervous system by activating \( \alpha_1 \) adrenoreceptors that are expressed in vascular smooth muscle cells, resulting in vasoconstriction (Flavahan, 2005). Urethral closure pressure is naturally
maintained by activation of $\alpha_1$ adrenoreceptors of the urethral smooth musculature in the dog by the sympathetic nervous system (Hashimoto et al., 1992), and therefore, PPA is effective when urethral closure pressure is not sufficient to maintain continence because it helps stimulate $\alpha_1$ adrenoreceptor activation. Currently, PPA is the most common therapy for treating USMI and is effective in 75-85% of cases (Arnold et al., 2009; Scott et al., 2002). PPA is FDA approved for use in dogs to treat urinary incontinence and is marketed in the United States under the trade name Proin® (PRN Pharmacal, Pensacola, FL).

While PPA is the most effective treatment for USMI currently available, it can produce side effects that makes the drug less appealing to some dog owners. PPA is not selective for the $\alpha_1$ adrenoreceptors within the urinary tract (Yakoot, 2012) and as a result, vasoconstriction of smooth musculature can occur in a variety of tissues. Side effects of PPA include hypertension, weight loss, emesis, proteinuria, anorexia, lethargy, and behavior changes (Pegasus Laboratories Inc., 2011). Furthermore, PPA's short half-life of approximately 4 hours (Hussain et al., 1987) requires frequent administration of the drug to maintain therapeutically effective vasoconstriction. In addition, drug treatment must be maintained for the dog's lifetime.

Ephedrine and pseudoephedrine are $\alpha$-adenergic agonists that have also successfully treated USMI. However, in addition to stimulated $\alpha_1$-adenergic receptors like PPA, ephedrine and pseudoephedrine also stimulate $\beta_1$, 2, and 3 receptors, resulting in an increase in side effects (Arnold et al., 2009; Byron et al., 2007; Vansal and Feller, 1999).
Estrogen therapy increases urethral closure pressure by increasing receptor sensitivity to catecholamines and has been used to treat USMI (Larsson et al., 1984). Estrogen therapy also decreases circulating concentrations of luteinizing hormone and follicle stimulating hormone (Chew and DiBartola, 2007), which are permanently elevated as a result of ovariectomy and may contribute to the development of urinary incontinence (Reichler et al., 2003). In the United States, there are two estrogens currently used in dogs. Diethylstilbestrol (DES) is a synthetic estrogen available from compounding pharmacies, and estriol is a natural estrogenic compound that is FDA approved under the trade name Incurin® (Merck Animal Health, Summit, NJ). While DES and estriol both rapidly associate with estrogen receptors, estriol has a fast rate of dissociation while DES forms a stable complex and therefore dissociates more slowly (Rich et al., 2002). The canine bone marrow is exquisitely sensitive to the suppressing effects of estrogens. As a result, bone marrow suppression is a potential adverse effect of estrogen treatment, and therefore, the shorter-acting and less potent estriol is preferred over longer-acting estrogen forms (Arnold et al., 2009; Sondern and Sealey, 1940).

Estrogen therapy for the treatment of USMI is 50-65% effective (Janszen et al., 1997; Mandigers and Nell, 2001; Nendick and Clark, 1987). Side effects include signs of estrus, attractiveness to male dogs, and swelling of the vulva and teats. However, decreasing the dose can prevent or reduce side effects in some cases (Mandigers and Nell, 2001). Reducing the dosing frequency with DES (administering a few times a week instead of daily) may reduce side effects in some cases. Estriol must be given daily to have an effect.
GnRH agonists have recently been investigated as a potential treatment for USMI. They decrease LH and FSH concentrations through downregulation of GnRH receptors in the anterior pituitary, and this mechanism of action has been hypothesized to restore continence to incontinent bitches. Treatment with GnRH agonists restores continence in approximately 50% of bitches (Reichler et al., 2003, 2006b), comparable to estrogen therapy. However, GnRH agonist treatment lasts significantly longer than other medical treatments available, ranging from 50-738 days after agonist administration with no adverse effects (Reichler et al., 2003, 2006b).

1.5.4 Combination Therapy with PPA

Successful combination therapies with PPA have been reported when the initial surgical or non-surgical treatment is not effective alone. Administering PPA after a surgical procedure to treat USMI can fully restore continence (Barth et al., 2005; Holt, 1990b; Rawlings, 2002). Administering PPA with estrogens (Arnold et al., 2009; Chew and DiBartola, 2007) or GnRH agonists (Reichler et al., 2003) is effective in treating USMI when any treatment alone is not efficacious.

1.5.5 Conclusions

Various surgical and non-surgical options exist for the treatment of urinary incontinence and combining any of these therapies may restore continence when one
treatment method individually is not efficacious. However, no single treatment is completely effective in treating urinary incontinence. This has stimulated further research interest into the underlying pathophysiology of USMI with the aim of developing more effective treatments without side effects (Ponglowhapan et al., 2012).
1.6 REFERENCES


Findlay, J.K. (1993). An update on the roles of inhibin, activin, and follistatin as local


incontinence with oestriol. Veterinary Record 149, 764–767.
Ponglowhapan, S., Church, D.B., Scaramuzzi, R.J., and Khalid, M. (2007). Luteinizing hormone and follicle-stimulating hormone receptors and their transcribed genes (mRNA) are present in the lower urinary tract of intact male and female dogs.


Veterinary Medical Association 198, 1180–1182.
CHAPTER II

PHYSIOLOGIC RESPONSES FOLLOWING GONADOTROPIN-RELEASING HORMONE IMMUNIZATION IN INTACT MALE DOGS

CE Donovan, M Greer, MA Kutzler

Reproduction in Domestic Animals
John Wiley & Sons Inc.
350 Main Street
Malden, MA 02148, USA
Volume 47, Supplement 6
2.1 ABSTRACT

We investigated the use of a commercial gonadotropin-releasing hormone (GnRH) vaccine as a method of temporary and reversible immunocastration in intact male dogs. Four privately-owned dogs were vaccinated twice at four week intervals. Blood samples were collected at 0, 4, 12, and 20 weeks following the initial vaccination. These samples were analysed for GnRH antibody titres, luteinizing hormone (LH) and testosterone concentrations. Scrotal measurements were made at the time of sample collection, and testicular volume was calculated using the formula of an ellipsoid. As a result of vaccination, dogs displayed an elevated GnRH antibody titre, decreased LH and testosterone concentrations and decreased testicular volume, which reversed by the end of the study period. Therefore, these results suggest that immunizing against GnRH may be a possible choice for temporary and reversible immunocastration.

2.2 INTRODUCTION

Nonsurgical sterilization methods are highly desirable for domestic animals for population control purposes as well as for animals that are not candidates for surgery. The hypothalamic-pituitary-gonadal axis, which is the regulator of mammalian reproductive function, has specifically been a target of manipulation for immunocontraception development. Briefly, gonadotropin releasing hormone (GnRH), a
trophic decapeptide secreted from the hypothalamus, controls the release of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary. In turn, LH and FSH act upon the testes in the male to stimulate testosterone production, which then negatively feeds back on the hypothalamus and anterior pituitary to regulate GnRH secretion. By diminishing GnRH production, the production of LH, FSH and testosterone are diminished as well.

It has been shown previously in intact male dogs that vaccinating with a GnRH analogue effectively decreases testosterone concentration (Sabeur et al., 2005; Walker et al., 2007). A commercially manufactured GnRH vaccine in the United States (Canine Gonadotropin Releasing Factor Immunotherapeutic®; Pfizer Animal Health, Exton, PA, USA; referred to in this manuscript as a 'commercial canine GnRH vaccine'), labelled for the twice-annual management of benign prostatic hyperplasia in intact male dogs, may also be an ideal option for a reversible means of immunocastration. The vaccine elicits antibodies that bind to GnRH and render it nonfunctional, ultimately decreasing testicular testosterone production (Pfizer Animal Health, Package Insert).

The objective of this study was to measure endocrine responses in intact male dogs after administering a commercial canine GnRH vaccine. Furthermore, because testosterone production is necessary for spermatogenesis and testicular volume largely reflects spermatogenesis (Gouletsou et al., 2008), testicular volume measurements were also recorded. It was expected that immunization against GnRH would temporarily elicit
a GnRH antibody titre, decrease LH and testosterone concentrations and decrease testicular volume in intact males.

2.3 MATERIALS AND METHODS

2.3.1 Animals, Vaccination, Sample Collection, and Scrotal Measurements

Four privately-owned intact male dogs were used for this study. Dogs were vaccinated subcutaneously with 1 ml of commercial canine GnRH vaccine twice at 4-week intervals (at week 0 and 4). Animals were closely monitored for adverse reactions following each vaccination. Venous blood samples were collected prior to each vaccination (0 and 4 weeks) and at 12 and 20 weeks following initial treatment. Sera were separated, aliquoted, and frozen at -20°C until analysis.

Scrotal length, width and height were measured using calipers at weeks 0, 4, 12, and 20. Testes were aligned side by side and forced down into the scrotum as far as possible for each measurement. This is the standard method for testis measurement in dogs and causes no observable pain to the animal. Measurements were used to calculate testicular volume using the formula of an ellipsoid, length \( \times \) width \( \times \) height \( \times \) 0.5236, as described by Gouletatsou et al. (2008).

2.3.2 Hormone Assays
Gonadotropin-releasing hormone antibody titres were determined by enzyme-linked immunosorbent assay (ELISA) using a technique modified from Elhay et al. (2007). Briefly, 96-well microtitre plates were coated with 100 µl of 5 µg/ml of LH-RH (71447-49-9, Sigma, St. Louis, MO, USA) in sodium bicarbonate buffer (pH 8.0) at 4°C overnight. After incubation, plates were washed with phosphate buffered saline containing 0.05% Tween-20 (TPBS) (pH 8.0) and were then blocked with a serum dilution buffer (phosphate buffered saline containing 0.5% bovine serum albumin, pH 8.0). After 1-h incubation period at room temperature, plates were washed with TPBS and serum samples were diluted in the serum dilution buffer, yielding final serum dilutions ranging from 1:2 to 1:128. After washing with TPBS, antibodies were detected using horseradish peroxidase protein G conjugate (HRP) (10-1223, Invitrogen, Camarillo, CA, USA) diluted as 1:2000 in serum dilution buffer for 1 h at room temperature. After a final wash with TPBS, HRP was visualized with ABTS peroxidase substrate (50-66-01, KPL, Gaithersburg, MD, USA). Absorbances were read at 405 nm using a spectrophotometer (Bio-Tek Instruments Inc., Winooski, VT, USA). The antibody titre was expressed as an endpoint titre for each sample, which was calculated from a regression line of optical density against the sample dilution with a threshold of 0.200 optical density using a software program (KinetiCalc, Bio-Tek Instruments Inc., Winooski, VT, USA).

Serum samples were analysed for LH concentrations using an ELISA kit for canine serum (LH-Detect®, Repropharm, Nouzilly, France) and performed according to the manufacturer’s instructions. The limit of detection was 0.12 ng/ml, and all assays
were performed in duplicate. Serum samples were also analysed for testosterone at the Animal Health Diagnostic Center at Cornell University using Coat-A-Count® Total Testosterone radioimmunoassay (Diagnostics Products Corporation, Los Angeles, CA, USA), validated for dogs by Reimers et al. (1991), and performed according to the manufacturer’s instructions. The detection limit of the assay was 0.04 ng/mL. The coefficients of variation for the intra-assay and inter-assay variability were 4-21% and 6-27%, respectively, depending on the testosterone concentration.

2.3.3 Statistical Analysis

Gonadotropin-releasing hormone antibody titres, LH concentrations, and testosterone concentrations, and the per cent change in testicular volume were analysed as a repeated measure in time design using PROC MIXED in SAS (Version 9.2, SAS Institute Inc., Cary, NC, USA). Significance was defined as p≤0.05. LH concentrations are expressed as mean ± SEM and GnRH antibody titres, testosterone concentrations and per cent change in testicular volume are expressed as mean ± SD.

2.4 RESULTS

None of the dogs vaccinated experienced an adverse reaction to the vaccine and remained clinically healthy for the duration of the study. All dogs were seronegative for antibodies against GnRH prior to the first vaccination and were seropositive for antibodies against GnRH after receiving the first vaccination. Antibody titres peaked at
12 weeks post-vaccination and were significantly greater at week 12 compared to week 0 (p<0.001), week 4 (p=0.008), and week 20 (p=0.01) (Fig. 2.1).

Luteinizing concentrations remained at basal levels (<0.8 ng/mL) post-vaccination (Fig. 2.1). All dogs also experienced a decrease in testosterone concentration following vaccination (Fig. 2.1). Concentrations of testosterone were below the detection limit of the assay at week 12, a significant decrease (p=0.02) compared to pre-vaccination testosterone levels. At week 20, there was a slight but insignificant rise in testosterone concentrations.

All dogs experienced a decrease in testicular volume following GnRH vaccination (Fig. 2.2). Testicular volume was significantly decreased at week 12 compared to week 0 (p=0.05), week 4 (p=0.02), and week 20 (p=0.01). At week 20, there was a slight but insignificant rise in testicular volume, consistent with the increase in testosterone concentrations.
Figure 2.1. Serum GnRH-Ab titre (±SD; left axis) as compared to testosterone (±SD) and luteinizing hormone (±SEM) concentrations (ng/ml; right axis) at weeks 0, 4, 12, and 20 following GnRH vaccination. \(^a\)p<0.05 compared to GnRH-Ab titres at weeks 0, 4, and 20. \(^b\)p<0.05 compared to testosterone concentration at week 0
Figure 2.2. Change in testicular volume (%) at weeks 0, 4, 12, and 20 following GnRH vaccination. *p<0.05 compared to weeks 0, 4, and 20
2.5 DISCUSSION

An alternative to surgical castration is needed for population control in dogs that are not surgical candidates because of health limitations and other reasons. A vaccine that elicits antibodies against GnRH to effectively decrease steroid hormone production is a potential option for a temporary, reversible means of immunosterilization. Although this commercial canine GnRH vaccine has been studied to treat benign prostatic hyperplasia in intact male dogs, it has not been thoroughly studied as an immunocontraceptive, and therefore, the objective of this study was to measure endocrine responses and testicular volume changes in intact male dogs as a result of vaccination.

As approximately 70-80% of testicular mass consists of seminiferous tubules (Gouletsou et al., 2008), measurement of testicular volume is largely reflective of the capacity for spermatogenesis. The dogs used in this study all responded to the vaccine by developing a GnRH antibody titre, and this resulted in a significant decrease in testosterone production and also a significant decrease in testicular volume.

Luteinizing concentrations remained at basal levels (<0.8 ng/ml) in all dogs post-vaccination, but this was not significant compared to week 0, perhaps because of the cyclical secretion pattern of LH. The time interval in between LH peaks in the intact male dog is approximately 103 min (Olson et al. 1992). Therefore, collecting multiple samples over a 2-h time interval is needed to determine if LH secretion was suppressed as a result of vaccination. This frequency of sampling was not feasible in the current study because the dogs used were privately owned.
In conclusion, vaccinating intact male dogs with a commercial canine GnRH vaccine induces a temporary and reversible humoral response that results in decreased testosterone concentration and decreased testicular volume. Further study using a double-blind placebo-controlled format that also evaluates sexual behaviour, semen quality and repeated immunization is a necessary next step.

2.6 REFERENCES


3.1 ABSTRACT

We have investigated gonadotropin-releasing hormone (GnRH) immunization for the treatment of urethral sphincter mechanism incompetence in ovariectomized bitches. It has been demonstrated that decreasing luteinizing hormone (LH) secretion through the use of GnRH depot agonists restores continence in some bitches. Therefore, decreasing circulating LH concentrations through immunization against GnRH may also maintain continence in incontinent dogs as well. Sixteen incontinent dogs that were using phenylpropanolamine (PPA) to control incontinence were recruited for this study. Eleven dogs were immunized against GnRH at week 0, and nine dogs were vaccinated again four weeks later. Five control dogs were vaccinated with a placebo twice at four week intervals. Vaccinated dogs discontinued PPA two weeks after re- vaccination, and control dogs remained on PPA for the duration of the study. Blood samples were collected before each injection and at 6, 8, 10, 12, 16, 20, and 24 weeks, and owners recorded episodes of incontinence throughout the study. Of the nine dogs that completed the vaccination series, four dogs remained continent after PPA was discontinued. For these four dogs, there was no difference in the episodes of incontinence when using PPA versus treatment.
with the vaccine. All nine vaccinated dogs developed a GnRH antibody titer, and LH concentrations decreased significantly in vaccinated dogs compared to controls. In conclusion, GnRH immunization was effective in maintaining continence in 44% of incontinent ovariectomized dogs, and in these dogs, treatment with the vaccine was comparable to treatment with PPA.

3.2 INTRODUCTION

The development of urethral sphincter mechanism incompetence (USMI) in female dogs is a prevalent sequelae after ovariectomy or ovariohysterectomy (hereafter referred to as ovariectomy), with incidences reported between 5.7-20% in ovariectomized bitches (Arnold et al., 1992, Thrusfield 1985). In comparison, only 0-1% of intact bitches develop USMI (Thrusfield 1985). After ovariectomy, urethral closure pressure decreases, even in bitches who remain continent (Arnold et al., 1997). However, urethral closure pressure is significantly more reduced in bitches with USMI (Richter and Ling, 1985).

Urethral closure pressure is normally maintained by sympathetic activation of α₃ adrenoreceptors in the urethral smooth muscle (Hashimoto et al., 1992). Therefore, the most common current method of treating USMI is with α-adenergic agonists, specifically phenylpropanolamine (PPA). PPA mimics the effect of catecholamines by activating α₃ adrenoreceptors in the urethral smooth muscle, effectively increasing urethral closure pressure and restoring continence. Unfortunately, PPA is not completely effective in the treatment of USMI (Arnold et al., 2009, Scott et al., 2002). In addition, PPA is not
selective for α1 adrenoreceptors within the urinary tract. Undesired vascular smooth muscle contraction from PPA administration elsewhere in the body, including blood vessels, can result in hypertension (Flavahan, 2005). Other reported side effects of PPA in dogs include anorexia, emesis and weight loss, lethargy and behavior changes, and proteinuria (Pegasus Laboratories Inc., 2011). Another clinical difficulty with the use of PPA is the four hour half-life (Hussain et al., 1987) that requires dosing every 8-24 hours to maintain therapeutically effective urethral closure. This frequent administration can be frustrating for owners because treatment must be continued for the remainder of the dog’s life.

Ovariectomy results in elevated concentrations of pituitary luteinizing hormone (LH) because there is no gonadal negative feedback. The canine urinary tract has LH receptors (Coit et al., 2009, Ponglowhapan et al., 2007, Reichler et al., 2007), and it has been postulated that elevated gonadotropins may contribute to the development of USMI (Reichler et al., 2003). Treatment of bitches with GnRH agonists downregulate LH secretion for prolonged periods of time (Concannon, 1993) and restores continence to incontinent bitches for approximately 250 days (Reichler et al., 2003, Reichler et al., 2006a). Similarly to PPA, efficacy of GnRH agonists for the treatment of USMI is not completely effective. However, unlike PPA, no adverse effects to GnRH agonists have been reported.

There are other methods reported to decrease LH concentrations that may also treat USMI, such as immunization against GnRH. Immunization against GnRH elicits the synthesis of GnRH neutralizing antibodies, which prevent GnRH from binding to
GnRH receptors and consequently prevent the synthesis of LH (Fürst et al., 1994). In 2004, a commercial GnRH vaccine was launched in the United States (Canine Gonadotropin Releasing Factor Immunotherapeutic®; Pfizer Animal Health USA). This vaccine was labeled for the treatment of benign prostatic hyperplasia in intact male dogs, but it has also been shown to decrease testosterone concentrations in intact male dogs for approximately 20 weeks (Donovan et al., 2012) and to safely terminate pregnancy in bitches (Chew and Purswell, 2010).

The objectives of this study were to determine (a) whether GnRH immunization will maintain continence in incontinent ovarioctomized bitches and (b) whether GnRH immunization controls USMI as effectively as phenylpropanolamine. It was hypothesized that GnRH immunization would effectively maintain continence for a prolonged duration and that it would be as effective as phenylpropanolamine for the treatment of USMI.

3.3 MATERIALS AND METHODS

3.3.1 Animals and Vaccination

Sixteen privately-owned ovarioctomized bitches were enrolled at Oregon State University's Veterinary Teaching Hospital for this study. A diagnosis of incontinence following ovarioctomy was confirmed through veterinary medical records. At the time the bitches were recruited for the study, all were being treated with PPA (Proin®; PRN Pharmacal, Pensacola, FL) to maintain continence. Clinical health was confirmed in all
dogs by a complete blood count, biochemistry panel, urinalysis, and urine culture at the beginning and end of the study.

Vaccinated dogs (n=11) received 1 mL Canine Gonadotropin Releasing Factor Immunotherapeutic® (Pfizer Animal Health) subcutaneously over the lateral thorax and were re-immunized 4 weeks later. Control dogs (n=5) received 1 mL saline over the lateral thorax and were injected again 4 weeks later. Animals were closely monitored by their owners for adverse reactions. One treatment dog developed tachypnea for 24 hours after initial vaccination and another treatment dog demonstrated impaired movement due to soreness for one week after initial vaccination. These two dogs did not receive a second vaccination and were excluded from further study.

3.3.2 Study Design and Sample Collection

Venous blood samples were collected from all dogs prior to each injection (0 and 4 weeks) and again at 6, 8, 10, 12, 16, 20, and 24 weeks after initial vaccination. Blood samples were divided into Vacutainer® clot tubes (02-685-A, Fisher Scientific Co.) to obtain serum and Vacutainer® EDTA tubes (02-683-99A, Fisher Scientific Co.) to obtain plasma. After centrifugation, serum and plasma were separated and frozen at -20°C until analysis.

Vaccinated dogs discontinued PPA use two weeks after the second injection, and control dogs remained on PPA for the duration of the study. All vaccinated dogs did not take PPA for at least one week after PPA was discontinued; once the dog became incontinent again, PPA administration was resumed.
Owners reported the frequency of incontinent episodes before any treatment for incontinence was initiated, and they also recorded all episodes of incontinence for the duration of the study. For vaccinated dogs that maintained continence after PPA discontinuation, the frequency of incontinent episodes before any treatment was initiated, during treatment with PPA (week 0 through week 6 of the study), and during treatment with the GnRH vaccine (week 7 through week 13 of the study) were compared to determine the efficacy of each treatment type.

3.3.3 Sample Assays

Serum samples were used to measure GnRH antibody titers, determined by ELISA using a technique modified from Elhay et al. (2007). Briefly, 96-well microtiter plates were coated with 100 µL of 5 µg/mL of LH-RH (71447-49-9, Sigma, St. Louis, MO, USA) in sodium bicarbonate buffer (pH 8.0) at 4ºC overnight. After incubation, plates were washed with phosphate-buffered saline containing 0.05% Tween-20 (TPBS) (pH 8.0). Plates were then incubated for 1 hour at 20ºC with serum samples in duplicate diluted in a buffer containing 0.5% bovine serum albumin (9048-46-8, Sigma, St. Louis, MO, USA) to yield final serum dilutions ranging from 1:8 to 1:1024. After tapping dry, antibodies were detected using horseradish peroxidase protein G conjugate (HRP) (10-1223, Invitrogen, Camarillo) diluted at 1:2000 in serum dilution buffer for 1 hour at 37ºC. After a final wash with TPBS, HRP was visualized with 100 µL of ABTS peroxidase substrate (50-66-01, KPL, Gaithersburg, MD, USA). Absorbances were read at 405 nm using a spectrophotometer (FLUOstar Omega, BMG Labtech Inc., San Francisco, CA,
USA) and each serum sample was measured in duplicate. The cutoff for seropositivity, defined in this study as the upper limit of a 99% confidence interval above the mean negative control level, was calculated using the methods of Frey et al. (1998). Serological results were expressed as the reciprocal of the highest twofold serial dilution above the calculated cutoff and linearized using a base-2 logarithmic scale.

Plasma samples were analyzed for LH concentrations in duplicate using an ELISA kit for canines (LH-Detect®; Repropharm, Nouzilly, France) and performed according to the manufacturer's instructions. The limit of detection was 0.12 ng/mL. Optical densities of the scale of standards and the samples were linearized using a base-e logarithmic scale and then back-transformed to determine the ng/mL LH concentration of each sample using a standard curve.

3.3.4 Statistical Analysis

Serum GnRH antibody titers were compared between the vaccination and control group using Fisher's exact test (GraphPad QuickCalcs Software, La Jolla, CA, USA). Plasma LH concentrations were compared between the vaccination and control group as a repeated measure using PROC MIXED in SAS (Version 9.2, SAS Institute Inc., Cary, NC, USA). Fixed effects in the repeated measure model were whether the animal was vaccinated, time after first vaccination, and the interactions between vaccination and time. A first order heterogeneous autoregressive variance-covariance structure was fitted for repeated measurements within animals. The frequency of incontinent episodes before initial treatment, treatment with PPA, and treatment with the vaccine were compared as a
repeated measure using PROC MIXED in SAS. The fixed effect of the repeated measure model was the treatment type. Significance was defined as $p<0.05$.

3.4 RESULTS

The signalment, treatment, and result of treatment for all bitches is provided in Table 3.1. There were no differences between size, age, weight, age at ovariectomy, or interval between ovariectomy and incontinence in bitches from the vaccinated and control groups. There was no change in clinical health over the duration of the study as determined by a complete blood count, biochemistry panel, urinalysis, and urine culture (data not shown).

As mentioned in the methods, two dogs were withdrawn from the study after the initial vaccination due to adverse reactions (tachypnea and prolonged soreness). Eight of the nine remaining dogs experienced minor side effects, including lethargy, swelling and/or soreness at the injection site, and decreased appetite. Three dogs that experienced minor side effects following the initial vaccination were given diphenhydramine at 1 mg/kg intramuscularly in conjunction with the booster vaccine, and owners reported a decrease in adverse reactions following the second vaccination.

Four out of nine vaccinated dogs remained continent after discontinuation of PPA. One dog became incontinent again 14 weeks after PPA was discontinued and received a third GnRH vaccination. Incontinent episodes did not occur after the third vaccination.
and she remained continent through the end of the study. The other three dogs remained continent through the end of the study (24 weeks). All five control dogs on PPA remained continent for the duration of the study. Of the four dogs where the vaccine had an effect, episodes of incontinence before treatment was initiated, during PPA treatment, and during vaccine treatment were compared. There were significantly less accidents when the dog was treated with PPA (p=0.02) or the vaccine (p=0.01) compared to before treatment initiation. There was no significant difference in treatment between PPA and the vaccine (Figure 3.1).

All dogs were seronegative for antibodies against GnRH prior to the first vaccination and all control dogs remained seronegative for the duration of the study. All nine vaccinated dogs developed a GnRH antibody titer after initial vaccination that peaked at week 6, and all but one dog still had an antibody titer at the end of the study (Figure 3.2). At week 0, plasma LH concentration was 5.06 ng/mL ± 1.99 ng/mL. Following GnRH immunization, plasma LH concentration declined to basal levels (Figure 3.3). Control dogs maintained significantly higher LH concentrations (Figure 3.3)
Table 3.1. Signalment of 16 ovariectomized dogs diagnosed with USMI

<table>
<thead>
<tr>
<th>Dog</th>
<th>Breed</th>
<th>Weight (kg)</th>
<th>Current Age (Years)</th>
<th>Age at Ovariectomy (Years)</th>
<th>Interval from Ovariectomy to Incontinence (Years)</th>
<th>Study Treatment</th>
<th>Treatment Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Greyhound</td>
<td>26</td>
<td>9</td>
<td>1</td>
<td>5</td>
<td>Vaccine</td>
<td>Continent</td>
</tr>
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<td>2</td>
<td>Golden Retriever</td>
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<td>0.5</td>
<td>2.5</td>
<td>Vaccine</td>
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<tr>
<td>3</td>
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<td>0</td>
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<td>Incontinent</td>
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<tr>
<td>4</td>
<td>Pitbull</td>
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<td>Incontinent</td>
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<tr>
<td>5</td>
<td>Pitbull Mix</td>
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<td>1</td>
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<td>0.25</td>
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<td>Incontinent</td>
</tr>
<tr>
<td>6</td>
<td>Doberman Mix</td>
<td>35</td>
<td>8</td>
<td>0.5</td>
<td>3.5</td>
<td>Vaccine</td>
<td>Incontinent</td>
</tr>
<tr>
<td>7</td>
<td>Australian Cattle Dog</td>
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<td>7</td>
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<td>0</td>
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<td>Continent</td>
</tr>
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<td>9</td>
<td>Viszla</td>
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<td>PPA</td>
<td>Continent</td>
</tr>
<tr>
<td>11</td>
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<td>8</td>
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<td>PPA</td>
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<td>0.5</td>
<td>2.5</td>
<td>PPA</td>
<td>Continent</td>
</tr>
<tr>
<td>13</td>
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<td>4</td>
<td>3</td>
<td>PPA</td>
<td>Continent</td>
</tr>
<tr>
<td>14</td>
<td>Weimaraner</td>
<td>29</td>
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<td>2</td>
<td>3</td>
<td>PPA</td>
<td>Continent</td>
</tr>
<tr>
<td>15</td>
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<tr>
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<td>0.5</td>
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<td>Vaccine</td>
<td>Rxn; Withdrawn</td>
</tr>
</tbody>
</table>

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<th>Mean</th>
<th>Age at Ovariectomy (Years)</th>
<th>Mean</th>
<th>Interval from Ovariectomy to Incontinence (Years)</th>
<th>Mean</th>
<th>Study Treatment</th>
<th>Treatment Effect</th>
</tr>
</thead>
<tbody>
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<td>0.25-4.5</td>
<td>0-6.5</td>
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</tbody>
</table>
Figure 3.1. Comparison of the number of incontinence episodes (± SD) that occurred in four out of nine vaccinated dogs before any treatment was previously initiated, during treatment with phenylpropanolamine (PPA) from week 0-6 of the study, and during treatment with the vaccine from week 7-13 of the study. There were significantly fewer accidents when treated with PPA (p=0.02) and the vaccine (p=0.01) compared to before treatment initiation. There was no difference between treatment with PPA and treatment with the GnRH vaccine (p=0.88)
Figure 3.2. Serum GnRH antibody titer (Mean ± SEM) in control (□) and vaccinated (●) dogs prior to each injection (0 and 4 weeks) and at weeks 6, 8, 10, 12, 16, 20, and 24 following initial treatment. *p<0.05 compared to controls.
Figure 3.3. Plasma LH concentrations (Mean ± SEM) in control (□) and vaccinated (●) dogs prior to each injection (0 and 4 weeks) and at weeks 6, 8, 10, 12, 16, 20, and 24 following initial treatment. There was a significant effect of the vaccine on LH concentrations (p=0.0004). *p<0.05 compared to controls.
3.5 DISCUSSION

The development of USMI after ovariectomy in female dogs is a significant problem. Treatment with PPA is not completely effective, must be administered frequently to have a therapeutic effect, and may cause adverse side effects. Therefore, there is a need to find a safe therapy with a longer duration of effect for the treatment of USMI. In this study, we demonstrated that GnRH immunization was able to maintain continence in 44% of incontinent ovariectomized dogs for 14 weeks or longer with an efficacy comparable to PPA in those dogs.

Immunization against GnRH did not maintain continence in all vaccinated dogs, which is similar to the effects of GnRH agonists on USMI. Reichler et al. (2003, 2006a) demonstrated 41-54% efficacy for the treatment of USMI by reducing LH concentrations through the use of GnRH agonists. Reducing LH concentrations does not directly improve urethral pressure, but rather increases bladder threshold volume (Reichler et al., 2006b). While it is understood that the decrease in urethral pressure as a result of ovariectomy plays a role in the development of USMI (Arnold et al., 1997), it has also been determined that ovariectomy results in changes to the bladder, specifically an increased collagen content and a reduced response of the detrusor muscle to muscaniariac stimulation in vitro (Coit et al., 2008). Therefore, it is possible that the changes in the bladder as well as the urethra may both contribute to the development of USMI. It is not known why increasing bladder threshold volume through reducing LH concentrations is enough to restore continence in some but not all ovariectomized bitches, but in these
dogs, an increase in bladder threshold volume may not be enough to compensate for decreased urethral pressure.

This study did not determine how long the GnRH vaccine had an effect on urinary incontinence because three of the four continent dogs were still continent at the end of the study. A previous study looking at the effect of the GnRH vaccine in intact male dogs found that 20 weeks after initial vaccination, antibody titers were insignificant compared to pre-vaccination and testicular volume was back to pre-vaccination measurements, indicating the vaccine had lost its effect by that point (Donovan et al., 2012). Therefore, it was expected that the GnRH vaccine would also lose its effect around week 20 in the female dogs in this study. On the contrary, all vaccinated dogs except one still had a GnRH antibody titer and all dogs still had basal LH concentrations by week 24, indicating that the effect of the GnRH vaccine may be further prolonged in females compared to males.

Side effects were experienced by nearly all vaccinated dogs in this study. While most side effects were minor and resolved without treatment after 24 hours, two dogs experienced adverse reactions that resulted in withdrawal from the study. Safety of the GnRH vaccine at 1 mL (n=237) and 2 mL (n=24) doses was demonstrated in intact male dogs with no significant reactions or adverse events observed (Pfizer Animal Health, 2004). However, mild injection site swelling occurred in 10% of the intact male dogs at the 1 mL dose and in 8.3% of the intact male dogs at the 2 mL dose. Minor side effects were also reported when the vaccine was used for pregnancy termination in bitches (Chew and Purswell, 2010).
In conclusion, GnRH immunization was effective in maintaining continence in 44% of incontinent ovariectomized dogs, and in these dogs, treatment with the vaccine was comparable to treatment with PPA. Because of low product sales, the GnRH vaccine investigated in this study is no longer commercially available. Efforts should be made to bring an available GnRH vaccine to market for the treatment of urethral sphincter mechanism incompetence.
3.6 REFERENCES


CHAPTER IV

CONCLUSION AND FUTURE STUDIES

We investigated two clinical applications of GnRH immunization in dogs. In intact males, GnRH immunization decreased testosterone concentrations and testicular volume for approximately 20 weeks. In ovariectomized incontinent females, GnRH immunization was sufficient to maintain continence in 44% of the bitches studied. Some minor and two major reactions occurred after immunization, which were reported to the vaccine manufacturer. Despite these side effects, owners were generally satisfied with this treatment option. It is important to mention that since ending the clinical investigation, there have been several requests from owners participating in the study as well as new owners and veterinarians interested in purchasing the vaccine to treat their incontinent spayed dogs. Unfortunately, this is not possible as the manufacturer has chosen not to continue GnRH vaccine production. With regards to urinary incontinence, future studies should investigate methods to prevent this sequelae of ovariectomy from developing. Specifically, it would be interesting to ovariectomize bitches following GnRH immunization to observe changes in gonadotropin concentration as well as changes in the urethral sphincter mechanism.

A problem of all GnRH vaccines investigated to date is that the effects of vaccination last for a short duration (weeks to months), which vary considerably based upon individual variations in the humoral response to immunization. Developing a GnRH
vaccine that would last for a long duration (months to years) or be permanent (irreversible) would be especially useful not only for treating urinary incontinence and other conditions resulting from ovariectomy (such as puppy coat syndrome), but also for non-surgically sterilizing dogs (which would completely prevent these post-ovariectomy complications). We have demonstrated the efficacy of such a vaccine in male dogs, albeit for short term immunosterilization.
APPENDICES
APPENDIX A: ANIMAL CARE AND USE PROPOSAL

ACUP ID 4001

PARTICIPANTS

1. Principal Investigator (PI): Michelle Kutzler, DVM
2. Department: Clinical Sciences
3. Principal Investigator’s signature and date:
4. Project title or course name and number: GnRH immunization for treatment of hormone-responsive urinary incontinence in spayed bitches
5. Start date: February 1, 2010 Completion date: January 31, 2012
6. Funding agency if applicable: Morris Animal Foundation
7. Check all items that apply:
   - Death as an end point
   - Procedures without the use of appropriate anesthetic, analgesic, or tranquilizing drugs.
   - Multiple survival surgery
   - Prolonged or unusual restraint or deprivation
   - Endangered species
8. Provide the following for the PI and all participants who will be working with the animals. Proposal will not be approved if the information is incomplete. For class activities list only the instructional staff.
   - Participant Name: Jana Gordon Date: 12/8/09
     Department: Clinical Sciences
     Job Title: Assistant Professor
     E-mail Address: jana.gordon@oregonstate.edu
     Work Telephone: 7-4812
     Education: Yes Occupational Health: Yes

   Participant Name: Michelle Kutzler Date: 12/8/09
   Department: Clinical Sciences
JUSTIFICATION FOR USE OF LIVE VERTEBRATES

1. Common name of animal(s) used: Dog

2. Number of animals needed: First year: 40 Second year: 40 (same animals) Third year: 40 (same animals)

3. What is the scientific justification for the number of animals proposed for this ACUP (e.g. number of students in the class, data sources, experimental design, etc.)?

   The numbers requested are based on the minimum numbers needed for statistical analysis purposes. There will be two groups consisting of 20 vaccinated dogs and 20 control-treated dogs receiving traditional treatment of daily Phenylpropanolamine (PPA) medication. Prior to treatment, we anticipate that LH concentrations will range between 0.5 and 31.2 ng/mL (median 7.3 ng/mL) based upon the data reported by Reichler et al (2006). In the 2006 Reichler study, following treatment with a GnRH analogue (leuprolide acetate), median LH concentrations were 0.72 ng/mL. Assuming similar values with equal variances and a standard deviation of 1.0 ng/mL in the proposed research, twenty dogs per group are needed for the study to have a power of 5% (alpha (1-tailed) set at 0.05).

I expect the vaccine to have similar results to leuprolide because the effect of treatment is the same (decreased LH concentrations), however the mechanism of action by which the LH concentrations are decreased is different. With leuprolide, the GnRH receptors are overstimulated and then down-regulated, resulting in an
eventual decrease in LH concentrations. With the GnRH vaccine, antibodies against GnRH are formed which block the ability for GnRH to bind to its receptor and thereby prevent LH secretion. Leuprolide is a human drug that is used in an extra-label manner in veterinary medicine. It is effective at treating LH-responsive incontinence but it is also very expensive (because it is a human drug), the efficacy is short-lived (must be repeated several times a year) and has some side effects associated with the initial stimulation of GnRH receptors (predominately within the cardiovascular system as described with humans (http://www.drugs.com/pro/leuprolide.html). None of these would be the expected with the GnRH vaccine being tested as it is labeled for use in dogs, is expected to last at least 6 months and probably for much longer than that (similar to other vaccines we use in dogs) and does not have any side effects.

4. Why are live vertebrates used and what non-animal alternatives were considered for this research or course?

There are no non-animal alternatives. Live animals are needed to determine if the response to vaccination alleviates the symptoms of their disease (specifically urinary incontinence).

What is the justification for use of this species?

The study must be performed in the target species (dogs) because this disorder (postovariohysterectomy hormone-responsive urinary incontinence) occurs spontaneously in this species following spaying.

6. Does this proposal duplicate a previous use of animals in teaching or research? No
If yes, why is duplication necessary (e.g. new group of students)?

7. Will animals experience discomfort, distress or pain during the proposed use, according to the following categories as defined by the Animal Welfare Act?
No discomfort, distress or pain involved (e.g. routine short term procedures involving manual or mechanical restraint; injections to administer substances, implant ID tags or collection of biological samples, such as blood, urine, stomach contents, etc.)

**ANIMAL CARE**

1. Where will the animals be housed? List building, floor, room, barn, corral, paddock, or pasture as applicable.
   
   Dogs will be housed with their owners.

2. Who will care for the animals? List person’s name and contact information e.g. work phone, pager or e-mail.
   
   Dogs will be cared for by their owners.

3. If other than LARC, who will provide veterinary care of sick, or injured animals? Provide name of individual(s) and their contact information.
   
   Dogs will receive regular veterinary care from their regular veterinarian and will receive additional care specific to their hormone-responsive urinary incontinence as it pertains to this research by Dr. Jana Gordon, small animal internal medicine specialist.

**ANIMAL USE**

1. What are the objectives and significance of the proposed research or instruction using animals? Avoid the use of acronyms and undefined abbreviations. Summarize in terms that a non-scientist would understand.

   Objective 1: To measure serum GnRH antibody titers and luteinizing hormone (LH) and follicle-stimulating hormone (FSH) concentrations in spayed bitches following treatment with Canine Gonadotropin Releasing Factor
Immunotherapeutic® (Pfizer Animal Health) vaccine or control (phenylpropanolamine (PPA))-treated. PPA is the current recommended treatment for hormone-responsive urinary incontinence in dogs.

Objective 2: To measure the efficacy of each treatment (vaccine versus control (PPA)) in restoring continence to incontinent, spayed bitches.

Phenylpropanolamine (PPA) is a non-hormonal medication commonly used in male and female dogs to treat hormone-responsive urinary incontinence. In 1999, the federal Food and Drug Administration banned PPA for human use, which significantly limited the veterinary sources for this drug. Currently, PPA is only available through veterinary compounding pharmacies. Dogs with hormone-responsive incontinence need to remain on either PPA for the rest of their lives.

The significance of the proposed study is to prove the effectiveness of a commercially available vaccine labeled for use in intact male dogs with benign prostatic hyperplasia in the treatment of the previously mentioned conditions in neutered dogs. These conditions impact the overall health and well being of companion animals, and also the owners of such pets. In addition to the aforementioned benefits on animal health and longevity, an additional future application of this vaccine in canine population control.

The Canine Gonadotropin Releasing Factor Immunotherapeutic vaccine is developed for use and has been in male client dogs. There is no risk to using this vaccine in client-owned female spayed dogs that would not be observed in the male dogs it has already been used in. The justification for using it in client-owned dogs is that this disease (urinary incontinence) is spontaneously occurring in the client-owned dog population. Research dogs are not available that have this problem. This disease is naturally-occurring and warrants treatment with a product that is already being used in client-owned male dogs. Although there has not been any published research using this vaccine in female dogs yet, a scientific
abstract by Chew & Purswell reporting the safety and efficacy of this vaccine in reducing serum progesterone concentrations in intact female dogs will be presented at the 2010 Society for Theriogenology meeting.

2. Where will the animal procedures be conducted? List all sites including building, room or any other location.

Animal procedures will be conducted at the Small Animal Veterinary Teaching Hospital at Oregon State University under the supervision of Dr. Jana Gordon.

3. Describe in detail all routine procedures involving the animal(s). Routine procedures are those that cause minimal discomfort or pain to the animal. Examples include: restraint techniques, tagging, dosing (list agents, dosages and routes of administration).

   Venous blood (3-5 mL) will be drawn from all participant animals to determined baseline serum GnRH antibody titers along with LH and FSH concentrations. Blood samples will be collected prior to subcutaneous administration of Canine Gonadotropin Releasing Factor® vaccine (1 mL). Dogs in the control treated group will receive phenylpropanolamine (PPA) at a dose of 1 mg/kg of body weight orally twice daily. Only dogs in the vaccine group will receive a vaccine treatment. Four weeks post initial administration of the Canine Gonadotropin Releasing Factor vaccine, a second dose will be administered to the dogs in the vaccine group. Both doses will be administered aseptically subcutaneously, as per the manufacturer’s directions. Venous blood (3-5 mL) will be drawn from all participant animals for the determination of serum GnRH antibodies along with LH and FSH concentrations every 8 weeks for the duration of the study (two years).

Twenty privately-owned, spayed bitches exhibiting symptoms of hormone-responsive urinary incontinence will be vaccinated as described above. Twenty privately-owned, spayed bitches with the same diagnosed condition will serve as
controls as described above. In addition to the measurement and comparison of GnRH antibody titers and gonadotropin concentrations (as described in the previous paragraph), the owners of participating animals will be heavily relied upon to determine the frequency of incontinent episodes. In order to reduce contributing factors in the occurrence of incidences of incontinence, a survey will be completed by bitch owners prior to the start of the study and on all follow-up visits. The survey will include such information as the animal’s feed and water intake, daily activities, changes in environment, and average number of incontinent episodes along with situations surrounding such episodes.

The risks and the potential consequences for the dogs participating in this study will be explained to the owners through the owner consent form. Understanding these risks and the potential consequences will be implied with the owner’s signature on the owner consent form. Owners are encouraged to contact Dr. Gordon or Dr. Kutzler if they have any questions or concerns regarding participation in this research. Dr. Kutzler will confirm that all dogs enrolled in the study have a signed owner consent form. Dr. Gordon will also confirm that owners complete the owner survey forms at each monthly office visit with their regular veterinarian during their participation in this study.

4. Describe method of euthanasia and carcass disposal.

   Not applicable

5. Check any of the following that will involve the animal. Complete and submit all applicable supplements.

   Discomfort, Distress, or Pain (non-surgical). (Complete Discomfort, Distress, Pain Supplement)

   Antibody production (Complete Antibody Production Supplement)
Breeding colony or sentinel animals (Complete Breeding Colony & Sentinel Animal supplement)

Death of the animals as an indicator or data point in the research or instruction (Complete the Death as an End Point Supplement)

Field studies (Complete the Field Studies Supplement)

Surgery (Complete the Surgery Supplement)

6. The appropriate compliance committee will be notified if any of the following agents are used:
   - Biohazards administered to the animal
   - Radioactive isotopes administered to the animal
   - Infectious agents administered to the animal
   - Carcinogens administered to the animal
   - Recombinant DNA products administered to the animal

**DISCOMFORT, DISTRESS, AND PAIN SUPPLEMENT (DDP)**

**(FILL OUT ONLY IF APPLICABLE)**

1. Describe in detail all procedures that may cause discomfort, distress or pain.

We do not anticipate that any of the procedures described will cause discomfort, distress or pain. This vaccine is licensed for use in dogs but not labeled for treatment of these conditions. Safety of this product has been demonstrated when administered according to the label directions. According to the vaccine package insert, when administered as a 2X overdose (2 mL), no significant systemic reactions or adverse events were observed over a 14-day post-treatment observations period. Preliminary safety trials conducted by the vaccine manufacturer involving 237 treatments resulted in approximately 10% mild injection site swellings that were only apparent by palpation, and resolved without treatment. No pain was reported upon injection in a controlled repeated overdose study, in which 24 adult dogs received a 2X (2 mL) followed by a second 2X
overdose 4 weeks later. A total of 4 injection site reactions out of 48 administrations were observed during the 21-day observation period, and resolved without treatment. These mild injection site swellings were only apparent by palpation. As with any vaccine, owners should be aware that more serious, anaphylactic reactions could occur.

2. Describe all signs of discomfort, distress or pain that will be monitored, and frequency of monitoring.

Owners will be instructed to monitor the dogs for changes following vaccination. We anticipate that the changes noticed by the owners will be associated with the beneficial effects of vaccination; namely reduced urinary incontinence. Owners will complete a survey at the time of each veterinary visit regarding any symptoms noticed following vaccination (injection site reactions). If signs of discomfort occur, owners are asked to contact their regular veterinarian and to notify Dr. Gordon.

3. List all medications (anesthetics, analgesics or tranquilizers) including, dosages, route and frequency of administration.

No medications will be given other than the vaccine in the vaccine-treated group unless there is a vaccine reaction. If anaphylaxis develops, epinephrine at a dose of 0.1 mL/10 pounds of body weight will be administered once intramuscularly or intravenously. Additional veterinary care for the treatment of a vaccine reaction will be determined by Dr. Gordon. For the control group, PPA (1 mg/kg) will be orally administered by the owners twice daily for the customary treatment of hormone-responsive urinary incontinence.

4. What refinement(s) to procedures were considered to minimize discomfort, distress or pain to the animals?
Patients will be followed on a every other month basis to minimize discomfort or distress involved with more frequent veterinary office visits. Every other month visits is the minimum number of observations (blood sample collections and owner survey results) necessary to demonstrate efficacy of this immunologic treatment for post-ovariohysterectomy hormone-responsive urinary incontinence.

5. What information source(s) or literature searches were used to locate alternatives to procedures involving pain or distress to the animal? The OSU-IACUC requires documentation of methods and sources to find acceptable alternatives including: a list of data bases searched, dates of search and key words used.

On December 7, 2009, a literature search of PUBMED was conducted. Using the keywords "dog, GnRH, vaccine or vaccination", there were a total of 12 hits and of these only 9 actually pertained to dogs. The most recent article describes the widespread application of a combined GnRH and rabies vaccine for both population control and rabies prevention in dogs (Wu X et al 2009). Two of the nine are review articles (Kutzler MA et al 2006 and Naz RK et al 2005). The remaining six articles all refer to original research in male dogs (not female dogs) and half of these (3 of 6 articles) were written by the research and development team that created the GnRH vaccine to be tested (Walker J et al 2007; Zeng W et al 2007 and Yu M et al 2005).

References:


APPENDIX B: ANIMAL CARE AND USE PROPOSAL AMENDMENTS

Animal Care and Use Amendment Form
Oregon State University Institutional Animal Care and Use Committee

Use this form to make a change to an Animal Care and Use Proposal. Return completed form to IACUC@oregonstate.edu or send to IACUC, Office of Research Integrity, 308 Kerr Administration, Corvallis, OR 97331

Principal Investigator: Michelle Kutzler Telephone: 7-1401
E-mail: michelle.kutzler@oregonstate.edu

Department/College: Animal Sciences Granting Agency: Collie Health Foundation

ACUP Number: 4001
Project Title: GnRH immunization for treatment of hormone responsive urinary incontinence in spayed bitches

Which type(s) of changes in protocol are being proposed? Check all that apply.
Addition/deletion of procedure

(Describe)

We would like to change the frequency of blood samples collection from weeks 0, 4, 8, 16, 24, 32, 40, 48, 56 to the weeks 0, 4, 6, 8, 10, 12, 16, 20, 24. The rationale for this change is based upon our preliminary results that show the highest titer at week 8 and no titer at week 24. The change in frequency will allow us to more accurately determine when the peak titer occurs. There is no increase in the number of blood samples collection as this remains a total of 9 blood samples. In addition, the change in protocol proposed reduces the study duration for each participant.

Describe all proposed change(s) in detail. If change in personnel, identify personnel no longer involved and new personnel. New personnel must satisfy educational and occupational health requirements before approval will be granted. If change(s) proposed add any procedures that have the potential to cause animal pain or distress, then you must describe the methods and sources by which alternatives to these procedures have been sought. Please review the original protocol and confirm that all principal investigator assurances apply to proposed change(s). (Attach additional sheets if necessary.)
Justify the need for these proposed changes.

We would like to change the frequency of blood samples collection from weeks 0, 4, 8, 16, 24, 32, 40, 48, 56 to the weeks 0, 4, 6, 8, 10, 12, 16, 20, 24. The rationale for this change is based upon our preliminary results that show the highest titer at week 8 and no titer at week 24. The change in frequency will allow us to more accurately determine when the peak titer occurs. There is no increase in the number of blood samples collection as this remains a total of 9 blood samples. In addition, the change in protocol proposed reduces the study duration for each participant. IACUC Amendment Form July 21, 2003

IACUC Action: VR Approved OSU Attending Veterinarian 6/11/12

Animal Care and Use Amendment Form
Oregon State University Institutional Animal Care and Use Committee

Change in Personnel

Use this form to make a change to an Animal Care and Use Proposal. Return completed form to IACUC@oregonstate.edu or send to IACUC, Office of Sponsored Programs and Research Compliance, 312 Kerr Administration, Corvallis, OR 97331

Principal Investigator: Michelle Kutzler Telephone: 7-1401
E-mail michelle.kutzler@oregonstate.edu

Department/College: Animal Sciences Granting Agency: Collie Health Fdtn

ACUP Number(s): 4001

Project Title: GnRH immunization for the treatment of hormone-responsive urinary incontinence in spayed bitches

Copy and paste the box for all new staff that will be added. Complete as noted in the instructions:
<table>
<thead>
<tr>
<th><strong>Name</strong></th>
<th>Caitlin Donovan</th>
<th><strong>Department</strong></th>
<th>Animal Sciences</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Work Address</strong></td>
<td>315 Withycombe Hall</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Work email</strong></td>
<td><a href="mailto:donovaca@onid.orst.edu">donovaca@onid.orst.edu</a></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Work phone number</strong></td>
<td>978-994-2754 (cell)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Role (PI, laboratory staff, post-doc, student, etc.)</strong></td>
<td>Master's student</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Animal Welfare Education Completed (Y/N)</strong></td>
<td>Y</td>
<td><strong>OHS Enrollment Completed (Y/N)</strong></td>
<td>Y</td>
</tr>
<tr>
<td><strong>Experience and Qualifications</strong></td>
<td>For this project, she will be assisting Dr. Gordon by restraining dogs for sample collection. She has completed her training in the human use of animals in research and the animal contact and occupational health questionnaire. She received a BS in Animal Science from the University of Massachusetts (Amherst). For her undergraduate Honor's thesis, she was involved with research on alpacas which she performed reproductive ultrasound exams, collected blood samples, and general herd health maintenance. She was also involved with FAMACHA research with sheep and goats. She is a multi-decade dog, cat, and horse owner, with experience in pet husbandry and basic veterinary care. She has worked as a Teaching Assistant both at UMass and at Oregon State University in Camelid Studies and Introduction to Animal Science, respectively.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

IACUC Approval 6/11/12
APPENDIX C: OWNER CONSENT FORM

INFORMED CONSENT FORM FOR ANIMAL OWNERS

PROJECT TITLE:
Efficacy of GnRH vaccination for the treatment of urinary incontinence in spayed female dogs

INVESTIGATORS:
Dr. Jana Gordon, DVM, DACVIM; Dr. Michelle Anne Kutzler, DVM, PhD, DACT

PURPOSE OF STUDY:
The purpose of this study is to investigate the effectiveness of a commercially available canine GnRH vaccine in suppressing the production of LH in spayed female dogs. We hypothesize that this vaccine will induce serum neutralizing antibody titers against GnRH and that a decrease in LH concentrations will correlate with a decrease in symptoms associated with urinary incontinence. To test these hypotheses, spayed female dogs will be vaccinated twice or treated with phenylpropanolamine (PPA; traditional treatment as prescribed by the animal’s veterinarian) and serum GnRH antibody titers as well as LH concentrations will be measured nine times over 6 months.

PURPOSE OF FORM:
You are being asked to give your consent to have your dog participate in a research study. This consent form gives you the information you will need to help you decide whether to allow your animal to participate. Please read the form carefully. You may ask any questions about the research, the possible risks and benefits, rights as a volunteer participant, and anything else that is not clear. When all of your questions have been answered, you can decide if you will allow your animal to be in this study or not.

WHAT WILL HAPPEN IN THE STUDY:
The GnRH vaccine used for this research is the Canine Gonadotropin Releasing Factor Immunotherapeutic® (Pfizer Animal Health). Ten spayed female dogs will receive two subcutaneous injections of this GnRH vaccine. Another ten spayed female dogs will be treated with PPA (as prescribed by the animal’s veterinarian). Venous blood samples (6-12 mL) will be collected at the Oregon State University Small Animal Hospital prior to each vaccination and then at 6, 8, 10, 12, 16, 20, and 24 weeks from the initial visit.

RISKS OF THE STUDY:
This vaccine is licensed for use in dogs but not labeled for treatment of these conditions. Safety of this product has been demonstrated when administered according to the label directions. According to the vaccine package insert, when administered as a 2X overdose (2 mL), no significant systemic reactions or adverse events were observed over a 14-day post-treatment observations period. Preliminary safety trials conducted by the vaccine
manufacturer involving 237 treatments resulted in approximately 10% mild injection site swellings that were only apparent by palpation, and resolved without treatment. No pain was reported upon injection in a controlled repeated overdose study, in which 24 adult dogs received a 2X (2 mL) followed by a second 2X overdose 4 weeks later. A total of 4 injection site reactions out of 48 administrations were observed during the 21-day observation period, and resolved without treatment. These mild injection site swellings were only apparent by palpation. Although there has not been any published research using this vaccine in female dogs yet, a scientific abstract by Chew & Purswell reporting the safety and efficacy of this vaccine in reducing serum progesterone concentrations in intact female dogs was presented at the 2010 Society for Theriogenology meeting. As with any vaccine, owners should be aware that more serious, anaphylactic reactions could occur. You should contact your regular veterinarian and the Oregon State Small Animal Hospital if any discomfort is observed following vaccination.

**BENEFITS OF THE STUDY:**
The significance of the proposed research will be to determine if this treatment will provide a long-term, inexpensive, simple and effective method for the treatment of hormone-responsive urinary incontinence that results from spaying female dogs. However, GnRH vaccination may not be effective for the treatment of urinary incontinence.

**IS THERE COMPENSATION FOR PARTICIPATING:**
No. There is no compensation or therapy for injuries included in participation of this study.

**ARE THERE ANY COSTS FOR PARTICIPATING:**
There will be no direct financial costs to the owner for participating. However, owners are required to bring their dog to 9 veterinary visits over 6 months, which will be paid for by the investigators.

**ALTERNATIVE TREATMENT:**
You should discuss alternative treatment options for your pet’s optimum health and well being with your regular veterinarian as this will differ on a case-by-case basis.

**EXTENT OF CONFIDENTIALITY OF RECORDS:**
Veterinary-Client records will remain confidential. However, results from this study will be reported in a qualitative and quantitative manner with the animal’s and owner’s names withheld.
WITHDRAWING MY ANIMAL FROM THE STUDY:
Your participation in this study is entirely voluntary and you may withdraw your animal at any time. You also understand that your animal may be withdrawn from the study if the investigators find it necessary. If your animal is withdrawn from the study for any reason, data already collected may continue to be used for research purposes. You will not be treated differently if you decide to not participate in this study. Your decision to participate, not participate, or withdraw from the study will not affect your relationship with OSU.

WHAT IF I HAVE QUESTIONS?
If you have any questions regarding this study, you may contact Dr. Jana Gordon (541-737-4812) or Dr. Michelle Kutzler (541-737-1401) or your regular veterinarian. I have read and understand the foregoing statements and agree to allow my animal to participate in this study. My questions about the experiments have been answered to my satisfaction. Your signature indicates that this research study has been explained to you, that your questions have been answered, that you understand the risks, potential consequences and outcomes, and that you agree to allow your animal to be in the study. You will receive a copy of this form.

Animal's Name:__________________________________

Owner’s Printed Name:___________________Signature:_____________________
Date:_____

Investigator's Printed Name: ________________Signature:_____________________
Date:______
APPENDIX D: Presented at the Willamette Valley Veterinary Medical Association Meeting, December 2011 (Corvallis, OR)
An Alternate Treatment

Hypothalamus

GnRH
GnRH-r
LH
FSH
GnRH-ant

Preliminary Studies

- 3 incontinent spayed female dogs were vaccinated
  - No significant systemic reactions or adverse effects were observed
  - Incontinence resolved completely for up to 6 months
- Based on the success of the preliminary studies, we are looking to further expand this study

Study Objective & Hypothesis

- Objective: To establish a more effective, safer & convenient treatment for urinary incontinence
- Hypothesis: Based on our preliminary results, we expect that vaccinated dogs will be continent for up to 6 months without PRA

We Need Your Help!

- We need patients willing to participate in this study!
- Qualifying patients must:
  - Be spayed females diagnosed with urinary incontinence
  - Be currently treated with Proin® (Pfizer Pharmaceutical, Fortwo FL) chewable tablets to restore continence
  - Have been treated with Proin® for at least six months

What Is Needed From Participants

- Patients will receive a physical exam & have a blood sample collected nine times over a period of 6 months
  - Must be conducted at the OSU Small Animal Teaching Hospital due to IACUC restrictions
  - GnRH antibody and LH will be measured from samples collected
  - Owners will need to complete a survey at the beginning of the study & keep a simple daily log
  - There is no cost to the owner for participation
  - Owners will receive a 6-month supply of Proin® for participating

We Need Your Help!

- We would really appreciate spreading the word about the study to recruit patients that qualify
- We have handouts & more information available for owners looking to participate in the study
- My contact information:
  - donovaca@onid.orst.edu
  - 541-737-1920
Appendix E: Presented at the Chintimini Kennel Club Meeting, November 2012
(Corvallis, OR)
What We Can Deduce About Incontinence

- Ovary removal and the consequent hormonal changes may contribute to the development of incontinence

Treatment Options for the Incontinent Dog

Diethylstilbestrol (DES)

- Synthetic estrogen that restores the hormonal feedback loop
- Administered daily when dosing regimen begins; then reduced to once or twice a week
- Oral administration

Diethylstilbesterol (DES)

- Effective ~60-65% of cases
- Side effects include vulvar swelling and attractiveness to male dogs
- Can be suppressive to bone marrow at higher doses
**Estriol (Incurin®)**
- Now FDA-approved estrogen treatment
- Administered once to twice a day
- Oral administration
- Estriol is shorter-acting than DES
  - Benefits: shorter retention time limits undesirable side effects, more uniform dosing amongst dogs
  - Detriments: dosing must be frequent, side effects still prevalent in 5-9% of treated dogs

**Phenylpropanolamine (Proin®)**
- Binds to alpha-1 receptors on the urethral smooth musculature to induce contraction
- Effective in 75-85% of cases
- Doses administered once to twice daily
- Most common treatment used today for urinary incontinence

**Phenylpropanolamine (Proin®)**
- Side effects can occur
  - Because alpha-1 receptors are located in all smooth muscle throughout the body, effects are not localized to the urinary tract
  - Side effects include high blood pressure, restlessness, anxiety, irritability, weight loss, increased heart rate

**Our Study: GnRH Immunization for the Treatment of Urinary Incontinence**

**Study Goal**
- Develop a:
  - Safe
  - Convenient
  - Effective treatment for urinary incontinence
- Objective: Restore continence by immunologically lowering LH and FSH concentrations

**The Treatment**
- GnRH vaccine marketed for the treatment of benign prostatic hyperplasia in intact male dogs
Study Design

- Healthy bitches diagnosed with incontinence post-spay and are currently well-controlled using Proin® have been recruited for the study.
- Dogs attend 9 visits over the period of 6 months at the Oregon State Small Animal Hospital.

Study Design

- Recruited dogs are separated into two groups:
  - Group 1 will be immunized with the GnRH vaccine twice at four-week intervals.
    - These dogs discontinue Proin® use two weeks after their second vaccination.
  - Group 2 will be immunized with a placebo.
    - These dogs remain on Proin® as usual for the duration of the study.

Study Design

- Week 0
  - Dogs receive a physical exam, urinalysis, urine culture, complete blood count, and biochemistry profile to evaluate overall health.
  - Baseline blood sample is taken for hormone analysis.
  - Owners fill out a survey and are given a daily log to keep for the duration of the study.
  - GnRH vaccine or placebo is administered.

Study Design

- Week 4
  - Blood sample is collected.
  - GnRH vaccine or placebo administered.
- Week 6
  - Blood sample is collected.
  - If immunized with the GnRH vaccine, Proin® administration is discontinued after this visit.
  - Weeks 8, 10, 12, 16, 20
    - Blood samples are collected.
Study Design

- Week 24
  - Dogs receive a physical exam, urinalysis, urine culture, complete blood count, and biochemistry profile to confirm they have remained in good health
  - Additional blood sample is taken for hormone analysis
  - Owners return daily log

What do We Need all This Blood For?!

- Blood samples taken at all nine visits are analyzed for:
  - The development of GnRH antibodies
  - LH concentrations
- We expect that vaccinated dogs will develop a GnRH antibody titer and have reduced LH concentrations compared to their baseline sample
- We expect that control dogs will not develop a GnRH antibody titer and will have consistently high LH concentrations

The Daily Log

Date: __________________________

How many accidents did your dog have today? __________________________

If she had accidents, what was she doing at the time? (Humping around? Peeing in the field?)

The amount of urine expelled was: (check one):
- Hardly
- A little
- A few drops

Have you noticed any changes in energy?
- [ ] Less energetic
- [ ] More energetic
- [ ] Energy level hasn't changed

Preliminary Results

- Dog 1 became incontinent one week after Proin® was discontinued
- Dog 2 remained continent for 13 weeks after Proin® was discontinued
- Dog 3 remained continent for 14 weeks after Proin® was discontinued
  - Was revaccinated at Week 20 and has remained continent
- Dog 4 remained continent for 14 weeks after Proin® was discontinued

We STILL Need Incontinent Dogs!!!

- We are still looking for dogs to be a part of the control group!
- Again, dogs must be:
  - Diagnosed incontinent AFTER spay
  - Currently well-controlled with Proin®
  - Free of any health issues that could affect continence
- If you have a dog or know of a dog that can participate, come chat with me!
- Benefits to enrolling:
  - 6 months of FREE Proin® upon study completion
  - Free bloodwork evaluation and urine analysis
  - You’re helping me graduate!
Acknowledgements
Collie Health Foundation
Dr. Michelle Kutzler, Department of Animal and Rangeland Sciences
Dr. Jana Gordon, College of Veterinary Medicine

Questions?
APPENDIX F: URINARY INCONTINENCE STUDY ADVERTISEMENTS

Does your dog suffer from urinary incontinence?
If so, we need you!

The Oregon State University Animal and Rangeland Sciences department is currently recruiting SPAYED, FEMALE dogs of any age or breed to participate in a study looking at an alternative treatment for urinary incontinence. All dogs MUST be:

- Currently CONTROLLED using Proin (phenylpropanolamine hydrochloride) oral tablets for at least 6 months
- Be free of any health problems that may affect continence
- Be diagnosed incontinent AFTER spaying by a licensed veterinarian

All dogs enrolled in the study will need to come to the Oregon State Small Animal Teaching Hospital for a total of 9 visits over a period of 6 months. On visits 1 and 9, dogs will receive a physical exam and have blood and urine collected for urinalysis, urine culture, complete blood count, biochemistry profile, and hormone analysis. On visits 1 and 2, the dog will receive a vaccine or placebo and vaccinated dogs will discontinue Proin use on the third visit. On visits 3-8, only a blood sample will be taken. Throughout the study period of 6 months, owners will be given a simple log to fill out daily regarding episodes of incontinence.

All veterinary work performed is free to the owner and 6 months of Proin will be given to all participants upon study completion.

For more information or to enroll your dog, take a tab and contact us! Email is the best form of communication.

donovaca@onid.orst.edu
978-994-2754
We Need Your Help!

If you have a spayed female dog that has been diagnosed with urinary incontinence and has been taking Proin® chewable tablets to restore continence for at least 6 months, we would love for you to participate in our study.

All dogs will receive a physical exam and have a blood sample collected a total of 9 times over a period of 6 months. This must be performed at the Oregon State University Small Animal Hospital (OSUSAH) but will be at no cost to you. In addition, you will be reimbursed with Proin® at the end of the study.

Owners will need to bring their dog to the OSUSAH for all 9 visits as well as keep a daily log of changes in continence during the study.

Questions? Want to Participate?

Contact us by email:

donovaca@onid.orst.edu

Or by phone:

541-737-1920

Thank you for considering our study, and we look forward to hearing from you!

Michelle Kutzler, DVM PhD DACT
Jana Gordon, DVM DACVIM
Caitlin Donovan, MS Candidate

Department of Animal Sciences
Oregon State University
315 Wycliffe Hall
Corvallis, OR 97330
Phone: 541-737-1920
The Problem

Urinary incontinence, or involuntary leakage of urine, is a disorder that affects up to 20% of spayed female dogs. Incontinence is a serious problem that can result in relinquishment or euthanasia due to hygienic implications. Therefore, an effective treatment is necessary to ensure stable futures for dogs that experience incontinence.

A common treatment for urinary incontinence is oral administration of phenylpropanolamine (PPA), usually given at least once a day. PPA is a stimulant designed to increase the function of vesical sphincter muscles. However, PPA is only effective in about 70% of dogs with incontinence. Side effects associated with PPA treatment include restlessness, irritability, and excitability.

Because urinary incontinence is a significant problem and the current treatment is not always effective, researchers at Oregon State University have been funded by the Collie Health Foundation to investigate an alternate treatment that has already shown promising results.

Why Does Incontinence Happen?

Gonadotropin releasing hormone (GnRH), a hormone released from the hypothalamus in the brain, is responsible for the regulation of reproductive function. The production of GnRH signals the pituitary gland in the brain to release luteinizing hormone (LH) and follicle stimulating hormone (FSH). FSH stimulates ovarian follicle growth and LH signals the follicles to produce estrogen. When a certain amount of estrogen is produced, this triggers GnRH release to stop, resulting in a negative feedback loop that keeps these hormones regulated.

When a dog has her ovaries removed, this feedback loop is disturbed. There are no ovaries to produce estrogen, so therefore there is no estrogen to stop the production of GnRH. As a result, GnRH, LH, and FSH are constantly being produced at high levels in spayed female dogs.

Previous studies have shown that constant high levels of LH results in a decreased bladder threshold volume. It has been hypothesized that urinary incontinence in spayed female dogs results from not being able to hold as much urine in the bladder due to the large amount of LH in circulation. The solution, therefore, would be to decrease production of LH.

The Solution

Canine Gonadostrophin Releasing Factor Immuno-therapeutic®, a product of Pfizer Animal Health, is a vaccine that suppresses LH production by inducing GnRH antibody production. GnRH antibodies prevent GnRH from signaling the pituitary to release LH. This vaccine has been demonstrated to be safe by the manufacturer and is labeled for the treatment of benign prostatic hyperplasia in intact male dogs.

In preliminary studies, three GnRH vaccinated spayed females have had incontinence resolved completely for up to 6 months. Based upon this success, we are looking to expand this study further with the goal of establishing a more effective, safer, and easier method for treating urinary incontinence.

Dogs will receive either a placebo or vaccine injection given subcutaneously twice at 4 week intervals. The placebo treated dogs will continue using PPA to control urinary incontinence whereas the vaccinated dogs will be weaned off of PPA 2 weeks after the second injection. Blood samples will be collected a total of 9 times over a 6 month period.
APPENDIX G: OWNER SURVEY

PET ID: _________________________________________________________________

Owner Information

Name:__________________________________________________________________

Email:________________________________________________________________

Address:________________________________________________________________

Phone #: ______________________________________________________________

Regular Veterinarian: ___________________________________________________

Q1. Pet Name ____________________________________________________________

Q2. Breed: _______________ _________________________________

Q3. Age: _________________

Q4. At what age was your pet spayed: *(Circle one number)*

0  Less than 3 months  2  6 months to 1 year  4  2 to 4 years  6  Over 6 years

1  3 to 6 months  3  1 year to 2 years  5  4 to 6 years  7  Don’t know

Q5. Is your pet an indoor or outdoor animal? *(Circle one number)*

1  Indoor  2  Outdoor  3  Both
Q6. How many other pets are present in your household? *(circle one number)*

0   NONE   2   TWO   4   FOUR   6   SIX
1   ONE    3   THREE  5   FIVE  7   SEVEN OR MORE

Q7. Are there health issues with the other pets present in your household? *(Circle a number)*

1   Yes    2   No

Q8. If yes to Q7, please specify:
__________________________________________________________

Q9. Please describe the overall health of your pet:
____________________________________________________________

Q10. At what age did symptoms of urinary incontinence develop?
______________________________

Q11. If symptoms of urinary incontinence were present at time of pet acquisition, at what age did you acquire your pet?
______________________________
Q11. Before you began treatment, how often did your pet experience episodes of incontinence or inappropriate urination? *(Circle one number)*

1. Several times per day
2. At least once a day
3. At least once every other day
4. At least once a week

Q12. Do your pet’s episodes of incontinence generally occur while your pet is awake or while sleeping? *(Circle one number)*

1. While awake
2. While sleeping
3. While awake and while asleep
4. Does not have a general time of occurrence
APPENDIX H: OWNER DAILY LOG

Date: ____________________________

How many accidents did your dog have today? ______________
If she had accidents, what was she doing at the time? (Jumping around? Sleeping? Other?)

The amount of urine expelled was (circle one):
A lot   A little   A few drops

Have you noticed any changes in energy?

☐ Less energetic

☐ More energetic

☐ Energy level has not changed
APPENDIX I: PARTICIPATING DOG VACCINE REACTIONS

Thirteen total dogs were vaccinated and twelve experienced side effects as a result of vaccination. Most side effects were minor and included lethargy, tenderness at the injection site, and decreased appetite that lasted approximately 24 hours. Other more adverse reactions included edema at the injection site, tachypnea, and difficulty moving. Vaccine reactions experienced by each dog are summarized in Table I.1. Towards the end of the study, diphenhydramine was intramuscularly administered concurrently with the vaccine at a dose of 1 mg/kg to reduce side effects.
Table I.1. Summary of vaccine reactions experienced by participating dogs. * denotes the administration of diphenhydramine concurrently with the vaccine

<table>
<thead>
<tr>
<th>DOG</th>
<th>FIRST INJECTION</th>
<th>BOOSTER INJECTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lilly Marcum</td>
<td>Lethargic 72 hours</td>
<td>Lethargic 24 hours</td>
</tr>
<tr>
<td>Kassie Reyes</td>
<td>Lethargic, soreness at injection site 24 hours</td>
<td>Lethargic 24 hours</td>
</tr>
<tr>
<td>Bonnie Corse</td>
<td>Lethargic, soreness at injection site 24 hours</td>
<td>Lethargic, soreness at injection site 24 hours</td>
</tr>
<tr>
<td>Ruby Hasenstein</td>
<td>No change</td>
<td>Lethargic 24 hours</td>
</tr>
<tr>
<td>Tess Pearce</td>
<td>No change</td>
<td>No change</td>
</tr>
<tr>
<td>Koda Langston</td>
<td>Tachypnea and distress 24 hours</td>
<td>Removed from study</td>
</tr>
<tr>
<td>Kaia Owen</td>
<td>No change</td>
<td>Edema and soreness at injection site 36 hours</td>
</tr>
<tr>
<td>Branson Blount</td>
<td>No change</td>
<td>Lethargic and soreness at injection site 24 hours</td>
</tr>
<tr>
<td>Azaria Acosta</td>
<td>No change</td>
<td>Edema and soreness at injection site 24 hours</td>
</tr>
<tr>
<td>Penny Moquin</td>
<td>Lethargy 24 hours</td>
<td>*Lethargy and soreness at injection site 24 hours</td>
</tr>
<tr>
<td>Lily Gassner</td>
<td>Lethargy and decreased appetite 24 hours</td>
<td>*Lethargy 24 hours</td>
</tr>
<tr>
<td>Mia Wanser</td>
<td>Lethargy, stiff, painful movement and soreness at injection site for 144 hours</td>
<td>Removed from study</td>
</tr>
<tr>
<td>Lena Mills</td>
<td>Lethargy and soreness at injection site 24 hours</td>
<td>*Lethargy 24 hours, alopecia at injection site</td>
</tr>
</tbody>
</table>
APPENDIX J: GNRH ANTIBODY ELISA PROTOCOL - CANINE

The GnRH antibody ELISA protocol was developed using the technique of Elhay and coworkers (2007) with some modifications. This is an indirect ELISA that uses horseradish peroxidase (HRP;101223, Life Technologies Corporation, Carlsbad CA) as the enzyme-labeled detection antibody and the reagent ABTS (50-66-00, KPL Inc., Gaithersburg MD) as the colorimetric substrate that visualizes HRP. ABTS is a less sensitive substrate than others available and is therefore the ideal choice for this already-sensitive ELISA; a more sensitive substrate choice would cause false positive results. A schematic summarizing this ELISA protocol can be seen in Figure J.1. To run three plates with five samples per plate, the time commitment on the first day of the protocol is approximately half an hour and the time commitment on the second day of the protocol is approximately five hours.

Human LH-RH, 1MG (71447-49-9, Sigma, St. Louis, MO) is utilized as the antigen in this ELISA; it is delivered in a powdered form and reconstituted in a 1 mg/mL solution. To reconstitute, add 1000 μL fresh distilled water total to the powder in increments no greater than 500 μL to prevent overflow. Briefly, add 500 μL water to the LH-RH vial, cap the vial and invert to mix, and pipette the solution into an empty centrifuge tube. Add another 500 μL water back to the vial, cap the vial and invert to mix, and pipette the resulting solution into the same centrifuge tube. This ensures all LH-RH sediments are reconstituted. Finally, aliquot LH-RH was into microcentrifuge tubes. To maintain coating of 5 μg/mL per well, as needed for the canine ELISA protocol, 50 μL LH-RH is needed per
96-well plate. An experienced assay operator can reliably run three plates at once and therefore, 160 μL of LH-RH (extra to allot for pipetting error) is the standard aliquot. Reconstituted LH-RH remains stable for approximately one month at -20°C.

Coating the plates is performed on Day 1 of the protocol. While reconstituted LH-RH is thawing on ice, make sodium bicarbonate buffer, which is composed of sodium bicarbonate and distilled water, to dilute the LH-RH. To maintain coating of 5 μg/mL per well, 10 mL of sodium bicarbonate buffer is needed per plate. Measure the appropriate amount of sodium bicarbonate (Table J.1) into a beaker with a stir bar containing half the amount of desired water (Table J.1) and mix using a stirring plate. Once thoroughly mixed, read the pH of the buffer using a pH meter. The buffer must be at or close to 8.0, so HCl or NaOH are added to adjust pH.

If the pH meter has not been used recently, it should be calibrated using pre-made samples. When using the meter, the crystal must always remain wet, stored in a water-filled cap, and rinsed between substances with distilled water. Once the buffer is adjusted to 8.0, pour the contents of the beaker into a graduated cylinder and add distilled water to measure to the exact amount of water needed.

Measure the exact amount of sodium bicarbonate buffer needed and pour into a reservoir. For example, if three plates are being run, 30 mL of buffer is needed minus 150 μL LH-RH, so 29,850 μL buffer should be measured into the reservoir. Vortex 150 μL thawed LH-RH and then pipette into the reservoir. Mix the solution by pipetting the buffer up and down a few times with a multi-channel pipettor, then pipette 100 μL of the LH-RH
in the sodium bicarbonate buffer into each well. Finally, place a plastic cover over the plate, pressed around the edges for a strong seal, and refrigerate overnight.

On Day 2 of the protocol, perform the ELISA. To begin, make two buffers, the bovine serum albumin (BSA) dilution buffer and the phosphate buffered saline with tween (TPBS) wash buffer. For the BSA buffer, measure the appropriate amount of NaCl, NaH$_2$PO$_4$, and Na$_2$HPO$_4$ (Table J.2) into a beaker with a stir bar containing half the amount of desired water (Table J.2) and mix using a stirring plate. Once thoroughly mixed, read the pH of the buffer and adjust to 8.0. Next, measure BSA (Table J.2) and add to the beaker to mix. Pour the contents of the beaker into a graduated cylinder and add distilled water to measure to the exact amount of water needed. Store the BSA buffer in the refrigerator; it can be stored for use for approximately 2 weeks.

TPBS wash buffer stores for a long period of time and therefore, large quantities are made at once. To make the TPBS wash buffer, measure the appropriate amount of NaCl, NaH$_2$PO$_4$, and Na$_2$HPO$_4$ (Table J.3) into a 4000 mL flask with a stir bar containing half the amount of desired water (Table J.3) and mix using a stirring plate. Once thoroughly mixed, the pH of the buffer and adjust to 8.0. Next, add distilled water to the flask to measure to the exact amount of water needed. Finally, add Tween-20 to the flask (Table J.3) and mix on the stirring plate. TPBS is stored at room temperature with parafilm covering the flask opening.

The 96-well plate layout is depicted in Figure C.1. Briefly, 5 serum samples fit on one plate and each is diluted from a 1:8 - 1:1024 dilution. To perform the "dilution train," place dilution tubes in five rows of 8 on a tube rack and label according the serum sample. Pipette 700 μL of BSA dilution buffer into the first tube of each row; pipette 400 μL of BSA
dilution buffer into the remaining tubes. Next, pipette 100 μL of the thawed, vortexed serum sample into the first tube of each row (the tube with 700 μL of BSA dilution buffer in it). Vortex the tube and remove 400 μL from the first tube and place into the second tube. After mixing the contents of the second tube via aspiration with the pipette, remove 400 μL from the second tube and place into the third tube. Repeat this process until the end of the row is reached. After each dilution train is performed, there is 400 μL of diluted serum in each tube except for the very last tube, which will have 800 μL of diluted serum. The first tube is a 1:8 dilution, the second a 1:16 dilution, and so on.

Next, calculate the positive and negative controls. The negative control is made at a 1:8 dilution, 100 μL in each well, and 8 wells per plate. In this study, negative control serum was collected from a spayed, unvaccinated female. To calculate, multiply the number of plates being run × 8 wells × 100 μL, and this equals amount of total solution needed for the negative control. Round this number up to allot for pipetting error, then divide by 8 (to account for the 1:8 dilution) to determine the amount of negative control serum needed. Subtract the amount of negative control serum needed from the total amount of solution calculated, and this equals the amount of BSA dilution buffer to add to the negative control serum. For an example of this calculation, see Figure J.3.

The positive control in this study was created by pooling positive-titer intact male dog serum samples obtained from a previous study (Donovan et al., 2012). The amount available was limited, and therefore, positive controls were calculated at a 1:27 dilution, 100 μL in each well, and 8 wells per plate. To calculate, multiply the number of plates being run × 8 wells × 100 μL, and this equals amount of total solution needed for the positive control.
Round this number up to allot for pipetting error, then divide by 27 (to account for the 1:27 dilution) to determine the amount of positive control serum needed. Subtract the amount of positive control serum needed from the total amount of solution calculated, and this equals the amount of BSA dilution buffer to add to the positive control serum. For an example of this calculation, see Figure J.4.

After all test serum samples are diluted and the controls are made, the plate is ready to be loaded with samples. Remove the coated plates from the refrigerator, allow to reach room temperature, and then tap-dry and rinse with TPBS. Briefly, the solution remaining in the plates was emptied into the sink and the plate was then vigorously slammed onto dry paper towels (tap-drying) until no more liquid was coming off onto the paper towels. The plate wells were then filled with TPBS, emptied into the sink, and then vigorously slammed onto paper towels until no more liquid was coming off. Vigorous slamming is necessary to remove all unbound material; any left behind will result in background and false positive results.

Next, load the plate with controls and samples, 100 μL per well, following the layout of Figure J.2 with each sample being vortexed a new pipette tip being used for every new sample. Cover the plate with adhesive film and incubate for one hour at room temperature.

During the incubation, calculate the amount of HRP solution needed to add to the plates. The HRP solution is made at a 1:2000 dilution, 100 μL in each well, and 96 wells per plate. To calculate, multiply the number of plates being run × 96 wells × 100 μL, and this equals amount of total solution needed for the HRP solution. Round this number up to allot for pipetting error, then divide by 2000 (to account for the 1:2000 dilution) to
determine the amount of HRP needed. Subtract the amount of HRP needed from the total amount of HRP solution calculated, and this equals the amount of BSA dilution buffer to add to the HRP. For an example of this calculation, see Figure J.5.

After the incubation period was over, empty the plate contents into the sink, tap-dry, rinse with TPBS, tap-dry, rinse and empty with TPBS three times, and tap-dry again. Mix the HRP solution in a well using a multichannel pipettor and add to the plate, 100 μL per well, cover with adhesive film, and incubate for one hour.

After this incubation period is over, add ABTS to the plate and immediately read in a spectrophotometer (FLUOstar Omega, BMG Labtech Inc, San Francisco, CA). The spectrophotometer available for use is located in the Linus Pauling Science Center in Dr. Gerd Bobe's lab, so all materials needed must be moved to his laboratory before adding ABTS. Once incubation is over, empty the plate contents into the sink, tap-dry, rinse with TPBS, tap-dry, rinse and empty with TPBS three times, and tap-dry again. Next, put 11 mL of ABTS into a well using the Drummond pipette-aid. ABTS is light-sensitive, so it must be exposed to light for as short of a period as possible. Pipette ABTS quickly into the plate, 100 μL per well, using a multichannel pipettor.

After ABTS is added, the colorimetric reaction is almost immediate. Insert the plate immediately into the plate reader and read at 405 nm. Repeat readings immediately for up to 7 minutes; a successful reading is defined as positive and negative control lanes having little variability between wells, duplicates having little variability between wells, and the positive control lane being around 1.3 - 1.5 OD. For an example of a successful plate, see Figure J.6.

Table J.1. Sodium bicarbonate buffer composition

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Table J.2. BSA dilution buffer composition

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Table J.3. TPBS wash buffer composition

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Figure J.1. GnRH antibody ELISA protocol summary

1. Wells are coated with GnRH, the antigen, in a sodium bicarbonate buffer.

2. Plates are washed; controls and serum samples are diluted in BSA dilution buffer and added to the plate. **Incubation overnight**

3. Plates are washed; Protein G conjugated to HRP enzyme in BSA dilution buffer is added to the plate. **Incubation for 1 hour**

4. Plates are washed; ABTS substrate is added to the plate. Substrate and enzyme interaction create a color change. **Incubation for 1 hour**

5. Plate is immediately read in a spectrophotometer.
Figure J.2. GnRH antibody ELISA 96 well plate layout

Neg = negative control, Pos = positive control, S = test sample

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Figure J.3. GnRH antibody ELISA negative control solution calculation

If 3 plates are being run:

3 plates run \times 8 wells \times 100 \mu L = 2400 \mu L negative control solution needed

Round up to 3000 \mu L to allot for pipetting error

3000 \mu L / 1:8 dilution = 3000/8 = 375 \mu L negative control serum needed

3000 \mu L - 375 \mu L = 2625 \mu L

Therefore, the negative control solution needed for three plates is comprised of 2626 \mu L BSA and 375 \mu L negative control serum
Figure J.4. GnRH antibody ELISA positive control solution calculation

If 3 plates are being run:

3 plates run × 8 wells × 100 μL = 2400 μL positive control solution needed

Round up to 2538 μL to allot for pipetting error while wasting as little positive control serum as possible

2538 μL / 1.27 dilution = 2444/27 = 94 μL positive control serum needed

2538 μL - 94 μL = 2444 μL

Therefore, the positive control solution needed for three plates is comprised of 2444 μL BSA and 94 μL positive control serum
Figure J.5. GnRH antibody ELISA HRP solution calculation

If 3 plates are being run:

3 plates run \times 96 wells \times 100 \ \mu\text{L} = 28,800 \ \mu\text{L} \ HRP \ solution \ needed

Round up to 30,000 \ \mu\text{L} to allot for pipetting error

30,000 \ \mu\text{L} / 1:2000 \ dilution = 30,000 / 2000 = 15 \ \mu\text{L} \ HRP \ needed

30,000 \ \mu\text{L} - 915 \ \mu\text{L} = 29,985 \ \mu\text{L}

Therefore, the HRP solution needed for three plates is comprised of 29,985 \ \mu\text{L} BSA and 15 \ \mu\text{L} HRP
Figure J.6. GnRH antibody ELISA successful plate reading. For layout, see Figure J.2.

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APPENDIX K: ANALYSIS OF GNRH ANTIBODY ELISA RESULTS

The results of the GnRH antibody ELISA are reported as endpoint titers, which are the reciprocal of the highest dilution of serum that gives a reading above a cutoff (Frey et al. 1998). The cutoff is derived from the negative controls and are calculated by the equation Cutoff = $\bar{x} + \text{SD}f$, where $\bar{x}$ is the mean of all negative control optical densities (ODs), SD is the standard deviation of all negative control ODs, and $f$ is a standard deviation multiplier (Frey et al. 1998). The standard deviation multiplier is derived from the chosen confidence level and number of negative controls used; see Table K.1 for the values of $f$ corresponding to confidence levels and negative controls.

To begin the conversion from raw OD to endpoint titer, first determine the cutoff. For this study, there were eight wells of negative control samples per plate and the chosen confidence level was 99%, so $f = 3.180$. The mean and standard deviation of the negative controls were calculated, and the cutoff was then derived from these values. For an example calculation, see Figure K.1.

Next, compare samples to the cutoff. Because all samples are run in duplicate, average the ODs, then subtract the calculated cutoff value from the averaged OD. If the resulting number is positive, the sample is considered positive. If the resulting number is negative, the sample is considered negative. For visual clarity, positive values are highlighted. For an example of this calculation, see Figure K.2.

The final endpoint titer is expressed as the reciprocal of the greatest dilution that yielded a positive result. Therefore, using Figure K.2 as an example, the Week 4 titer
would be 32, the Week 6 titer would be 512, and so forth. However, these dilutions are not linear and therefore not normally distributed. For example, the titer difference between 8 and 16 is the same as the titer difference between 512 and 1024, but data analysis programs will not treat the titers as such. Therefore, titers have to undergo logarithmic transformation so titers can be represented linearly for statistical analysis, calculation of standard deviations, graphing, etc. In this study, 2-fold dilutions were used and therefore, titers were log-transformed to the base 2. See Table K.2 for the log-2 transformations of the reciprocals of the serial dilutions.

References

Table K.1. Standard deviation multipliers ($f$) (Frey et al. 1998)

<table>
<thead>
<tr>
<th>Number of Controls ($n$)</th>
<th>Confidence level ($1 - \alpha$)</th>
<th>95.0%</th>
<th>97.5%</th>
<th>99.0%</th>
<th>99.5%</th>
<th>99.9%</th>
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<td>3.668</td>
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Table K.2. Log-2 transformed GnRH antibody titers

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<th>Reciprocal of the Serial Dilution</th>
<th>Log-2 Transformation</th>
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<td>8</td>
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<td>64</td>
<td>6</td>
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<td>128</td>
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<td>256</td>
<td>8</td>
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<tr>
<td>512</td>
<td>9</td>
</tr>
<tr>
<td>1024</td>
<td>10</td>
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</tbody>
</table>
Figure K.1. Calculation of a cutoff

<table>
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<tr>
<th>96</th>
<th>1</th>
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<tr>
<td>A</td>
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<tr>
<td>B</td>
<td>0.108</td>
</tr>
<tr>
<td>C</td>
<td>0.124</td>
</tr>
<tr>
<td>D</td>
<td>0.146</td>
</tr>
<tr>
<td>E</td>
<td>0.128</td>
</tr>
<tr>
<td>F</td>
<td>0.118</td>
</tr>
<tr>
<td>G</td>
<td>0.096</td>
</tr>
<tr>
<td>H</td>
<td>0.090</td>
</tr>
</tbody>
</table>

Average of negative controls: 0.112

Standard deviation of negative controls: 0.020785

\[ f = 3.180 \] (8 wells at 99% confidence, see Table J.1)

Cutoff = \( \bar{x} + SDF \)

Cutoff = (0.112) + (0.020785)(3.18)

Cutoff = 0.178095
Figure K.2. Example calculation of sample ODs minus calculated cutoffs

Averaged ODs:

<table>
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<tr>
<th>Dilution</th>
<th>Wk 0</th>
<th>Wk 4</th>
<th>Wk 6</th>
<th>Wk 8</th>
<th>Wk 10</th>
<th>Wk 12</th>
<th>Wk 16</th>
<th>Wk 20</th>
<th>Wk 24</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:8</td>
<td>0.211</td>
<td>0.9505</td>
<td>1.5925</td>
<td>1.0955</td>
<td>1.142</td>
<td>1.6955</td>
<td>1.355</td>
<td>0.9525</td>
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<tr>
<td>1:16</td>
<td>0.1895</td>
<td>0.71</td>
<td>1.6195</td>
<td>1.2355</td>
<td>1.153</td>
<td>1.61</td>
<td>1.254</td>
<td>0.679</td>
<td>0.404</td>
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<tr>
<td>1:32</td>
<td>0.2025</td>
<td>0.492</td>
<td>1.35</td>
<td>1.2065</td>
<td>1.027</td>
<td>1.231</td>
<td>0.732</td>
<td>0.402</td>
<td>0.325</td>
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<tr>
<td>1:64</td>
<td>0.186</td>
<td>0.3985</td>
<td>0.78125</td>
<td>1.131</td>
<td>0.783</td>
<td>1.0855</td>
<td>0.5885</td>
<td>0.304</td>
<td>0.248</td>
</tr>
<tr>
<td>1:128</td>
<td>0.203</td>
<td>0.303</td>
<td>1.239</td>
<td>0.9745</td>
<td>0.5945</td>
<td>0.6595</td>
<td>0.376</td>
<td>0.235</td>
<td>0.2245</td>
</tr>
<tr>
<td>1:512</td>
<td>0.119</td>
<td>0.187</td>
<td>0.701</td>
<td>0.501</td>
<td>0.344</td>
<td>0.3045</td>
<td>0.216</td>
<td>0.169</td>
<td>0.1575</td>
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<tr>
<td>1:1024</td>
<td>0.1115</td>
<td>0.129</td>
<td>0.4085</td>
<td>0.374</td>
<td>0.279</td>
<td>0.154</td>
<td>0.136</td>
<td>0.1435</td>
<td>0.1465</td>
</tr>
</tbody>
</table>

Calculated cutoffs:

Wk 0-10: 0.408467
Wk 12-24: 0.34505

Average ODs minus calculated cutoffs:

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Wk 0</th>
<th>Wk 4</th>
<th>Wk 6</th>
<th>Wk 8</th>
<th>Wk 10</th>
<th>Wk 12</th>
<th>Wk 16</th>
<th>Wk 20</th>
<th>Wk 24</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:8</td>
<td>-0.197</td>
<td>0.542</td>
<td>1.184</td>
<td>0.687</td>
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<td>1.009</td>
<td>0.607</td>
<td>0.113</td>
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<td>1:16</td>
<td>-0.218</td>
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<td>0.744</td>
<td>1.264</td>
<td>0.908</td>
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<tr>
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<td>0.083</td>
<td>0.941</td>
<td>0.798</td>
<td>0.618</td>
<td>0.885</td>
<td>0.386</td>
<td>0.056</td>
<td>-0.020</td>
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<tr>
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<td>0.372</td>
<td>0.722</td>
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<td>0.740</td>
<td>0.243</td>
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<tr>
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<td>0.186</td>
<td>0.314</td>
<td>0.030</td>
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<td>-0.120</td>
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<tr>
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<td>0.379</td>
<td>0.001</td>
<td>0.114</td>
<td>-0.035</td>
<td>-0.159</td>
<td>-0.159</td>
</tr>
<tr>
<td>1:512</td>
<td>-0.289</td>
<td>-0.221</td>
<td>0.292</td>
<td>0.092</td>
<td>-0.064</td>
<td>-0.040</td>
<td>-0.129</td>
<td>-0.176</td>
<td>-0.187</td>
</tr>
<tr>
<td>1:1024</td>
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<td>-0.279</td>
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<td>-0.03</td>
<td>-0.129</td>
<td>-0.191</td>
<td>-0.209</td>
<td>-0.201</td>
<td>-0.198</td>
</tr>
</tbody>
</table>
APPENDIX L: LH-DETECT® FOR CANINES ELISA PROTOCOL

LH-Detect® for Canines (Repropharm, Nouzilly, France) is a sandwich-type ELISA that uses a scale of standards for quantitative results and is a more affordable alternative to measuring LH compared to a radioimmunoassay. Serum, plasma, or whole blood can be used in this assay. The assay was performed according to the manufacturer's instructions and for the contents of the kit, see Figure L.1.

Briefly, remove the ELISA plate, the dilution (DIL) substrate, and scale of standards (referred to as GAM in Figure L.1) from the kit. Thaw the plate at room temperature, thaw the DIL in tepid water, and thaw the scale of standards on ice. At this time, also thaw the test samples on ice. Each plate fits 40 samples; to see a plate layout design, see Figure L.2.

While thawing occurs, label forty centrifuge tubes with the test sample dog name and date of sampling. Once the DIL is thawed, add 200 μL of DIL to each centrifuge tube. Vortex the thawed test sample and add 50 μL to the correctly labeled centrifuge tube. Repeat this for all 40 samples. Vortex the centrifuge tube and deposit 100 μL of the diluted sample into the specified wells. Finally, vortex the scale of standards were individually and deposit into the specified wells. All samples and standards are deposited in duplicate. Cover the plate with an adhesive film and incubate at room temperature for one hour. During incubation, thaw the second antibody, AC2, from the kit in tepid water.

After the incubation period ends, rinse the plate five times with phosphate buffered saline with tween (TPBS) wash buffer (See appendix J, table J.4 for the wash buffer
composition), and tap-dry in between each rinse. Pour the thawed AC2 into a well and pipette 100 μL into each well using a multichannel pipettor. Re-cover the plate with adhesive film and incubate at room temperature for another hour. During this second incubation, the enzyme-conjugated antibody, AC3, was thawed in tepid water.

After the second incubation, rinse the plate again with TPBS five times and tap-dry between each rinse. Pour thawed AC3 into a well and pipette 100 μL into each well using a multichannel pipettor. Re-cover the plate with adhesive film and incubate at room temperature for another hour. During this third incubation, thaw the colorimetric substrate (SUB) and stop solution (STOP) in tepid water. For this study, the SUB solution was TMB, recommended by the manufacturer for a quantitative results, and STOP was a sulfuric acid solution.

Shortly before the third incubation ended, supplies need to be brought to the laboratory housing the spectrophotometer (FLUOstar Omega, BMG Labtech Inc, San Francisco, CA). After incubation, rinse the plate with TPBS five times and tap-dry between each rinse. Pour thawed TMB was then into a well and pipette 100 μL into each well using a multichannel pipettor. Insert the plate immediately into the spectrophotometer and read at 650 nm repeatedly until the 8 ng/mL standard reaches 0.35 OD. Once this occurs, remove the plate and pipette 50 μL of STOP into each well using a multichannel pipettor. Then place the plate back into the spectrophotometer and read at 450nm repeatedly for 30 minutes. A successful reading is defined as the scale of standards increasing in optical density as the concentrations increase as well as duplicates having little variability between wells. For an example of a successful plate, see Figure L.3.
Figure L.1. Contents of LH-Detect® for canines kit

Reagents and samples preparation:

- GAM or POS
- sample
- AC2
- AC3
- DIL
- SUB
- STOP

ELISA plate:
- to defrost for 30 min at room temperature

You can stock them again at -20°C (1 to 2 times maximum for AC2 & AC3)

- to defrost
- to defrost
- to defrost, SUB has to be protected from light
Figure L.2. LH-Detect® ELISA plate layout

Columns 1 and 2 represent the scale of standards, S = test sample

<table>
<thead>
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<th></th>
<th>1</th>
<th>2</th>
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<th>4</th>
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<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
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<tbody>
<tr>
<td>A</td>
<td>0 ng/mL</td>
<td>0 ng/mL</td>
<td>S1</td>
<td>S1</td>
<td>S9</td>
<td>S9</td>
<td>S17</td>
<td>S17</td>
<td>S25</td>
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<td>S33</td>
<td>S33</td>
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<tr>
<td>B</td>
<td>0.12 ng/mL</td>
<td>0.12 ng/mL</td>
<td>S2</td>
<td>S2</td>
<td>S10</td>
<td>S10</td>
<td>S18</td>
<td>S18</td>
<td>S26</td>
<td>S26</td>
<td>S34</td>
<td>S34</td>
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<tr>
<td>C</td>
<td>0.25 ng/mL</td>
<td>0.25 ng/mL</td>
<td>S3</td>
<td>S3</td>
<td>S11</td>
<td>S11</td>
<td>S19</td>
<td>S19</td>
<td>S27</td>
<td>S27</td>
<td>S35</td>
<td>S35</td>
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<tr>
<td>D</td>
<td>0.5 ng/mL</td>
<td>0.5 ng/mL</td>
<td>S4</td>
<td>S4</td>
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<td>S12</td>
<td>S20</td>
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<td>E</td>
<td>1 ng/mL</td>
<td>1 ng/mL</td>
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<td>S5</td>
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<td>S21</td>
<td>S21</td>
<td>S29</td>
<td>S29</td>
<td>S37</td>
<td>S37</td>
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<tr>
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<td>2 ng/mL</td>
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<td>S6</td>
<td>S14</td>
<td>S14</td>
<td>S22</td>
<td>S22</td>
<td>S30</td>
<td>S30</td>
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<td>S38</td>
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<tr>
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<td>4 ng/mL</td>
<td>S7</td>
<td>S7</td>
<td>S15</td>
<td>S15</td>
<td>S23</td>
<td>S23</td>
<td>S31</td>
<td>S31</td>
<td>S39</td>
<td>S39</td>
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<tr>
<td>H</td>
<td>8 ng/mL</td>
<td>8 ng/mL</td>
<td>S8</td>
<td>S8</td>
<td>S16</td>
<td>S16</td>
<td>S24</td>
<td>S24</td>
<td>S32</td>
<td>S32</td>
<td>S40</td>
<td>S40</td>
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</table>
Figure L.3. Successful LH-Detect® plate reading. For layout, see figure L.2.

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<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
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<tbody>
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<td>0.666</td>
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<td>0.647</td>
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</tr>
<tr>
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<td>0.765</td>
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<td>0.568</td>
<td>0.571</td>
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<tr>
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<td>0.514</td>
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<td>0.702</td>
<td>0.685</td>
<td>0.478</td>
<td>0.470</td>
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<td>0.492</td>
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<tr>
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<td>0.584</td>
<td>0.446</td>
<td>0.451</td>
<td>0.543</td>
<td>0.535</td>
<td>0.789</td>
<td>0.774</td>
<td>0.496</td>
<td>0.484</td>
<td>0.596</td>
<td>0.576</td>
</tr>
<tr>
<td>G</td>
<td>0.646</td>
<td>0.630</td>
<td>0.467</td>
<td>0.460</td>
<td>0.611</td>
<td>0.557</td>
<td>0.740</td>
<td>0.729</td>
<td>0.489</td>
<td>0.490</td>
<td>0.488</td>
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<tr>
<td>H</td>
<td>0.745</td>
<td>0.748</td>
<td>0.463</td>
<td>0.456</td>
<td>0.555</td>
<td>0.556</td>
<td>0.572</td>
<td>0.573</td>
<td>0.528</td>
<td>0.507</td>
<td>0.540</td>
<td>0.496</td>
</tr>
</tbody>
</table>

Finished cycle: 1
Display cycles: 1
APPENDIX M: ANALYSIS OF LH-DETECT® ELISA RESULTS

For this assay, optical density (OD) is converted into concentrations (ng/mL) based upon the manufacturer's provided scale of standards. This is typically achieved by developing a standard curve, plotting the OD of the standard (y axis) against the given concentration of the standard (x axis) and comparing test sample ODs to the curve to determine the concentration. However, the scale of standards for this assay does not increase linearly, so logarithmic transformation is needed to develop an accurate standard curve.

For this study, the development of the curve and consequent calculations to determine test sample concentrations were derived using an Excel® (Microsoft Corporation, Redmond WA) template developed by Dr. Katsumi Wakabayashi (2008). Briefly, average the ODs of each standard and subtract the OD of the 0 ng/mL (blank) standard from all other standards. If a standard OD does not increase accordingly with increasing concentration, remove it. The resulting values (ΔBlank) are then log transformed to the base e (ln(ΔBlank)). The given concentrations of the scale of standards are log-transformed to the base e and plotted against ln(ΔBlank) to develop the standard curve. Finally, add a 3rd order polynomial trendline to derive the equation of the line. An example of these calculations and the derived standard curve can be seen in Figure M.1.

To compare test samples to the curve, they also must be log-transformed. Briefly, subtract the OD of the blank from the OD of each sample, and these values are then log-transformed to the base e. This resulting value (ln(ΔBlank)) is inserted into the equation.
of the standard curve, giving the value Cal(ln). Take the inverse natural logarithm from Cal(ln), and this results in a calculated concentration of the test sample (CalCon).

Because each sample was run in duplicate, the CalCon values of duplicate samples are averaged to determine the final concentration of the sample in ng/mL (FinCon). An example of this calculation process is seen in Figure M.2.

References

Figure M.1. Development of a standard curve

<table>
<thead>
<tr>
<th>Scale of Standard Concentrations, ng/mL (Conc.)</th>
<th>ln(Conc.)</th>
<th>OD</th>
<th>OD</th>
<th>Averaged ODs</th>
<th>Averaged OD minus averaged OD of blank (ΔBlank)</th>
<th>ln(ΔBlank)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>2.079442</td>
<td>0.745</td>
<td>0.748</td>
<td>0.7465</td>
<td>0.2655</td>
<td>-1.32614</td>
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<tr>
<td>4</td>
<td>1.386294</td>
<td>0.648</td>
<td>0.63</td>
<td>0.639</td>
<td>0.158</td>
<td>-1.84516</td>
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<tr>
<td>2</td>
<td>0.693147</td>
<td>0.57</td>
<td>0.584</td>
<td>0.577</td>
<td>0.096</td>
<td>-2.34341</td>
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<td>1</td>
<td>0</td>
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<td>0.522</td>
<td>0.5415</td>
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<tr>
<td>0.25</td>
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<td>0.514</td>
<td>0.515</td>
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<tr>
<td>0.12</td>
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<td>0.483</td>
<td>0.514</td>
<td>0.4985</td>
<td>0.0175</td>
<td>-4.04555</td>
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<tr>
<td>Blank (0)</td>
<td></td>
<td>0.465</td>
<td>0.497</td>
<td>0.481</td>
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<td></td>
</tr>
</tbody>
</table>

Plot ln(Conc.) (y axis) against ln(ΔBlank) (x axis). Add a polynomial trendline, order 3, to derive the equation of the line.

\[
y = -0.1975x^3 - 1.6517x^2 - 2.6917x + 0.9115
\]

\[R^2 = 0.9948\]
Figure M.2. Calculation of test samples from OD to ng/mL concentrations

<table>
<thead>
<tr>
<th>OD1</th>
<th>OD2</th>
<th>(ΔBlank)1</th>
<th>(ΔBlank)2</th>
<th>ln(ΔBlank)1</th>
<th>ln(ΔBlank)2</th>
<th>Cal(ln)1</th>
<th>Cal(ln)2</th>
<th>CalCon1</th>
<th>CalCon2</th>
<th>FinCon</th>
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<tbody>
<tr>
<td>0.806</td>
<td>0.791</td>
<td>0.325</td>
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<td>-1.123</td>
<td>-1.171</td>
<td>2.134</td>
<td>2.119</td>
<td>8.454</td>
<td>8.328</td>
<td>8.391</td>
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<tr>
<td>0.765</td>
<td>0.744</td>
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<td>0.263</td>
<td>-1.258</td>
<td>-1.335</td>
<td>2.080</td>
<td>2.034</td>
<td>8.008</td>
<td>7.650</td>
<td>7.829</td>
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<tr>
<td>0.747</td>
<td>0.722</td>
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<td>-1.324</td>
<td>-1.422</td>
<td>2.042</td>
<td>1.970</td>
<td>7.707</td>
<td>7.173</td>
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<td>0.749</td>
<td>0.741</td>
<td>0.268</td>
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<td>0.308</td>
<td>0.293</td>
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<td>-1.227</td>
<td>2.117</td>
<td>2.096</td>
<td>8.308</td>
<td>8.134</td>
<td>8.221</td>
</tr>
<tr>
<td>0.74</td>
<td>0.729</td>
<td>0.259</td>
<td>0.248</td>
<td>-1.350</td>
<td>-1.394</td>
<td>2.024</td>
<td>1.99</td>
<td>7.571</td>
<td>7.336</td>
<td>7.453</td>
</tr>
</tbody>
</table>
Appendix N: LH Concentration Comparison with Continent Bitches

Venous blood samples were taken from twenty-seven medium-sized (25-49 pounds of body weight) and large-sized (50-100 pounds) healthy, continent ovariecitomized bitches. These samples were used to compare LH concentrations to the sixteen incontinent bitches previously sampled (see Chapter III). All continent bitches were ovariectomized (OE) at least one year prior to sampling.

LH concentration comparisons were made between and within groups with regard to continence, size and age at OE using PROC TTEST in SAS (Version 9.2, SAS Institute Inc., Cary, NC). Results are summarized in Table N.1. In general, incontinent bitches had lower LH concentrations compared to continent bitches ($p=0.04$), consistent with findings reported previously by Reichler et al. (2005). However, when the size of bitch was included in this comparison, LH concentrations were only significantly different between medium-sized bitches ($p=0.02$) but not between large-sized bitches ($p=0.79$). It is important to note that in general, medium-sized bitches had higher LH concentrations than large-sized bitches.

Overall, age at ovariectomy did not significantly affect LH concentrations. However, there was a trend for incontinent bitches ovariectomized under one year of age to have LH concentrations lower than continent bitches ovariectomized at a similar time ($p=0.06$) and for LH concentrations to be higher than in incontinent bitches who were ovariectomized over one year of age ($p=0.07$).
These findings support our hypotheses that 1) the development of incontinence in ovariectomized females is related to significantly lower LH concentrations; and 2) early age ovariectomy increases LH concentrations compared to older age ovariectomy.

References

Table N.1. LH concentration comparisons between continent and incontinent ovariectomized bitches.

<table>
<thead>
<tr>
<th>VARIABLE</th>
<th>N</th>
<th>MEAN LH CONCENTRATION (ng/mL)</th>
<th>SEM</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>Continent bitches</td>
<td>27</td>
<td>7.85</td>
<td>1.05</td>
<td>0.04</td>
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<tr>
<td>Incontinent bitches</td>
<td>16</td>
<td>4.78</td>
<td>0.51</td>
<td></td>
</tr>
<tr>
<td><strong>SIZE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium bitches</td>
<td>23</td>
<td>8.56</td>
<td>1.07</td>
<td>0.004</td>
</tr>
<tr>
<td>Large bitches</td>
<td>20</td>
<td>4.56</td>
<td>0.69</td>
<td></td>
</tr>
<tr>
<td>Medium continent bitches</td>
<td>15</td>
<td>10.34</td>
<td>1.40</td>
<td>0.02</td>
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<tr>
<td>Medium incontinent bitches</td>
<td>8</td>
<td>5.22</td>
<td>0.72</td>
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</tr>
<tr>
<td>Large continent bitches</td>
<td>12</td>
<td>4.71</td>
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<td>0.79</td>
</tr>
<tr>
<td>Large incontinent bitches</td>
<td>8</td>
<td>4.32</td>
<td>0.73</td>
<td></td>
</tr>
<tr>
<td>Medium continent bitches</td>
<td>15</td>
<td>10.34</td>
<td>1.40</td>
<td>0.005</td>
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<tr>
<td>Large continent bitches</td>
<td>12</td>
<td>4.71</td>
<td>1.07</td>
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<tr>
<td>Medium incontinent bitches</td>
<td>8</td>
<td>5.22</td>
<td>0.72</td>
<td>0.39</td>
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<tr>
<td>Large incontinent bitches</td>
<td>8</td>
<td>4.32</td>
<td>0.73</td>
<td></td>
</tr>
<tr>
<td><strong>TIME OF OVARIECTOMY</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Bitches ovariectomized ≤ 1 yr</td>
<td>30</td>
<td>7.12</td>
<td>0.90</td>
<td>0.37</td>
</tr>
<tr>
<td>Bitches ovariectomized &gt; 1 yr</td>
<td>13</td>
<td>5.72</td>
<td>1.12</td>
<td></td>
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<tr>
<td>Continent bitches ovariectomized ≤ 1 yr</td>
<td>17</td>
<td>8.57</td>
<td>1.47</td>
<td>0.06</td>
</tr>
<tr>
<td>Incontinent bitches ovariectomized ≤ 1 yr</td>
<td>13</td>
<td>5.22</td>
<td>0.54</td>
<td></td>
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<tr>
<td>Continent bitches ovariectomized &gt; 1 yr</td>
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<td>6.59</td>
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<td>Incontinent bitches ovariectomized &gt; 1 yr</td>
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<td>2.86</td>
<td>0.71</td>
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<tr>
<td>Continent bitches ovariectomized ≤ 1 yr</td>
<td>17</td>
<td>8.57</td>
<td>1.47</td>
<td>0.37</td>
</tr>
<tr>
<td>Continent bitches ovariectomized &gt; 1 yr</td>
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<td>6.59</td>
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<td>Incontinent bitches ovariectomized ≤ 1 yr</td>
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APPENDIX O: PUBLICATIONS, ABSTRACTS, AND POSTERS

Appendix O.1. Summary

Throughout my graduate career, I have been privileged to be able to collaborate with other researchers as well as present my research at regional, national, and international conferences.
Phage-peptide chemical fusions, GnRH peptides were conjugated to phage particles using 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) chemistry (Hernanson 1996). The EDC method couples peptides and phage via the free carboxyl group of the peptide C-terminal amino acid and the N-terminus of the phage coat proteins, producing conjugates joined by stable amide bonds. To evaluate antigenic potentials of the conjugates, they were extensively tested for recognition by different types of commercial anti-GnRH antibodies and experimentally produced feline and canine anti-GnRH sera as well as for ability to stimulate specific anti-GnRH immune responses in mice.

Materials and Methods
Preparation of phage-peptide conjugates
A filamentous phage vector F5-8 (provided by Dr. Petrenko) with wild-type phage coat proteins (Petrenko and Smith 2005) was used as a carrier for construction of chemical conjugates based on the sequence of GnRH peptide. Phages were propagated and purified using standard techniques as described in Brigati et al. (2008). To generate the constructs, a peptide with exact GnRH sequence and three rationally designed peptides modified for chemical conjugation to phage were synthesized. For peptide sequences and their designs, see Table 1. For three of the peptides, a non-immunogenic spacer Ahx (6-aminohexanoic acid) was added to the peptide C-terminus to produce conjugates (named PepEGAhx, PepEKGAhx, and PepGFAhx) that stimulate responses focused on the peptide immunogens rather than phage proteins. The fourth peptide (named PepEG) was synthesized with amidated C-terminus without a spacer. Such design allowed conjugation via the carbonyl group of a glutamic acid, which occupies position one in the GnRH peptide sequence. The peptides were synthesized and purified to 95% by Pl Proteomics, LLC (Huntsville, AL, USA). For phage-peptide conjugation, 500 μl of phage (1 mg/ml by NanoDrop 1000; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was mixed with 50 μl of 10% (weight) EDC. This step was followed by adding 500 μl of a peptide diluted in water at the concentration of 1 mg/ml. The mixture was incubated for 4 h at room temperature (RT) and then dialysed against PBS, pH 7.0 in Slide-A-Lyzer G2 Dialysis Cassettes 10K MWCO (Thermo Fisher Scientific, Inc.) for 24 h.

Phage spectroscopy
To validate formation of phage-peptide conjugates, absorption profiles of the following four preparations were obtained by spectrophotometry: (i) phage particles alone, (ii) phage-GnRH peptide chemical conjugates, (iii) free GnRH peptide and (iv) a physical mixture of phage particles and free GnRH peptide. Absorbance maximums of phage-peptide preparations were determined by NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc.).

Electron microscopy
Phage preparations diluted to 1 x 10^12 virions/ml (vit/ml) were pipetted onto 300 nickel mesh, carbon coated formvar grids (Electron Microscopy Sciences, Hatfield, PA, USA) to incubate at RT. All experimental sample grids were incubated in rabbit anti-GnRH primary antibody diluted 1 : 200 (Abcam, Cambridge, MA, USA) for 1 h. Each grid was rinsed, washed and suspended on drops of donkey anti-rabbit 6 nm gold-labelled antibody diluted 1 : 20 (Jackson Immunoresearch Laboratories, Inc., West Grove, PA, USA) for 1 h, followed by negative staining with 2% phosphotungstic acid. Using a Philips 301 transmission electron microscope (Philips Electronics North America Corporation, Andover, MA, USA), photomicrographs were taken at 60 kV voltage acceleration at 57 000x magnification.

Phage ELISA
Phage ELISA was used in this study in four separate experiments: (i) to test interactions of a phage-GnRH conjugate with anti-GnRH antibody and anti-phage serum (to provide evidence that phage-peptide conjugates are composed of two components, phage and GnRH peptide); (ii) to evaluate interactions of phage-peptide conjugates with different types of commercially available anti-GnRH antibodies; (iii) to detect anti-GnRH antibodies in sera of mice injected with the conjugates; and (iv) to evaluate interactions of phage-peptide conjugates with sera collected from cats and dogs immunized with different GnRH-based vaccines. Anti-phage serum was obtained in an unrelated study by immunization of an animal with a preparation of phage particles that had coat proteins identical to those used in this work. For the second experiment, four types of commercial anti-GnRH antibodies were tested: (i) rabbit polyclonal ab16216 generated to pyro-EHWGSRG synthetic peptide and affinity purified against the immunogen (Abcam); (ii) rabbit polyclonal ab5617 generated to EHWGSRG synthetic peptide and purified via ammonium sulphate precipitation (Abcam); (iii) rabbit polyclonal raised against N-terminal amino acids of human GnRH and peptide affinity purified (Decapheran Biotechnology LLC, Aurora, CO, USA); and (iv) mouse monoclonal ab62432 generated to purified human GnRH and protein G purified (Abcam). For all four separate experiments, phage preparations were diluted at 1.3 x 10^2 in TBS buffer and added to 96-well plates (80 μl/well) for overnight

<table>
<thead>
<tr>
<th>Peptide name</th>
<th>Peptide sequence</th>
<th>Spacer</th>
<th>Peptide description</th>
</tr>
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<tr>
<td>PepEGAhx</td>
<td>EHWGSRG</td>
<td>Ahx</td>
<td>Direct GnRH peptide; acid on C-terminus</td>
</tr>
<tr>
<td>PepEKGAhx</td>
<td>EHWGSRG</td>
<td>Ahx</td>
<td>GnRH peptide modified with K in position six; acid on C-terminus</td>
</tr>
<tr>
<td>PepGFAhx</td>
<td>GPRLGSWYH</td>
<td>Ahx</td>
<td>Reverse GnRH peptide; acid on C-terminus</td>
</tr>
<tr>
<td>PepEG</td>
<td>EHWGSRG</td>
<td>No</td>
<td>Direct GnRH peptide; amidite on C-terminus</td>
</tr>
</tbody>
</table>
immobilization at 4°C. After that, unbound phages were removed from the plates and 150 μl of 1% (weight) BSA in TBS was added to the wells for 1 h at 37°C to block non-specific binding. Subsequently, the blocking solution was removed, and wells were washed three times with TBS containing 1% BSA, 0.05% (volume) Tween-20. Serial twofold dilutions of the commercial antibodies or serum samples from immunized mice, cats and dogs were added to the phage-coated wells, and the plates were incubated for 2 h at 37°C. The wells were then washed three times with TBS containing 0.05% Tween-20. Peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) diluted 1: 2000 in TBS containing 1% BSA and 0.05% Tween-20 was added to the wells for 1 h incubation at RT. The wells were washed as previously, reacted with o-phenylene diamine substrate solution (Acros Organics, Geel, Belgium), and absorbance measured at 490 nm. Each sample was evaluated in triplicate. Antibody responses were presented as means of OD₆₉₀ ± SD.

**Immunization of mice and evaluation of mouse sera for specific antibody responses by ELISA**

CD-1 outbred 6-week-old male mice (n = 6) (Charles River, Wilmington, MA, USA) were used in immunization experiments in accordance with the approval of the Auburn University Animal Use and Care Committee. Each mouse was immunized with 4 × 10⁷ virions of phage in 150 μl PBS administered subcutaneously (six mice total). No adverse reactions were observed in any of the mice. Blood samples were collected via tail vein puncture prior to immunization with phage-GnRH peptide conjugate and again 2 weeks after the immunization. For detection of anti-GnRH antibodies, serial twofold dilutions of mouse sera were prepared and reacted with phage as described in the Phage ELISA section above. Data presented at sera dilution 1: 400.

**Cat and dog sera**

Samples of cat and dog sera used in this study were obtained in previously performed independent experiments from two different sources. Animals were used in accordance with protocols approved by Animal Care Committees of the corresponding institutions. The first source was the laboratory of Dr. Henry Baker, Scott-Ritchey Research Center, Auburn, AL. Sexually mature male domestic cats (cats 1, 2 and 3 in Fig. 5) were immunized twice with 100 μg of a leukotoxin-GnRH recombinant protein. The second source was the laboratory of Dr. Michelle Kutzler, Oregon State University, Corvallis, OR. Male cats and dogs (cats 4 and 5 and dogs 1, 2 and 3 (see Fig 5) received 1 ml Canine Gonadotropin Releasing Factor Immunoaffinity (Pfizer Animal Health, Exton, PA, USA) twice. Testosterone concentrations in post-immunized sera from both sources were non-detectable by Coat-A-Count Total Testosterone radioimmunoassay (Diagnostics Products Corporation, Los Angeles, CA, USA).

**Statistical analysis**

ELISA measurements are presented as means of three repeats ± SD. Antibody responses in mice were compared using one-way ANOVA at a significance value of p ≤ 0.05 using Origin 7.5 data analysis and graphing software (OriginLab, Northampton, MA, USA).

**Results**

**Characterization of phage-GnRH peptide conjugates by spectrophotometry**

To validate formation of phage-peptide conjugates, their spectroscopic profiles were compared to those of phage particles alone, free GnRH peptide, and a physical mixture of phage particles with free GnRH peptide. The absorption profile of the 'phage alone' preparation was typical for filamentous phage with a broad plateau in the area of 260-280 nm and a maximum at 269 nm. The absorption profiles of all four phage-peptide chemical conjugates (see Table 1 for their descriptions) were similar to the 'phage alone' preparation except the absorption maximum shifted to 273-nm position.

To prove that these phage-peptide preparations are conjugates, but not a physical mixture of the two components, spectroscopic profiles for the free GnRH peptide as well as the free peptide mixed with the phage were obtained and their maximums measured. The absorption maximum for the free peptide was 280 nm as expected for a protein-like compound. The physical mixture of phage particles with the free peptide (without chemical conjugation) demonstrated the maximum at 277 nm that was higher than for the conjugates, but lower than for the free peptide, indicating that all preparations tested are different and have individual characteristics.

**Characterization of phage-GnRH peptide conjugates by electron microscopy**

To demonstrate specificity of the phage-peptide conjugates as well as the integrity of phage particles after the chemical conjugation process, the conjugates were analysed by electron microscopy. A sample containing PepEQAhx conjugate was absorbed on grids, reacted with specific anti-GnRH primary antibody followed by secondary antibody labelled with gold. A sample of i5-8 phage (without GnRH peptide) was included in the experiment as a negative control. The experimental sample containing the conjugate had very prominent immunolabelling of 'normal-looking' filamentous phage particle morphology (Fig. 1). In contrast, the negative control had little if any gold labelling associated with phage. Thus, formation and specificity of phage-GnRH peptide conjugates were confirmed by electron microscopy. It was shown as well that the chemical conjugation procedure used in the study did not change the morphology of phage particles. Density of the immunolabelling indicated that GnRH peptide was conjugated to the phage at high copy numbers.
Characterization of phage-GnRH peptide conjugates by ELISA; immune responses to PepEGAlx conjugate in mice

To provide additional evidence that phage-peptide conjugates are composed of two components (phage and GnRH peptide), we evaluated ELISA interactions of one of the conjugates, PepEGAlx, with anti-GnRH antibody as well as with anti-phage serum. The ELISA signals from the conjugate binding to antibody or serum were decreasing proportionally with each antibody or serum dilution, indicating specificity of binding (Fig. 2). In contrast, control samples containing dialysis washes obtained at different stages of the conjugate purification did not produce any signals in ELISA (not shown), indicating that detected an antibody binding was attributed to the conjugate, but not because of free peptide remaining in the conjugate preparation.

Next, specificity of all four phage-GnRH conjugates (PepEGAlx, PepEKGAlex, PepEAlx and PepEG) was tested in ELISA, using several types of anti-GnRH antibodies obtained from different sources. As shown in Fig. 3, all four conjugates demonstrated strong binding with three types of polyclonal antibodies at the tested antibody dilutions (1:25 600 for binding to Pep-

Fig. 2. Interaction of PepEGAlx phage-peptide conjugate with anti-GnRH antibody or anti-phage serum at increasing twofold antibody or serum dilutions. The solid line represents binding of the conjugate to anti-GnRH antibody. The dashed line represents binding of the conjugate with anti-phage serum. Data were obtained in ELISA format. Means of three repeats ± SD are shown

Fig. 3. Interaction of PepEGAlx, PepEKGAlex, PepEAlx and PepEG phage-peptide conjugates with different types of commercial anti-GnRH antibodies. Black columns indicate binding to Abcam ab16216; dark grey — Abcam ab6617; light grey — Decipheragen Biotechnology; white — Abcam ab62432. To assay binding to PepEGAlx, PepEKGAlex and PepEAlx conjugates, antibodies were diluted 1:25 600; for binding to PepEG conjugate, antibodies were diluted 1 : 51 200

EGAlx, PepEKGAlex, PepEAlx and 1 : 51 200 for binding to PepEG). The monoclonal antibody provided appreciable signal only with the PepEG conjugate.

The ability of PepEGAlx conjugate to stimulate production of anti-GnRH antibodies in vivo was evaluated in mice. Serum samples were collected from mice before and after the immunization and compared for the presence of anti-GnRH antibodies. Figure 4 shows antibody responses to the phage-GnRH conjugate in six individual mice. In each mouse, the ELISA signal obtained from post-immunization serum was significantly higher (p ≤ 0.05) than that of baseline signal generated from pre-immunization serum, suggesting that the conjugate stimulated production of anti-GnRH antibodies.

Interaction of phage-GnRH peptide conjugate with sera collected from GnRH-immunized cats and dogs

As shown in the section above, all phage-GnRH peptide conjugates demonstrated strong ELISA signals when reacted with purified anti-GnRH antibodies obtained
A filamentous phage vector f5-8 with wild-type phage coat proteins was used as a carrier in this study as an example. Theoretically, any other filamentous phage with wild-type phage coat proteins or even with specifically designed recombinant coat proteins might be used for conjugation purposes. In a study by van Houten et al. (2006), the F1K filamentous phage was engineered to have an additional lysine residue near the N-terminus of the major coat protein VIII. This allowed not only efficient conjugation of antigenic peptides to the phage particles, but also focused the antibody responses against the displayed peptides. To our knowledge, this is the only study that has utilized filamentous phage for chemical conjugation of peptides for vaccine development. Several studies reported the use of chemically modified filamentous phage for delivery of drugs. For instance, Bar et al. (2008) created drug-carrying phage particles for targeted anti-cancer therapy. First, the phage was genetically modified to display cell-specific antibodies as targeting moieties. Next, the phages were loaded with the cytotoxic drug doxorubicin by EDC chemical conjugation. When compared to the free drug, the resulting phage particles were shown to cause a superior inhibition effect on the growth of the target cancer cells.

Chemical conjugation could be a valid alternative to phage-based vaccines obtained via genetic manipulations, which have limitations defined by phage biology. Such limitations include length, number and sequence composition of the peptides displayed by phage as well as low propagation rates of genetically modified phages. To improve immune responses against phage-peptide constructs, one could develop mosaic phage-peptide particles, which are conjugates of phage with more than one type of peptide, for example, various B- and T-cell epitopes. Sequences of immunogenic peptide epitopes (if not known) for various targets can be identified, for example, via selections from biological display libraries. Once the peptide sequences are identified, they can be chemically synthesized and conjugated to the thousands of copies of the phage coat proteins on the surface of filamentous phage particles produced at commercial scale. Interestingly, chemical conjugation of GnRH peptides to phage proteins in this study resulted in complete loss of phage infectivity as tested by phage titration (not shown). Such phage conjugates have no ability to propagate and will degrade if released into the environment. This feature would be important for vaccine preparations used for feral and wild animals in field conditions where environmental safety is paramount.

Conclusion

To conclude, phage-GnRH peptide conjugates with high-density peptide fusions were obtained in this study using EDC chemistry. The chemical conjugation procedure did not change the morphology of phage particles. The ability of GnRH peptides fused to the phage particles to recognize different antibody types varied depending on modifications of the peptides as well as antibody types. GnRH peptides conjugated to the body of filamentous phage particles were able to stimulate
anti-GnRH immune responses in mice. Recognition of the conjugates by neutralizing antibodies that caused immunocastration in cats and dogs suggests that vaccines formulated to include phage-GnRH peptide chemical conjugates might be viable alternatives to current methods of population control of feral animals.

Acknowledgements

This study was supported by the Animal Health and Disease Research grant from College of Veterinary Medicine and by the Scott-Ritchey Research Center, Auburn University. We thank Pfizer for donating the GnRH vaccine for the immunization study performed at Oregon State University.

References


Submitted: 1 Jun 2012; Accepted: 8 Jul 2012

Author's address (for correspondence): T Samoylova, Scott-Ritchey Research Center, College of Veterinary Medicine, Auburn University, Auburn, AL 36849, USA. E-mail: samotl@auburn.edu
Appendix O.3. Presented at the 2013 Annual Conference for the Society for Theriogenology (SFT; Louisville, KY)

**GnRH Immunization for the Treatment of Urinary Incontinence in Spayed Bitches**

C.E. Donovan, J.M. Gordon, M.A. Kutzler

1Department of Animal and Rangeland Sciences, Oregon State University, Corvallis, OR

2College of Veterinary Medicine, Oregon State University, Corvallis, OR

**INTRODUCTION.** Because urinary incontinence is common in spayed bitches (4.9-20%) [1-2], it has been postulated that permanently elevated gonadotropin concentrations as a result of ovariectomy may contribute to the development of urinary incontinence [3]. Therefore, the purpose of this study was to determine if a gonadotropin releasing hormone (GnRH) vaccine, labeled for the management of benign prostatic hyperplasia in intact male dogs, would also be effective for the treatment of urinary incontinence in spayed bitches. It was hypothesized that spayed incontinent bitches immunized against GnRH would develop antibodies that would prevent gonadotropin elevation, resulting in a return to continence.

**METHODS.** Nine privately-owned bitches diagnosed with urinary incontinence following ovariohysterectomy were used for this study. All bitches had been receiving daily oral phenylpropanolamine (PPA; Proin®, PRN Pharmacal, Pensacola, FL) to control incontinence prior to the start of the study. Six bitches received two subcutaneous injections of Canine Gonadotropin Releasing Factor Immunotherapeutic® vaccine (Pfizer Animal Health, Exton, PA) at 4 week intervals, and three bitches received two placebo injections at 4 week intervals. Venous blood samples were collected pre-vaccination (week 0) and weeks 4, 6, 8, 10, 12, 16, 20, and 24. Vaccinated bitches discontinued PPA use two weeks after receiving the second injection; control bitches remained on PPA for the duration of the study. Owners recorded all episodes of incontinence. Serum GnRH antibody titers were determined by an enzyme linked immunosorbent assay and antibody titers were analyzed using Fisher's exact test (GraphPad QuickCalcs Software, La Jolla, CA). Significance was defined as $p<0.05$.

**RESULTS.** Side effects of the vaccine included soreness at the injection site (n=1), swelling at the injection site (n=1), lameness (n=1), and tachypnea (n=1, dog removed from the study). All side-effects resolved without treatment within 24 hours. All dogs were seronegative for GnRH antibodies at week 0 and control dogs remained seronegative for the duration of the study. Vaccinated dogs developed a GnRH antibody titer that was significant compared to control dogs at weeks 4-16 ($p=0.02$). Of the five vaccinated dogs that completed the study, two dogs remained continent for 112.5±23.3 days after PPA was discontinued, while three dogs became incontinent 3.6±2.1 days after PPA was discontinued despite having elevated GnRH antibody titers.

**CONCLUSION.** The pathophysiology of urinary incontinence following ovariectomy is not well understood. The results of this study show that reducing gonadotropin concentrations by GnRH immunization may be necessary but not sufficient to restore continence in all bitches. Plasma luteinizing hormone measurements are currently being performed to determine whether GnRH immunization vaccine effectively decreases gonadotropin concentrations.
REFERENCES.
INTRODUCTION. GnRH is responsible for the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the pituitary, which regulates reproductive steroid hormone synthesis. When vaccinated against GnRH, endogenous GnRH will be prevented from binding to its receptor in the pituitary, halting LH and FSH production. As a result, steroid hormones will not be synthesized. The objective of this study was to measure the endocrine responses to GnRH immunization in intact male dogs. Based upon the manufacturer’s label, we hypothesized that vaccinating against GnRH would elicit a GnRH antibody titer and decrease LH and testosterone concentrations in intact male dogs for six months.

METHODS. Four privately-owned intact male dogs were used for this study. All were vaccinated with Canine Gonadotropin Releasing Factor Immunotherapeutic® (Pfizer Animal Health, Exton PA) and boostered again four weeks later. Venous blood samples were collected at 0, 4, 12, and 20 weeks. Sera were separated, aliquoted, and frozen at -20°C until analyzed.

GnRH antibody titers were determined using an enzyme-linked immunosorbent assay (ELISA) using 1 μg/mL LH-RH as the antigen (71447-49-9, Sigma, St. Louis, MO, USA). LH samples were run in duplicate using a canine-specific ELISA (LH-Detect® for canines, ReproPharm, Nouzilly, France) and performed according to the manufacturer’s instructions. The sensitivity of the assay was 0.01 ng/mL and a value greater than 0.80 ng/mL was determined to be a positive result. Testosterone concentrations were determined using a double antibody radioimmunoassay (Diagnostic Products Corporation, Los Angeles, CA). The detection limit of the assay was 0.04 ng/mL.

Mean±SEM were determined for LH concentrations and compared between animals at the four time points using ANOVA with Bonferroni corrections. Mean±SD were determined for testosterone and GnRH antibody concentrations and compared between animals using ANOVA with Bonferroni corrections. Analysis was performed using Stata statistical software (Version 12; StataCorp. 2011, College Station, TX). Significance was defined as p<0.05.

RESULTS. GnRH titers peaked 12 weeks following initial vaccination, which differed significantly from weeks 0 and 20 (Figure 1). Although LH and testosterone
concentrations appeared to lower at week 4 and week 12, respectively, these changes were not significant.

**DISCUSSION.** GnRH immunization is an ideal candidate for nonsurgical contraception in dogs and cats because a single product should be effective in both males and females. However, GnRH is a weak immunogen and must be coupled to a large protein and combined with an adjuvant to enhance its antigenicity (1). The vaccine used in the current study was prepared commercially and our laboratory has previously used this vaccine to suppress estrus in female horses (2). It is not clear why these four male dogs responded weakly to vaccination. However, using a different GnRH vaccine construct, Levy and coworkers (2004) reported that one-third of the male cats vaccinated against GnRH had a partial response with low positive GnRH antibody titers accompanied by equivocal serum testosterone concentrations (3). The small sample size in the current study is a limitation. Although assuming the differences observed in this study would be similar in a large population, 19 dogs would have needed to be studied to demonstrate significance.

**REFERENCES.**


**Figure 1.** Serum GnRH antibody titer as compared to testosterone and LH concentration (ng/mL) at weeks 0, 4, 12, and 20 of four intact male dogs vaccinated with Canine Gonadotropin Releasing Factor Immunotherapeutic®.
Physiologic Responses To Gonadotropin Releasing Hormone (GnRH) Immunization In Intact Male Dogs

Caitlin Donovan1, Martha Green2, Michelle Kutscher1

1Department of Animal Sciences, Oregon State University, Corvallis, Oregon, USA
2Veterinary Medicine, International College of Veterinary Medicine, Nashville, Tennessee, USA

INTRODUCTION

Gonadotropin releasing hormone (GnRH) and follicle stimulating hormone (FSH) from the anterior pituitary, which in turn regulate reproductive steroid hormone synthesis.

Objective Immunization prevents GnRH from binding to its receptor on the anterior pituitary, reducing LH and FSH production and therefore preventing steroid hormone synthesis (Figure 1).

In the male, testosterone is necessary for spermatogenesis. Furthermore, testicular volume is a reflection of spermatogenesis.

RESULTS

1. Four dogs produced GnRH antibodies following GnRH immunization (Figure 4).
   - Serum LH levels were significantly decreased at weeks 9, 12, 15, and 20 compared to week 1.

2. Four DESVOT treated dogs had a decrease in serum testosterone following GnRH immunization (Figure 5).
   - Testosterone levels were significantly decreased at week 12.

3. Four dogs displayed an apparent but insignificant decrease in LH concentrations following GnRH immunization (Figure 4).

4. Four dogs displayed a decrease in testicular volume following GnRH immunization (Figure 5).
   - The percent change was significantly decreased at week 12 compared to weeks 9, 12, 15, and 20.

   *Week 12 was significantly decreased compared to weeks 9 and 12.

DISCUSSION

The results of this study indicate that immunization against GnRH using Canine GnRH Inhibiting Factor (GIF) can successfully induce a temporary hormonal response and significantly decrease testicular volume.

Additional studies are necessary to evaluate changes in sexual behavior in response to the immunization.

REFERENCES


ACKNOWLEDGMENTS

This research was supported by a grant from the National Institute of Health (NIH) and the American Society for Reproductive Medicine (ASRM).