COMPOUND 6, A SYNTHETIC KISSPEPTIN ANALOG, INCREASES PLASMA LUTEINIZING HORMONE CONCENTRATIONS IN BEEF COWS UNDER LOW CIRCULATING PROGESTERONE CONCENTRATIONS

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ABSTRACT

Reproductive performance affects profitability in the food animal industry. Estrus synchronization has improved fertility, but hormonal tools like kisspeptin (KP) have potential for further enhancement. However, KP's short half-life limits its use in cattle. Compound 6 (C6), a synthetic KP analog, was created to improve stability and extend half-life. While C6 has stimulated reproductive responses in other species, its ability to induce LH secretion and ovulation in cattle is less understood.

The study investigated the effects of C6 on plasma LH, serum progesterone (P4), and ovarian dynamics in cattle. Ten cows underwent two treatments: control (CON; 2 mL saline IM; n=10) or C6 (100 nmol in 2 mL saline IM; n=10). Estrus synchronization was achieved using prostaglandin $F_{2\alpha}$, GnRH, and internal progesterone-releasing devices (CIDR), with treatments on Day 6 of follicular wave emergence. With CIDRs in place to keep circulating P4 low, blood samples were collected hourly for 12 hours post-treatment to measure plasma LH. Additional blood samples for P4 analysis and ultrasonography of ovaries were collected at -1 and +11 hours, twice daily for four days post-treatment, and once daily thereafter for three days. C6 significantly increased plasma LH concentrations ($p < 0.0001$) compared to CON, especially at Hours 4, 5, and 6. Luteinizing hormone levels remained elevated ($p < 0.0001$) from Hours 4 to 7 in the C6 group.

Serum P4 concentrations were higher in the CON group at baseline ($p < 0.0078$) and decreased significantly after CIDR removal (Hours 168, 192 and 216; $p < 0.0001$). C6 treatment led to a significant reduction in P4 at Hour 192 ($p = 0.045$). Ultrasonography did not show luteal tissue, but C6-treated cows were more likely ($p < 0.0001$) to lose a dominant follicle within 36 hours of treatment. Collectively, C6 caused an increase in plasma LH concentrations and

possibly promoted ovarian follicular turnover. Further research is warranted to determine if the C6 induced increase in LH consistently promotes ovarian follicular turnover and the effectiveness of integrating C6 into bovine breeding programs as an alternative or supplement to GnRH-based synchronization.

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CHAPTER ONE

LITERATURE REVIEW

Reproductive efficiency is a critical determinant of productivity and profitability in cattle operations. The ability of an individual in a herd to conceive, maintain pregnancy, and successfully calve within a defined breeding season directly impacts economic returns. Infertility and reproductive inefficiencies, including prolonged calving intervals, embryonic loss, and anestrus, pose significant financial burdens on beef and dairy producers [1]. In the United States alone, infertility-related losses in the beef industry are estimated to exceed $249 million annually due to increased maintenance costs, reduced weaning weights, and diminished lifetime productivity of affected animals [2]. As such, improving reproductive efficiency through strategic reproductive management is essential for optimizing herd performance and ensuring economic sustainability.

Hormonal regulation is fundamental to reproductive success in cattle, governing the estrous cycle, follicular development, ovulation, and corpus luteum (CL) function. The hypothalamic-pituitary-gonadal (HPG) axis orchestrates reproductive processes through the secretion of key hormones, including gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH), follicle-stimulating hormone (FSH), progesterone (P4) and estradiol (E2) [3]. These hormones function in a tightly regulated feedback system to coordinate ovulation and maintain pregnancy.

Gonadotropin releasing hormone, synthesized and released by the hypothalamus, serves as the primary regulator of reproductive endocrinology. It is secreted in a pulsatile manner, stimulating the anterior pituitary gland to release LH and FSH, which in turn regulate ovarian follicular growth and ovulation [4]. Luteinizing hormone plays a pivotal role in the final

maturation of the dominant follicle and triggers ovulation, whereas FSH supports the recruitment and growth of ovarian follicles [3]. As the dominant follicle develops, granulosa cells produce increasing concentrations of E2, which stimulates estrus behavior and provides positive feedback to the hypothalamus to induce the preovulatory LH surge [4]. Following ovulation, the remnants of the ovarian follicle, granulosa and theca cells, differentiate into the CL, which secretes P4, the dominant hormone of the luteal phase of the estrous cycle. Progesterone inhibits GnRH pulsatile release, thereby affecting LH secretion to prevent further ovarian follicular development and ovulation during pregnancy [5].If conception does not occur, the uterus releases prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), which induces luteolysis, leading to CL regression, a decline in circulating P4, and the initiation of a new estrous cycle. This cyclical hormonal interplay is essential for reproductive efficiency, influencing conception rates, embryo survival, and overall fertility outcomes.

Given the economic and biological significance of reproductive efficiency, substantial research has been dedicated to developing strategies that manipulate these hormonal pathways to optimize reproductive performance. Synchronization protocols, artificial insemination, and emerging reproductive biotechnologies leverage hormonal control to improve fertility and enhance reproductive outcomes in cattle [6]. As scientific advancements continue to refine our understanding of reproductive endocrinology, novel hormonal treatments, including kisspeptin (KP) analogs such as Compound 6 (C6), present promising opportunities for improving reproductive management in cattle. This literature review explores the fundamental hormonal mechanisms governing cattle reproduction, the historical development of hormonal interventions, and the potential of emerging reproductive technologies to enhance fertility and efficiency in livestock production.

The Hypothalamic-Pituitary-Gonadal Axis

The HPG axis is the central regulatory system controlling reproductive function in cattle. This axis integrates endocrine signals from the hypothalamus, anterior pituitary, and gonads to synchronize reproductive events, including ovarian follicular development, ovulation, and pregnancy maintenance [3]. The precise coordination of hormone secretion within the HPG axis is essential for reproductive efficiency, as disruptions in its regulation can lead to infertility, subfertility, or irregular estrous cycles [7].

At the top of the HPG axis, the hypothalamus serves as the primary regulatory center for reproductive endocrinology. Specialized neurons within the arcuate/infundibular nucleus and preoptic area of the hypothalamus secrete GnRH in distinct patterns: pulsatile secretion from the tonic center and surge secretion from the surge center. These patterns of GnRH release are essential for the regulated stimulation the anterior pituitary [8]. GnRH secretion is modulated by feedback mechanisms from ovarian hormones, particularly E2, inhibin, and P4, ensuring that reproductive events occur in a controlled and cyclic manner [9].

The anterior pituitary, the second component of the HPG axis, responds directly to hypothalamic GnRH signals and secretes LH and FSH, the two primary gonadotropins involved in regulating ovarian function [10]. Follicle stimulating hormone facilitates the recruitment and early development of a cohort ovarian follicles, while LH is involved in further maturation of dominant ovarian follicles, triggering ovulation, and is critical for CL formation [3]. In cattle, ovarian follicular development occurs in waves during both the luteal and follicular phases of the estrous cycle. During the luteal phase, high circulating P4 suppresses GnRH pulse frequency, leading to basal FSH release sufficient to recruit a new wave of ovarian follicles. However, due

to low LH pulse frequency and high P4, even dominant follicles undergo atresia without reaching ovulatory status. In contrast, during the follicular phase following luteolysis, P4 levels decline, GnRH pulse frequency increases, and dominant follicles are supported by both basal FSH and increasing LH stimulation, allowing them to reach ovulatory size [11]. Pre-dominant or recruited ovarian follicles are highly dependent on FSH for their initial growth and survival, while the dominant follicle, once selected, becomes increasingly LH dependent as it matures. The transition from dependence on FSH to LH occurs as the dominant ovarian follicle matures. This maturation is characterized by an increased expression of LH receptors on granulosa cells, in addition to the LH receptors already present on the theca cells, and allows the dominant follicle to continue steroidogenesis and growth. As this dominant follicle grows, it secretes increasing amounts of E2 and inhibin, which both have a negative feedback effect on FSH secretion at the anterior pituitary. In contrast, subordinate ovarian follicles, which do not gain LH receptors on their granulosa cells, remain highly dependent on FSH and as FSH levels decrease, these follicles lose their proliferative stimulus and undergo atresia. The increasing production of E2 by the dominant ovarian follicle not only suppresses FSH to maintain its competitive advantage but also plays a role in coordinating the next stages of the reproductive cycle.

The gonads, the final component of the HPG axis, serve as both targets and regulators of endocrine function. In females, the ovary produces two primary steroid hormones: E2 and P4, which modulate GnRH and gonadotropin secretion via feedback mechanisms. During follicular development, granulosa cells convert androgens into E2, which promotes ovarian follicular growth, induces estrus behavior, following a decline in circulating concentrations of P4, and, once it reaches a threshold concentration, triggers the preovulatory LH surge [10]. This surge initiates ovulation and the subsequent formation of the CL from the ruptured follicle [3].

Following ovulation, the endocrine focus shifts from the E2 driven ovarian follicular events to P4 dominated luteal activity, which prepares the system for a potential pregnancy.

After ovulation, the CL becomes the dominant ovarian structure, producing P4, the primary hormone of the luteal phase. Progesterone exerts strong negative feedback on the hypothalamus with additional effects on the anterior pituitary. High circulating P4 concentrations slow the frequency of GnRH pulses released from the hypothalamus. This reduced GnRH pulse frequency leads to a corresponding decrease in LH pulse frequency, which is important for supporting dominant ovarian follicle growth. Although FSH secretion still occurs at basal levels, the suppressed LH environment prevents further ovarian follicle maturation. As a result, new ovarian follicular waves may be initiated, but developing dominant follicles typically undergo atresia rather than progressing to ovulation. This hormonal suppression maintains the reproductive system in a transient state that is either preparing the uterus for pregnancy or regulating the cycle in the absence of conception [12]. If pregnancy does not occur, the uterus releases prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), which induces CL regression (luteolysis), leading to a decline in circulating P4, the removal of its inhibitory effect on pulsatility of GnRH release, and the initiation of a new estrous cycle [11]. The interplay between gonadotropins and ovarian hormones ensures the proper timing of reproductive events. Follicle stimulating hormone-dependent ovarian follicular recruitment and growth set the stage for ovulation, while E2-driven LH surges trigger ovulation and CL formation. The cyclic rise and fall of P4 and E2 regulate GnRH pulsatility, controlling the pattern of gonadotropin release and ensuring the estrous cycle progresses in a predictable and synchronized manner [11].

Understanding the regulation of the HPG axis is fundamental for improving reproductive efficiency in cattle. Modern reproductive technologies, including estrus synchronization

protocols, which aid in the use of artificial insemination and embryo transfer, manipulate this axis to enhance fertility and reproductive outcomes [12]. Further research into emerging reproductive hormones, such as KP and its analogs, may provide novel tools to fine-tune hormonal regulation and improve reproductive efficiency in cattle production systems. The earliest scientific research into using GnRH to control reproductive outcomes in cattle dates to the early 1970s. Following the identification and structural characterization of GnRH in the early 1970s, researchers began synthesizing GnRH analogs to address reproductive dysfunctions and enhance reproductive efficiency in farm animals [13]. These analogs were developed to improve the potency and efficacy of GnRH in regulating reproductive functions, leading to advancements in estrus synchronization protocols and overall reproductive management in cattle.

Bovine Estrous Cycle & Synchronization

The bovine estrous cycle is a tightly regulated reproductive process that governs ovarian follicular development, ovulation, and the maintenance or termination of pregnancy. This cycle lasts approximately 18–24 days in cattle and is divided into two major phases: the follicular phase and the luteal phase, each characterized by distinct hormonal patterns and physiological changes [3]. The follicular phase (~4–6 days in duration) begins with luteolysis, the regression of the CL, which leads to a decline in circulating P4 concentrations. As P4 concentrations decrease, frequency of GnRH pulses from the hypothalamus increases, stimulating the anterior pituitary to release LH [10]. At the same time, increased FSH promotes the recruitment of a cohort of ovarian follicles, initiating a new follicular wave. Early in the wave, all follicles are under the influence of FSH and grow larger. With growth, the dominant ovarian follicle begins to express more LH receptors on its granulosa cells, which reduces the follicle's dependance on FSH for growth and separates it from the subordinate follicles within the cohort. This growing follicle

releases increased concentrations of E2 and inhibin, which have a negative feedback effect on the anterior pituitary. This negative feedback will suppress FSH production further and this decline in FSH will cause the smaller follicles to discontinue their growth and undergo atresia [10]. When E2 produced by the dominant follicle reaches a threshold circulating concentration, it triggers a positive feedback loop on the hypothalamus and pituitary, leading to a preovulatory surge of GnRH and LH which leads to ovulation, or the rupture of the mature dominant follicle and the release of an oocyte for potential fertilization [3].

Following ovulation, the cycle transitions into the luteal phase (~14–18 days in duration), during which the ruptured follicle transforms into a functional CL that secretes P4 [14]. Progesterone exerts negative feedback on the hypothalamus and anterior pituitary, reducing GnRH pulse frequency and thereby reducing LH pulse frequency, but FSH secretion continues in basal pulses. As a result, new ovarian follicular waves are initiated even during the luteal phase. Small cohorts of ovarian follicles are recruited and begin to grow under the influence of FSH but because of the low LH pulse frequency and high circulating P4, these follicles cannot fully mature or ovulate. Typically, one dominant ovarian follicle emerges during a follicular wave but in the presence of high circulating P4 concentrations and insufficient LH stimulation, the development of another dominant follicle is prevented [6]. If pregnancy does not occur, the uterus releases $PGF_{2\alpha}$ which induces luteolysis, or regression of the CL, and a decline in circulating P4 concentrations. As P4 declines, GnRH pulse frequency increases, which increases LH pulse frequency in the next follicular phase of the cycle [9].

Synchronization protocols have been developed to manipulate the estrous cycle, aligning ovulation within a controlled timeframe to facilitate different advanced reproductive techniques, like timed artificial insemination (TAI) without the need for estrus detection and embryo

transfer, to name a few. These protocols, a few of which are described below, enhance reproductive efficiency by controlling GnRH, LH, FSH, and P4 concentrations to regulate ovarian follicular waves and ovulation [15].

1. **Ovsynch Protocol**: The Ovsynch protocol, first developed in the 1990s, utilizes a combination of GnRH and $PGF_{2\alpha}$ to synchronize ovulation [12].
    - Day 0: An initial GnRH administration stimulates the ovary to induce ovulation of a dominant follicle and initiate a new follicular wave.
    - Day 7: Administration of $PGF_{2\alpha}$ to induce luteolysis, reducing circulating concentrations of P4, and allowing follicular growth to continue.
    - Day 9: A second dose of GnRH is administered which triggers an LH surge, leading to ovulation.
    - Day 10: Timed artificial insemination is completed 16–20 hours after the final GnRH administration.

    This protocol is widely used in dairy cattle due to its ability to eliminate the need for estrus detection while achieving consistent pregnancy rates [6].
2. **CO-Synch Protocol:** The CO-Synch protocol is a variation of Ovsynch commonly used in beef cattle, designed to be simpler and more practical for field conditions [15]. It follows a similar GnRH- $PGF_{2\alpha}$ -GnRH sequence but combines TAI with the second GnRH administration on Day 10, rather than 16–20 hours later. CO-Synch has been particularly effective in postpartum beef cows, improving fertility rates while reducing labor-intensive estrus detection [12].
3. **CIDR-Based Protocols:** The Controlled Internal Drug Release (CIDR) system delivers exogenous P4 to regulate estrous cycles [16]. A CIDR device is inserted intravaginally

for 7 days, maintaining circulating P4 concentrations to prevent ovulation and possibly aid in control of timing of $PGF_{2\alpha}$ release from the uterus. On Day 7, $PGF_{2\alpha}$ is administered, followed by CIDR removal, leading to a rapid decline in circulating P4. Gonadotropin releasing hormone is administered to induce ovulation, with TAI occurring 48–56 hours later. CIDR-based protocols are especially beneficial for anestrous or non-cycling cows, as they increase the proportion of cows exhibiting estrous cyclicity before breeding [16].

The implementation of estrus synchronization protocols has provided substantial economic advantages in cattle production. By reducing the need for estrus detection and enabling TAI, synchronization protocols improve conception rates, shorten calving intervals, and enhance genetic selection through artificial insemination [17]. Studies have demonstrated that TAI programs lead to higher pregnancy rates, improved calving distributions, and increased weaning weights, all of which contribute to greater economic returns [15]. Despite these benefits, the adoption of synchronization protocols remains relatively low in some sectors, particularly among small- to mid-sized beef operations [1]. Several barriers to widespread adoption include cost, labor requirements, and variable response rates. Some protocols involve multiple treatments and handling events, making them impractical for large or extensively managed herds [17]. Hormonal treatments and the labor required for these multiple handling events can be expensive, particularly for operations with low reproductive efficiency [14]. While estrus synchronization improves pregnancy rates, factors such as body condition, nutrition, and postpartum anestrus influence the effectiveness of these protocols overall [2, 14]. To address these challenges, simplified synchronization protocols and longer-lasting hormonal treatments are being developed to improve reproductive outcomes with reduced labor inputs [14]. Emerging research and

continued advancements in reproductive technologies and alternative reproductive hormones, such as KP and its synthetic analogs, aims to enhance synchronization strategies by reducing variability in ovarian response and providing more targeted reproductive interventions.

Kisspeptin

Kisspeptin, a 54-amino acid neuropeptide encoded by the KISS1 gene, was initially discovered in the 1990s as a tumor metastasis suppressor in human melanoma cells [18]. However, its role in reproductive function was later discovered when mutations in KISS1R (formerly GPR54) were found to cause hypogonadotropic hypogonadism, a condition characterized by delayed puberty and infertility due to impaired gonadotropin release [19]. This pivotal discovery identified KP as a critical upstream regulator of GnRH secretion and reproductive function [20]. Kisspeptin plays a central role in the HPG axis, acting as a key modulator of GnRH pulsatility and, consequently, LH and FSH secretion [8]. Structurally, the full-length peptide (KP-54) can be processed into shorter active fragments like kisspeptin-13 (KP-13), kisspeptin-14 (KP-14) and kisspeptin 10 (KP-10). Of these, KP-10 is the smallest fragment that retains full biological activity at the KISS1 receptor [21]. The identification of KP as an essential regulator of puberty onset, reproductive hormone release, and fertility has since positioned it as a focal point in reproductive endocrinology research, with increasing interest in its potential applications in livestock reproductive management.

Kisspeptin plays its role in the regulation of reproductive function through two primary hypothalamic populations: kisspeptin/neurokinin β /dynorphin (KNDy) neurons, located in the arcuate nucleus (ARC) of the hypothalamus, and kisspeptin-only neurons located in the pre-optic area (POA) [22]. These two populations serve distinct yet complimentary roles in the control of GnRH release. KNDy neurons in the ARC integrate endocrine and environmental cues to govern

the pulsatile release of GnRH, which is critical for maintaining normal reproductive function [8]. Within this network, neurokinin β (NKβ) acts as a stimulatory signal, promoting KP secretion and facilitating GnRH pulse generation [23]. Estradiol has been shown to enhance NKβ expression in KNDy neurons during the follicular phase, increasing GnRH pulse frequency and leading to the preovulatory LH surge. In contrast, dynorphin (Dyn) serves as an inhibitory counterpart, preventing excessive GnRH secretion by modulating the frequency and amplitude of pulses [22]. During the luteal phase, rising circulating P4 concentrations stimulate Dyn expression, which suppresses KP activity and reduces GnRH pulse frequency. This inhibition contributes to the characteristic decline in LH pulse frequency observed during diestrus.

In contrast to the ARC population, POA KP neurons, which do not co-express NKβ or Dyn, are directly involved in initiating the preovulatory GnRH/LH surge. These neurons are sensitive to rising circulating E2 concentrations, and once a critical threshold is reached, E2 stimulates POA KP expression, triggering the GnRH surge required for ovulation [13, 24]. So, while ARC KNDy neurons regulate basal GnRH pulsatility, POA KP neurons modulate the positive feedback mechanisms that lead to ovulation.

The coordinated action between these two neuron populations is responsible for the proper timing and regulation of both tonic and surge modes of GnRH secretion. During the follicular phase of the estrous cycle, increased E2 levels enhance KP activity in both regions, resulting in more frequent LH pulses and ultimately the LH surge [4]. Conversely, during the luteal phase, increased circulating P4 concentrations stimulate Dyn, inhibits KP output from KNDy neurons, and suppresses GnRH/LH secretion. Disruptions in this system, such as alterations in KP expression or KNDy neuron activity, can lead to reproductive disorders, delayed puberty, or anovulation. Kisspeptin is also responsive to metabolic and environmental

cues, linking energy balance and reproductive function. Nutritional deficits or stress conditions can suppress KP expression, leading to reduced GnRH secretion and infertility [25]. This adaptive mechanism ensures that reproduction occurs only under favorable metabolic conditions, highlighting KP's role as a reproductive integrator of nutritional and environmental signals[7].

The discovery of KP as a key upstream regulator of GnRH secretion has generated significant interest in its potential applications for improving reproductive efficiency in cattle. Research on exogenous KP administration has demonstrated its ability to stimulate LH release, synchronize ovulation, and potentially enhance fertility [26]. However, the clinical use of native KP presents several limitations. Native KP, particularly Kisspeptin-10 (KP-10), is rapidly degraded in circulation, resulting in a short biological half-life [27]. KP-10 is a 10 amino acid peptide fragment of the full-length KP-54, and although both are biologically active, KP-10 has reduced stability and a shorter duration of action in vivo. Consequently, studies in cattle have required either continuous intravenous infusion or relatively high doses of KP-10 to achieve sufficient stimulation of LH release and ovulation [28]. Even under these conditions, the ovulatory response to exogenous native KP remains inconsistent and highly dependent on the animal's endocrine status, particularly circulating P4 concentrations. Early research demonstrated that peripheral administration of KP-10 significantly increased plasma LH concentrations in cows and heifers, with effects observed across different reproductive stages, suggesting that KP acts primarily by stimulating GnRH neurons [29, 30].

In addition to its well-established role in the hypothalamus, there is growing evidence that KP and its receptor, KISS1R, are also expressed locally within the ovary, suggesting the possibility of autocrine or paracrine roles in follicular development and ovulation. Both KISS1 and KISS1R transcripts have been detected in the ovarian tissue of multiple species, including

cattle, sheep, rodents and primates, indicating that KP may function in an autocrine or paracrine manner to influence ovarian physiology [31]. Studies in rodents and primates have shown that KP signaling within the ovary may influence oocyte maturation, steroidogenesis, and ovulatory mechanisms independent of hypothalamic GnRH stimulation [32]. Additionally, KP expression in the ovary appears to vary across the estrous cycle, with increased expression noted around the time of ovulation, further supporting its potential involvement in follicular development and ovulatory signaling [33]. While the relevance of ovarian KP in cattle is still under investigation, these findings suggest a broader role for KP signaling that extends beyond the hypothalamus and may complement central actions in regulating fertility.

Further studies investigated whether KP-10 administration could synchronize ovulation by mimicking the preovulatory LH surge. Leonardi et al. found that administration of KP-10 during the luteal phase of the estrous cycle induced a transient increase in circulating concentrations of LH but did not consistently trigger ovulation, suggesting that additional hormonal support, like P4 priming or co-administration of GnRH, may be necessary for consistent ovulatory responses [26]. More recently, Leonardi examined the mechanism by which peripherally administered KP-10 induces LH release in cattle [9]. Those findings indicated that KP-10 does not cross the blood-brain barrier, but instead acts at the median eminence to stimulate the release of pre-synthesized GnRH, leading to increased LH secretion. This mechanism is consistent with observations in other mammalian species, supporting the idea that KP's primary site of action is within the hypothalamus rather than directly on the pituitary gland [22].

The ability of KP to stimulate LH secretion has been a major focus of reproductive endocrinology research. Unlike GnRH, which causes an immediate and robust LH surge, KP

appears to produce a more prolonged LH secretion pattern, which may be beneficial for improving ovarian follicular development and synchronizing ovulation [30]. It has been demonstrated that KP administration in ewes induced synchronized preovulatory LH surges and ovulation, even during the non-breeding season, which suggests that KP can override seasonal anestrus. Ahmed et al. found that KP-10 stimulated both LH and FSH secretion in prepubertal calves, indicating its potential role in advancing puberty and improving fertility in heifers [34]. These findings suggest that while KP administration effectively enhances gonadotropin secretion, its ability to trigger ovulation remains variable. Factors such as the stage of the estrous cycle, serum P4 concentrations, and overall reproductive status may influence its effectiveness, necessitating further research into combination protocols with other reproductive hormones.

Despite its promising role in reproductive hormone regulation, natural or native KP faces several challenges that limit its practical application in cattle reproduction. KP-10 has a short half-life in circulation due to rapid degradation by proteolytic enzymes, making its effects transient and less predictable [35]. This necessitates frequent or continuous administration, which is not feasible for commercial cattle operations. Peripheral administration of KP does not directly activate GnRH neurons within the hypothalamus, as KP-10 does not efficiently cross the blood-brain barrier [9]. This limits its ability to fully mimic the endogenous KP-GnRH signaling pathway seen in natural reproductive cycles. While KP reliably stimulates LH secretion, its ability to induce ovulation is inconsistent in cattle, particularly in cows that are not at the appropriate stage of ovarian follicular development [4]. This suggests that KP alone may not be sufficient to replace GnRH-based synchronization protocols. The cost of synthetic KP peptides also remains relatively high, and efficient delivery methods for sustained release formulations have yet to be optimized for large-scale cattle production [35].

Compound 6

While native KP has demonstrated the ability to stimulate GnRH and LH secretion, its clinical and agricultural applications are limited by its rapid degradation and short biological half-life [35]. KP-10 is highly susceptible to proteolytic degradation, resulting in transient endocrine effects that require frequent administration to sustain LH secretion [36]. Additionally, because KP-10 does not efficiently cross the blood-brain barrier, its access to GnRH neuronal cell bodies within the hypothalamus is limited [9]. Instead, peripherally administered KP-10 interacts with GnRH nerve terminals within median eminence (ME), a circumventricular region lacking a blood brain barrier, by binding to the KISS1R [37]. Although this interaction can trigger GnRH release into the hypophysial portal circulation, it may bypass upstream neuronal regulation, possibly restricting the duration and coordination of downstream LH and FSH release. These challenges have prompted the development of synthetic KP analogs, designed to increase metabolic stability, prolong biological activity, and enhance receptor binding affinity while maintaining potent gonadotropic effects. Early efforts employed structural modifications to the KP-10 sequence to identify critical residues essential for receptor activation, such as a phenylalanine at positions 6 and 10, and arginine at position 9 [38]. Those findings guided subsequent design of analogs incorporating conservative substitutions at non-essential positions with the goal of improving half-life and resistance to enzymatic degradation. One such analog, FTM080, demonstrated the ability to stimulate LH release in anestrous ewes, although its effectiveness required higher dosing, and it's in vivo activity was shorter-lived than that of KP-10 [39]. The receptor agonist for kisspeptin-54, MVT-602, caused a therapeutic-level LH response in humans with a reported half-life of 1.5-3.5 hours [27]. Further innovations came from a rational design approach, which systematically addressed both proteolytic degradation

and renal clearance by introducing chemical modifications into the KP-10 backbone. This approach led to the synthesis and evaluation of a series of triazololipopeptide analogs with varying modifications, including N-terminal acetylation, triazole substitutions at protease sensitive sites, and different albumin binding motifs [35]. These analogs were tested for serum stability, receptor potency, and ability to stimulate prolonged LH release in vivo and while early versions showed mixed results, iterative modifications revealed that combining a single triazole substitution at the Gly7–Leu8 bond with a strategically placed albumin-binding group provided the best balance of bioactivity and extended half-life [35]. This process ultimately informed the development of Compound 6, a rationally designed synthetic KP-10 analog that incorporates chemical modifications to improve its resistance to enzymatic degradation and extend its duration of action [35]. These modifications allow C6 the ability to extend the duration of LH secretion following administration, potentially offering a more practical alternative to native KP for use in reproductive interventions for cattle.

Several studies have investigated the efficacy of C6 as a potential tool for stimulating LH release and inducing ovulation. During the initial development and validation of C6, researchers demonstrated that C6 induced a prolonged increase in circulating concentrations of LH in rodent models, lasting significantly longer than KP-10 [36]. In 2019, Parker et. al determined the acute and subacute effects of C6 on plasma concentrations of LH and FSH in prepubertal bull calves, where C6 not only significantly increased plasma LH concentrations compared to KP-10, but the concentrations were maintained for a longer duration [40]. This suggests that C6 might be useful in stimulating puberty onset and enhancing testicular development in bulls, with potential applications in human male fertility. Leonardi et al. investigated the mechanism by which KP-10 induces LH secretion in cattle [9] and found that kisspeptin primarily stimulates the release of

pre-synthesized GnRH. Similarly, C6 has been shown to trigger GnRH-mediated LH release, but with a significantly prolonged effect due to its enhanced metabolic stability and slower clearance from circulation [41].

In addition to its hypothalamic effects, emerging data suggest that KP and its analogs, such as C6, may exert direct local effects on ovarian tissues [42]. The presence of KISS1R in ovarian structures supports this hypothesis. This local action would be like that of human chorionic gonadotropin (hCG) in cattle and humans, or equine chorionic gonadotropin (eCG) gonadotropin in mares, which directly bind to LH receptors in ovarian cells to promote ovulation or luteinization. If KP and/or C6 act locally, they could influence ovarian follicular development or atresia independent of the indirect effects of KP and/or C6 on LH, offering a dual mechanism of action.

Preliminary research on KP administration in cows suggests that it may enhance follicular development and ovulatory responses, but its efficacy as a sole ovulation-inducing agent remains inconsistent [41]. C6 holds promise for practical applications in cattle reproduction, with potential uses in a variety of areas including treatment for reproductive endocrine disorders of cattle, like cystic ovarian disease (COD). COD is characterized by the persistence of anovulatory follicles often due to disrupted GnRH/LH signaling and failure to induce a normal preovulatory LH surge [43]. By reliably stimulating LH release, C6 may help resolve persistent follicles and support ovulatory function in affected animals. Beyond its potential use in treating reproductive disorders, C6 could serve as a substitute for or in combination with GnRH-based synchronization protocols, enhancing fertility in anestrous cows by maintaining LH secretion, and advancing puberty by stimulating gonadotropin release in prepubertal heifers and bull calves. However, further research is necessary to optimize C6

dosing, timing, and combination with other reproductive hormones to maximize its effectiveness in commercial cattle production systems.

CHAPTER TWO

INTRODUCTION

Reproductive efficiency, defined as the capacity of animals to successfully reproduce and produce offspring, is a cornerstone of productivity and profitability in food animal production. Effective management of reproductive cycles is crucial and widely practiced, with reproductive synchronization protocols used in at least 58% of beef cow-calf operations in the United States [2]. Improvements in the reproductive metrics of a beef herd (consistent pregnancy rates, live births, and shorter calving intervals) are major goals for beef producers as the costs associated with reduced fertility can be estimated to be $210 million [1]. Even modest improvements in the management of reproductive health can have significant economic and productive benefits, with just a 4% increase in pregnancy rates resulting in 1.1 million more beef calves born each year, substantially increasing herd productivity and profitability [5, 44].

The heart of regulating reproductive function is the hypothalamic-pituitary-gonadal (HPG) axis, a highly coordinated neuroendocrine system responsible for the regulation and release of key reproductive hormones [5]. Within this axis, gonadotropin releasing hormone (GnRH) from the hypothalamus signals the anterior pituitary gland to release luteinizing hormone (LH) and follicle stimulating hormone (FSH), hormones critical for reproductive success. Extensive research efforts since the 1990s, particularly those investigating estrus synchronization protocols [6], have deepened the scientific understanding of hormonal pathways and the role of upstream regulators, or molecules that initiate and modulate the activity of key hormonal pathways, such as the neuropeptide kisspeptin (KP). The role of KP in reproductive regulation was first recognized in 2003, when mutations in the kisspeptin receptor, known as KISS1R (formerly GPR54), were found to cause hypogonadotropic hypogonadism in humans

and in mice [19, 20]. Kisspeptin acts at the very beginning of the hormonal cascade, triggering the release of GnRH and setting off the chain of events that govern reproductive function. Exogenous administration of KP has been shown to stimulate the secretion of GnRH and subsequently LH in multiple species, including rodents, primates and livestock making it an important factor in reproductive physiology and a potential target for improving reproductive management and fertility treatments [45].

In the hypothalamus, KP-producing neurons located in the arcuate nucleus (ARC) co-express dynorphin (DYN) and neurokinin β (NKβ) and together are referred to as KNDy neurons. These KNDy neurons play a central role in mediating both the positive and negative feedback of estradiol (E2) on GnRH secretion, thereby regulating reproductive hormone dynamics throughout the estrous cycle [23]. Additionally, KP-only neurons are in the preoptic (POA) and anteroventral periventricular (AVPV) areas in livestock species and rodents, respectively [46]. These neurons are sensitive to increasing E2 levels and influence the GnRH/LH surge that induces a cascade of events within the mature ovarian follicle, culminating in follicular rupture and ovulation [8]. Kisspeptin is also involved in the initiation of puberty in both males and females. In females, KP is responsible for oocyte and ovarian follicular development and ovulation. In males, it helps regulate spermatogenesis, Leydig cell development, and other reproductive behaviors [7]. Theoretically, exogenous KP administration may serve as a potential addition to or replacement in estrus synchronization protocols to induce ovulation and a potential treatment for infertility or other disorders of reproductive endocrinology. One such disorder is cystic ovarian disease (COD), a major cause of infertility in cattle characterized by disrupted ovulation and abnormal follicular presence [47]. This condition shares several pathophysiological features with polycystic ovary syndrome (PCOS) in humans,

including elevated LH secretion, disrupted folliculogenesis, and altered metabolic profiles [43]. The ability of KP or its analogs to modulate GnRH and LH secretion makes it a promising target for therapeutic intervention in conditions involving HPG axis dysregulation.

The 54-amino acid structure of KP can be broken into shorter fragments that are biologically active: KP-14, KP-13 and KP-10. KP-10 is the smallest fragment that retains full biological activity and shows potent *in vitro* activity at the KISS1R [36]. However, KP-10's application clinically is limited, as it faces rapid renal clearance and proteolytic degradation in vivo [37]. Studies have reported that KP-10 has a half-life of approximately 4 minutes in mice and humans, compared to the observed 30-minute half-life of KP-54 (the full-length peptide) [37]. This rapid clearance limits the duration of its effects, which necessitates frequent administration [37]. Additionally, KP-10 does not effectively cross the blood-brain barrier, so peripheral administration further limits its effectiveness [48]. While it can stimulate GnRH release by acting on nerve terminals in the median eminence, it does not effectively activate GnRH neuronal cell bodies located within the hypothalamus [9].

To overcome the pharmacokinetic limitations of native KP peptides, research initially focused on developing synthetic KP analogs with enhanced stability and bioactivity. Continued optimization led to the development of Compound 6 (C6), a synthetic analog featuring specific structural modifications, including a triazole core and albumin binding motif. These changes were designed to increase the size, decrease the degradation, and reduce kidney clearance rates of the molecule [35]. Therefore, C6, with its increased stability and longer half-life compared to KP, holds potential for application in livestock reproduction management, where extended duration and consistency of therapeutic effects are critical. Previous studies have demonstrated C6's ability to advance puberty in rodents, promote consistent increases in LH concentration

increases in ewes during both the breeding and non-breeding seasons [36] and enhancing both the amplitude and duration of LH secretion in bull calves [40]. However, research on the effects of C6 on LH secretion, follicular development, and ovulation in non-seasonal, adult female livestock, particularly cows, remains limited. To address this gap, this study aimed to evaluate the effects of C6 on plasma LH concentrations and ovarian follicular dynamics in beef cows in a low progesterone environment. Investigating how C6 influences these reproductive parameters will contribute to a better understanding of its potential role in enhancing reproductive efficiency in cattle.

MATERIALS AND METHODS

The Institutional Animal Care and Use Committee of the University of Tennessee approved, in protocol numbers 2834-0421 and 2986-0623, all procedures and protocols throughout Experiment 1 and 2. The experimental design for this study is depicted in **Figure 1.**

Animals

Experiment 1 was completed using eight gonadal intact adult beef cows [4-5 years of age, 578 ± 50 kg (SD)] during the summer (June-August). Cows were housed on pasture at the University of Tennessee's East Tennessee Research and Education Center - Blount Unit (Alcoa, TN, USA, latitude and longitude coordinates are 35.841430° N, 83.951416° W, respectively). Experiment 2 was completed using six gonadal intact adult beef cows (3-5 years, 719 kg ± 71 kg) during the fall (September-November). Cows were housed on pasture at the University of Tennessee's Veterinary Research and Education Center (Knoxville, TN, USA, latitude and longitude coordinates are 35.942732° N, 83.944976° W, respectively). For both experiments, the animals were maintained in ambient climate and photoperiod for the duration of the experiments. A period of acclimation (over about 2 weeks) to housing facilities, handling and moving through

working areas of the facility, and transrectal ultrasonography was provided to cows before initiation of the studies. Cows had ad libitum access to water and grass pasture and grass hay.

Estrus Synchronization

Cows were administered a luteolytic dose of $PFG_{2\alpha}$ IM (30 mg dinoprost tromethamine injection; Lutalyse®, Zoetis Inc., Parsippany-Troy Hills, NJ, USA) at the beginning of the acclimation period. Fourteen days later, a second luteolytic dose of $PFG_{2\alpha}$ was administered to each cow and transrectal ultrasonography using broadband straight linear probe (EasiScan: Go; Scanner Operating Frequencies: 2412MHz-2462MHz; IMV Technologies, L'Aigle, France) of their ovaries was completed. Four days later, ovulation (defined as "the disappearance of a large (>8mm) ovarian follicle seen in the previous examination followed by the development of a CL") was confirmed via transrectal ultrasonography. Three days later, after ovulation was confirmed, follicles ≥5 mm in diameter on both ovaries were ablated by transvaginal ultrasound-guided follicle aspiration. A dose of GnRH IM (100 ug gonadorelin diacetate tetrahydrate Injectable Solution; Cystorelin®, Boehringer Ingelheim Animal Health USA Inc., Duluth, GA, USA) was administered to induce ovulation or luteinization of any remaining large (>8 mm) ovarian follicles and initiate emergence of a new ovarian follicular wave. In the first replication, cows were monitored through the protocol until Day 20 of the follicular wave emergence. There was a 30-day wash-out period between replications. To re-enroll in the second replication, cows received a luteolytic dose of $PGF_{2}\alpha$ to regress the existing CL, thereby synchronizing their reentry to Day -8 of a new follicular wave.

Immediately after, a progesterone-releasing device was placed in the vagina (1.38 g, progesterone intravaginal insert; EAZI-BREED™ CIDR®, Zoetis Inc., Parsippany-Troy Hills, NJ, USA) on follicular wave emergence Day 0 to maintain circulating progesterone (P4)

concentrations at a low and consistent concentration. New ovarian follicular wave emergence (Day 0) was expected ~1.5 days after ablation and/or GnRH, and cows were administered a luteolytic dose of $PFG_{2\alpha}$ IM on Days 3.5 and 4 (i.e., 8 and 8.5 days after previous ovulation) (***Figure 1***). This scheme, modeled from a previous study, was designed to specifically allow a dominant ovarian follicle to continue growing without ovulating while preserving the ability to ovulate later by careful regulation of circulating P4 concentrations [26]. This approach helps ensure that observed changes in plasma LH concentration and/or ovarian follicular dynamics were most likely due to experimental treatment administration with less influence from endogenous hormone fluctuations.

Experimental Treatments

For Experiment 1, cows received 1 of 2 treatments [Control (n = 4; **CON**; saline) or C6 (n = 4; **C6**; 100 nmoles compound 6; MW = 1361.63 g / mole)] administered intramuscularly once (~0800 h of Day 6 following ovarian follicular wave emergence) in 2 mL of 0.9% sodium chloride (sterile, non-pyrogenic, isotonic, and preservative free; 50 mL Single-dose 0.9% Sodium Chloride Injection, USP; Hospira Inc. Lake Forst, IL) containing 1% dimethyl sulfoxide (90% DMSO; Mfr # 764782004907, Valhoma Corp Tulsa, OK). The dosage of C6 used here (100 nmoles) was determined from a previous study with ewes [41] and bull calves [40] where 15 and 20 nmoles were used, respectively. A second experiment was completed, with additional cows, to increase the sample size and power. Similarly, for Experiment 2, cows received 1 of 2 treatments [Control (n = 6; CON; saline) or C6 (n = 6; 100 nmoles compound 6)].

Blood Sampling and Processing

Before treatments, cattle were temporarily restrained in a cattle chute for jugular catheter placement and transrectal ultrasonography of the ovaries. For catheter placement, an area over

the right jugular vein was clipped and prepped with betadine scrub (Povidone iodine 7.5%, Item #51646, AllVet Supply Inc., Miami, FL) and isopropyl alcohol (Isopropyl Rubbing Alcohol, Item #MDS098003WH, Medline, Northfield, IL). The area over the jugular vein was desensitized by subcutaneous administration of approximately 3 mL of 2% lidocaine (Lidocaine HCL 2% Injection, Mfr # 16598832, Aspen Vet., Loveland, CO) before using a #15 blade to make an approximately 2 cm long skin incision through which a 16-gauge IV catheter (Extended Use MILACATH® catheter, Mila international Inc, Florence, KY, USA) was placed. The catheter was sutured in place on the cow's neck and a 13 cm extension set was attached. The catheters and extension sets were flushed with heparinized saline solution (20 U of heparin/mL; Heparin Sodium Injection, USP; 1 mL vial; Sagent Pharmaceuticals, Schaumburg, IL in 1000 mL Injectible 0.9% Sodium Chloride Injection, USP; Hospira Inc. Lake Forst, IL). Before collection of each blood sample to be used for plasma LH analysis, 3 mL of fluid was removed as waste from the catheter-extension set and after each sample was drawn, the catheter-extension set was flushed with 3 mL of the heparinized saline solution (20 U). A 3 mL blood sample was collected via syringe from the catheter-extension set at -1 hour and -0.5 hours before treatments and every 60 min for 11 additional hours after treatments for plasma LH concentration analysis. At Hour 0 and Hour 11, an additional 3 mL blood sample was collected for subsequent serum P4 analysis. The sampling frequency is depicted in **Figure 2.** If IV catheters failed during the intense sampling period, blood was collected from the coccygeal vein. Each blood sample to be analyzed for plasma LH was placed into a 12 x 75 mm glass round bottom tube (Fisherbrand, Catalog #: 14-961-26) each containing 50 µl of 10,000 USP/mL heparin (Heparin Sodium Injection, USP; 1 mL vial; Sagent Pharmaceuticals, Schaumburg, Illinois 60195 USA). Blood samples for LH analysis were either immediately centrifuged or stored at 4°C for no more than 5 minutes before

being centrifuged at 3,000 relative centrifugal force for 10 minutes at 4°C. Following the day of treatment, a 3 mL blood sample, used to determine serum P4 concentrations, was collected from either the coccygeal or jugular vein every 12 hours for the first 72 hours (Days 1–3), and then once every 24 hours from 72 to 192 hours (Days 4–8) post-treatment (depicted in ***Figures 2 & 3***). Each blood sample to be analyzed for serum P4 was placed into 10 mL red conventional closure BD Vacutainer plastic tube (Linen Plus, SKU: LPI-BD367820). Blood samples for serum P4 analysis were stored at 4°C less than 24 hours before being centrifuged at 3,000 relative centrifugal force for 10 minutes at 4°C. Plasma and serum was aliquoted in duplicate into 1.5 mL microcentrifuge tubes (BioRad Laboratories, Catalog #: 2239390) and frozen at -20°C until they were assayed.

Plasma Luteinizing Hormone Analysis

Luteinizing hormone concentrations were determined in duplicate with a radioimmunoassay using 50-100 µl of plasma and reagents purchased from the National Hormone and Peptide Program (Torrance, CA) using methods previously described [22]. Luteinizing hormone assay sensitivity was 0.20 ng/tube with intra- and inter-assay coefficients of variation being 5.69 % and 13.89 %, respectively.

Serum Progesterone Analysis

Serum P4 concentrations were measured using chemiluminescence (IMMULITE1000, Siemens Healthcare Diagnostics, Tarrytown, NY) using methods previously described [49-51] with manufacturer's instructions. The assay sensitivity was 0.20 ng/tube with an inter-assay coefficient of variation of 26.3%

Ovarian & Follicular Ultrasonography

Transrectal ultrasonography of the ovaries for both Experiments 1 and 2 was conducted using the IMV Easi-Scan handheld ultrasound device. In both experiments, ultrasound examinations were completed at predetermined timepoints beginning on Study Day 1 (treatment day) and continuing through Study Day 10 post-treatment to monitor follicular development and assess ovulatory response. During each session, both ovaries were scanned systematically. For Experiment 1, the number of visible follicles per ovary and the estimated diameter (in millimeters) of each follicle were recorded using the caliper function displayed on the ultrasound screen. All measurements and observations were documented manually on standardized sheets containing printed schematics of both the right and left ovary immediately following each scan (***Figure 4***). Observers recorded changes in follicular size, structural appearance, and presence or absence in later scans. The location of observed follicles (left or right ovary), as well as the appearance of any new or emerging follicles following treatment, was noted. In Experiment 2, ultrasound scanning procedures were consistent with those used in Experiment 1; however, follicular data were recorded and analyzed digitally. Ultrasound video footage was captured using the Easi-Scan–compatible Go-Scan mobile application and archived for subsequent analysis. These recordings enabled the identification and longitudinal tracking of individual follicles on each ovary. Representative still images were extracted from the video recordings for each observation timepoint and imported into the GNU Image Manipulation Program (GIMP; created by Spencer Kimball and Peter Mattis). Each image was labeled with the corresponding cow identification number and ovary side (left or right) to ensure traceability. Follicle dimensions were measured using GIMP's built-in measurement tool, averaging the longest and widest diameters (the two measurements being 90° of one another) in millimeters. To correct for

scale discrepancies between the ultrasound display and the image processing software, all measurements were adjusted using a scaling factor of 2.5 (based on a 10 mm to 4 mm calibration ratio). All follicular measurements were systematically documented, and any instances of missing or unusable images were recorded.

Statistical Analysis

Plasma LH, serum P4, and ovarian follicle diameter were analyzed using mixed models appropriate for a crossover design with repeated measures. Fixed effects included treatment, time, and their interaction, with body weight included as a covariate for P4 analysis. When residuals violated assumptions of normality or homogeneity of variance (per Shapiro–Wilk and Levene's tests), rank data transformation was applied. Post hoc comparisons were adjusted using Tukey's method, and statistical significance was defined as $p < 0.05$. The effect of treatment, side, and time on ovarian follicle diameter was also evaluated using repeated measures mixed models. A pooled analysis of follicle status (persistence vs. turnover) from Experiments 1 and 2 was conducted post hoc to increase statistical power and assess treatment consistency across trials. Due to the absence of follow-up imaging and P4 data in Experiment 1, only observational follicle status classifications were included for that study. All analyses were performed using SAS 9.4 (TS1M8; SAS Institute Inc., Cary, NC).

CHAPTER 3

RESULTS

Plasma Luteinizing Hormone (Experiments 1 and 2)

Plasma LH concentrations were affected by treatment ($p < 0.0001$), time ($p < 0.0001$), and the interaction of treatment and time ($p = 0.0001$). Mean LH concentrations were greater ($p < 0.05$) in the C6-treated group (5.90 ± 1.04 ng/mL) compared to the CON group (3.86 ± 0.35 ng/mL). Additionally, plasma LH concentrations were elevated in the C6 group compared to the CON group at Hours 5 and 7 ($p < 0.05$) and were significantly increased from baseline (pre-treatment; Hours -0.5 or 0) in the C6 group from Hours 3 and 8 ($p < 0.05$; ***Figure 5***).

Serum Progesterone (Experiment 2)

Serum P4 concentrations were affected by time ($p < 0.0001$) and an interaction of time and treatment ($p = 0.01$), but not by treatment ($p = 0.08$). Serum P4 concentrations were lowest at Hours 168, 192, and 216, corresponding to the days following CIDR removal, compared to all other time points ($p < 0.05$; **Figure 6**). At baseline (Hour 0), serum P4 concentrations were greater ($p < 0.0078$) in the CON group compared to the C6 group. Following CIDR removal, all post-removal time points (Hours 168, 192, and 216) in the CON group had reduced P4 concentrations ($p < 0.0001$). In contrast, in the C6 group, only the Hour 192 time point was lower than baseline (Hour 0; $p = 0.045$; **Figure 6**). Additionally, cow body weight had a negative effect on serum P4 concentrations ($p = 0.004$), with greater body weight associated with lower P4 levels (slope = –0.1368).

Follicular Dynamics (Experiments 1 & 2)

In Experiment 1, hand recorded follicular observations indicated potential changes following C6 treatment. Three of 4 cows treated with C6 had follicles ≥10 mm that disappeared

within 36 hours of treatment administration, with notations suggesting possible ovulation or luteinization based on the presence of luteal tissue or a corpus hemorrhagicum. CON cows in Experiment 1 did not display the loss of a large follicle within 36-48 hours after treatments. In Experiment 2, ovarian follicle diameter was not affected by treatment ($p = 0.27$), time ($p = 0.14$), or animal body weight ($p = 0.96$), and no interaction of treatment and time was observed ($p = 0.37$). However, treatment did influence the persistence of the largest ovarian follicle present at the time of treatment administration ($p = 0.03$). In Experiment 2, 5 of 6 cows treated with C6 began the study with a dominant follicle ≥ 7.7 mm in diameter. In 4 of those 5 cows, the initial dominant follicle disappeared within 36 hours after C6 administration, and a new dominant follicle emerged and was tracked for the remainder of the study. Overall, for Experiment 2, cows treated with C6 were 46.8% more likely to lose the largest pre-treatment follicle before the study concluded. In contrast, CON cows did not display this same pattern, and the follicles present at the time of treatment generally persisted and were continuously tracked throughout the end of the study. When data from both Experiments 1 and 2 were pooled, C6 treatment was associated with significantly reduced follicle persistence compared to CON ($p < 0.0001$).

CHAPTER 4

DISCUSSION

In the present study, administration of C6 led to a significant increase in plasma LH concentrations in cows, with a peak observed around 5 to 7 hours and LH remaining elevated for up to 8 hours after treatment. This finding aligns with previous studies in ewes and bull calves, where C6 similarly stimulated the hypothalamic-pituitary axis [40, 41]. In the ewe model, it was demonstrated that 15 nmol of C6 induced a rapid rise in LH, peaking approximately 5 to 6 hours after treatment and returning to baseline levels by approximately 12 hours [41]. In this study, LH concentrations peaked around 6 hours after C6 administration and returned to near pre-treatment levels around 8 hours later. Similarly, in bull calves, 20 nmol of C6 produced an increase in plasma LH concentrations lasting up to 6 hours before beginning to decline, although the magnitude of the response varied [40]. The timing of the LH peak was similar across species, but the duration of the increase in LH differed, which suggests that while C6 stimulates LH release in multiple species, the duration of that effect varies.

Despite the robust LH response, no ovulations were observed in cows administered either C6 or saline during the study period. Although ovulation was not confirmed with serum P4 in Experiment 1, observational notes suggest that C6 treatment may have influenced follicular outcomes in atypical ways. In 3 of 4 cows treated with C6, dominant follicles present prior to treatment were later noted as having either disappeared or undergone apparent structural changes within 24 to 36 hours. In contrast, CON cows retained the same dominant follicle throughout the observation period, with no comparable signs of follicular disruption or turnover. While these observations were not validated through imaging or hormone profiles, they suggest treatment related alterations in follicular fate that are consistent with patterns observed in Experiment 2. In

Experiment 2, transrectal ultrasonography revealed that in 4 of 5 cows treated with C6 that began the study period with a dominant follicle ≥ 7.7 mm, that dominant follicle disappeared within 12 to 36 hours of treatment administration. However, this follicular disappearance was not accompanied by a corresponding rise in serum P4 concentrations or the formation of a CL, ruling out successful ovulation and subsequent luteinization. In contrast, a previous study in ewes reported both ovulation and CL formation following C6 administration [36]. These contrasting outcomes raise the possibility that C6 may exert effects at multiple levels of the reproductive axis, including local ovarian action. While C6 is primarily thought to act centrally by stimulating GnRH release, recent studies have shown that kisspeptin and its receptor (KISS1R) are also expressed in ovarian tissue, including granulosa and theca cells [52]. Local kisspeptin signaling has been implicated in regulating follicular development, steroidogenesis, and ovulation in several species, including cattle and humans [33]. Therefore, it is plausible that C6 may directly influence ovarian follicle fate, independent of or in addition to its systemic LH mediated effects. Further investigation is needed to determine whether C6 can mimic endogenous ovarian KP activity and modulate follicular turnover through local mechanisms. The absence of ovulation in the current study suggests that either the magnitude or duration of LH elevation was insufficient to trigger ovulation, or that the dominant follicles were not developmentally ready to respond to the increase in LH. Pre-treatment follicle size is a key determinant of LH responsiveness, as follicles smaller than 8 mm are generally considered unresponsive to increases in endogenous LH [11]. Interestingly, the rapid disappearance of dominant follicles in this study raises the possibility that C6 may have induced ovarian follicular turnover rather than typical atresia or ovulation. In general, atretic follicles tend to persist and regress gradually over several days, often maintaining a relatively large diameter and reduced echogenicity before fully disappearing

[53]. The follicles observed here were no longer detectable on ultrasound within a short 12- to 36-hour window, a timeframe that is inconsistent with typical atresia. Instead, it suggests a hormonally mediated, non-ovulatory effect attributable to C6, where C6 initiated a cascade beginning with GnRH and LH release. The resulting LH elevation may have disrupted the developing dominant follicle's trajectory, leading to structural compromise or premature regression without inducing ovulation [54]. This pattern more closely resembles follicular "turnover," wherein a dominant follicle is rapidly replaced by a subordinate follicle, initiating a new wave of follicular development [53]. If C6 can reliably induce follicular turnover without ovulation or full luteinization, it may represent a novel mechanism for resetting the follicular wave. This would offer a valuable tool in cattle reproductive management, particularly for estrus synchronization and fixed-time artificial insemination protocols, super ovarian follicular stimulation, or the treatment of ovarian follicular cysts. Traditional synchronization strategies rely on luteolysis or exogenous GnRH to control follicular dynamics, but a compound capable of directly inducing dominant follicle replacement could provide a unique, targeted approach to managing ovarian activity.

Serum P4 concentrations had a treatment by time interaction, suggesting the pattern of P4 decline differed modestly between C6 and CON cows following CIDR removal. While serum P4 concentrations were determined primarily to confirm and support any observed ovulatory events rather than to independently evaluate the effects of C6 on P4 secretion and no overall treatment effect was detected, is noteworthy that CON cows had significantly higher baseline P4 concentrations at the time of treatment compared to C6 cows. This difference in starting concentrations likely contributed to the observed variation in P4 decline, rather than any direct effect of the C6 treatment itself and, and concentrations in both groups approached the assay's

lower detection limit (0.20 ng/mL) by 168-192 hours after experimental treatment administration. These levels are consistent with the expected pharmacokinetics of a single CIDR in cows lacking a functional CL. Previous studies have demonstrated CIDRs typically maintain circulating P4 concentrations in the range of 0.3-1.0 ng/mL in non-cycling cows or heifers without endogenous luteal support [16]. The P4 concentrations observed here are within expected limits for animals reliant solely on exogenous progesterone, supporting the interpretation that the CIDR was the source of P4 during the study. In contrast to findings in ewes, where serum progesterone elevation followed C6-induced LH stimulation and ovulation [35], no significant increase in P4 was observed in the C6 group in this study, further supporting that there was no ovulatory response. Additionally, cow body weight was found to influence P4 profiles, with smaller animals tending to have higher P4 concentrations. This relationship may reflect differences in hormone absorption, distribution, or clearance: fixed-dose progesterone delivery via CIDR may result in relatively lower plasma concentrations in larger animals due to greater volume of distribution and metabolic clearance [55]. Studies have shown that body weight loss can lead to increased circulating P4 concentrations, suggesting that body composition and metabolic state can significantly influence hormone dynamics [56]. These findings suggest that individual variation in body size can meaningfully affect systemic hormone exposure in cattle, which may need to be considered when interpreting P4 dynamics in future studies using fixed-dose devices like CIDRs.

Compared to traditional GnRH treatments, C6 acts “upstream” by stimulating endogenous GnRH release rather than acting directly on the anterior pituitary. By triggering GnRH release in a more physiologically relevant manner, C6 offers a potential advantage over direct GnRH administration. Repeated administration of GnRH is known to cause receptor

desensitization, leading to reduced pituitary responsiveness [57]. While the long-term effects of C6 on KISS1R sensitivity have not been fully evaluated, its synthetic design may offer advantages in reducing desensitization risk. However, evidence from Parker et al. [40] indicates that repeated administration of C6 (20 nmoles IM daily) in prepubertal bull calves led to a reduced LH response by Day 4 compared to Day 1, along with suppressed FSH concentrations on Day 4, but not on Day 1. This pattern suggests that chronic exposure to C6 may begin to suppress the HPG axis, potentially through alterations in GnRH pulsatile release.

The extended LH response observed following C6 administration, likely due to its structural stability and slower metabolic clearance, may influence ovarian dynamics even in the absence of confirmed ovulation. In this study, several cows treated with C6 exhibited signs suggestive of follicular turnover, indicating that C6 may still promote meaningful changes in follicles. In support of this, data from both experiments showed that C6-treated cows experienced the disappearance of a dominant follicle within 36 hours of treatment, followed by emergence of a new dominant follicle despite no evidence of ovulation or luteinization. Saline treated cows, in contrast, retained the same dominant follicle throughout the study. After the turnover pattern first became clear in Experiment 2, the observational data from Experiment 1 was reevaluated in depth. Although that trial lacked follow-up imaging or P4 data, notes indicated that dominant follicles also disappeared within a similar time window after C6 treatment. These findings prompted the pooled analysis, which revealed that C6 treated cows were less likely to retain the same dominant follicle compared to controls. This suggests that C6 could induce follicular turnover, which is characterized by rapid replacement of a dominant follicle, not typically observed during the slow regression of atresia [53]. While this effect is likely mediated in part by extended LH secretion following C6 administration, the speed and

consistency of follicular “turnover” suggest that central stimulation alone may not fully account for the observed changes. C6 may also exert effects locally at the ovary, as KP and its receptor are expressed in bovine ovarian tissue, including granulosa and theca cells, and local KP signaling has been implicated in regulating follicular development, steroidogenesis, and ovulation [52] and this local signaling can influence several ovarian processes, including follicle maturation or structural remodeling during turnover. Given this, it is plausible that C6, as a KP analog, may influence follicular fate through both systemic (GnRH/LH mediated) and local (ovarian) pathways.

Limitations

Among the limitations faced with this study was the low circulating P4 concentrations in animals at the time of treatment. While this was intentional and allowed us to evaluate C6 effects in a GnRH-permissive environment, it may not fully represent physiological conditions during typical reproductive cycles. Low P4 environments are known to influence the sensitivity of the reproductive axis [52], and it is possible that different results may have been seen under higher P4 conditions or circulating P4 concentrations typical of the follicular phase of the estrous cycle. Future studies without the use of CIDR devices may yield different and possibly more meaningful reproductive outcomes, but they would likely require larger sample sizes and longer-term monitoring. In addition to the scientific considerations, logistical and regulatory factors constrained the scope of this study. Current U.S. regulations restrict the use of KP analogs like C6 in food producing animals unless conducted as terminal studies [58], thereby limiting sample availability and experimental design options. These restrictions may continue to prevent the comprehensive evaluation of C6’s potential for broader application in cattle reproduction.

Future Research

Future research should focus on validating C6's ability to induce reliable follicular turnover and assessing how this activity can be harnessed for reproductive management. If confirmed, this mechanism could offer a novel approach to resetting the follicular wave, providing producers with greater control over the timing of ovulation and enhancing the precision of synchronization protocols. Such improvements could reduce reliance on multi-step hormone regimens, lowering both labor demands and costs associated with timed artificial insemination programs. Beyond its potential use in synchronization protocols, C6 may also have therapeutic applications. Cystic ovarian disease (COD) remains a major cause of infertility in dairy cattle. Given the hypothalamic dysfunction implicated in COD [43] and KISS1R agonist therapeutic use in treating polycystic ovary syndrome (PCOS) in humans [59], KP analogs like C6 may offer a novel therapeutic avenue for COD in cows. Future studies should investigate whether C6 can restore normal cyclicity or induce follicular turnover in cows with cystic ovarian structures. This may provide not only a new tool for fertility management but also insights into the role of KP signaling in ovarian dysfunction.

Conclusions

Studying how C6 influences other circulating reproductive hormones, such as FSH, E2, and P4, and determining how it can be integrated into existing reproductive management protocols will be essential to fully characterize its role in cattle reproduction. The findings from this study demonstrate that C6 reliably stimulates a sustained increase in plasma LH concentrations, even under low circulating P4 conditions. While this LH response did not lead to ovulation in the current model, it was associated with notable changes in ovarian follicular

dynamics, including a possible induction of follicular turnover, which is an effect not typically seen with traditional hormonal treatments.

These results suggest that C6 could serve as a promising addition or alternative to traditional GnRH-based protocols for stimulating LH release, especially in synchronization schemes where precise control over follicular waves is desired. Additionally, the structural stability and prolonged activity of C6 may offer logistical advantages, such as reduced frequency of hormone administration or greater consistency in physiological response across animals.

As research continues, future studies should investigate optimal dosing strategies, timing of administration, and combination protocols with other reproductive hormones. Exploration of C6's role in special applications such as inducing follicular turnover, prepubertal heifers, or cows with COD may reveal further utility. Ultimately, understanding both the endocrine and ovarian-level effects of C6 will be critical to understanding its full potential as a reproductive management tool in cattle.

REFERENCES

[1] Moorey SE. Beef heifer fertility: importance of management practices and technological advancements. Journal of Animal Science and Biotechnology. 2020.

[2] Prevatt C. What Is the Economic Impact of Infertility in Beef Cattle? IFAS Extension2018.

[3] Gavernick A. Female Reproductive Physiology and Endocrinology of Cattle. Veterinary Clincs of North America: Food Animal Practice. 1995;9:223-47.

[4] Leonardi CEP, Dias FCF, Adams GP, Singh J. Effect of Kisspeptin-10 on plasma luteinizing hormone concentrations and follicular dynamics during the luteal phase in cattle. Theriogenology. 2018;119:268-74.

[5] Senger PL. Pathways to pregnancy & parturition. 3rd ed. Redmond, OR: Current Conceptions; 2012.

[6] Bo GA. EXOGENOUS CONTROL OF FOLLICULAR WAVE EMERGENCE IN CATTLE. Theriogenology. 1995;43:31-40.

[7] Xie Q. The role of kisspeptin in the control of the hypothalamic-pituitary-gonadal axis and reproduction. Frontiers in Endocrinology. 2022;13.

[8] Uenoyama Y. Role of KNDy Neurons Expressing Kisspeptin, Neurokinin B, and Dynorphin A as a GnRH Pulse Generator Controlling Mammalian Reproduction. Frontiers in Endocrinology. 2021;12.

[9] Leonardi CEP. Mechanism of LH release after peripheral administration of kisspeptin in cattle. PlOS one. 2022:18.

[10] Adams GP. Effect of progesterone on ovarian follicles, emergence of follicular waves and circulating follicle-stimulating hormone in heifers. Journals of Reproduction and Fertility. 1992;95:627-40.

[11] Crowe M. Regulation and Function of Gonadotropins Throughout the Bovine Oestrous Cycle. InTech. 2014.

[12] Bo GA. Synchronization of ovulation and xed-time arti cial insemination in beef cattle. Animal. 2014:144-50.

[13] Han SK, Gottsch ML, Lee KJ, Popa SM, Smith JT, Jakawich SK, et al. Activation of gonadotropin-releasing hormone neurons by kisspeptin as a neuroendocrine switch for the onset of puberty. Journal of Neuroscience. 2005;25:11349-56.

[14] Galbreath C. Effect of feeding flax or linseed meal on progesterone clearance rate in ovariectomized ewes. Domestic Animal Endocrinology. 2008;35:164-9.

[15] Lamb G. Synchronization and Artificial Insemination Strategies in Beef Cattle. Veterinary Clincs of North America: Food Animal Practice. 2016;32.

[16] Martinez M. The use of a progesterone-releasing device (CIDR-B) or melengestrol acetate with GnRH, LH, or estradiol benzoate for fixed-time AI in beef heifers. American Society of Animal Science. 2002.

[17] Force BRT. Protocols for Synchronization of Estrus and Ovulation. 2009.

[18] Lee D. Discovery of a receptor related to the galanin receptors. Federation of European Biochemical Societies. 1998:103-7.

[19] de Roux N. Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. PNAS. 2003;100:10972.

[20] Seminara SB. A locus for autosomal recessive idiopathic hypogonadotropic hypogonadism on chromosome 19p13.3. The Journal of Clinical Endocrinology & Metabolism. 2003;88:2947-50.

[21] George JT. KP-10 is a potent stimulator of LH and increases pulse frequency in Men. JCEM Online. 2011.

[22] Goodman R. Kisspeptin Neurons in the Arcuate Nucleus of. Endocrinology. 2007;148:5752-60.

[23] Wakabayashi Y. Neurokinin B and Dynorphin A in Kisspeptin Neurons of the Arcuate Nucleus Participate in Generation of Periodic Oscillation of Neural Activity Driving Pulsatile Gonadotropin-Releasing Hormone Secretion in the Goat. The Journal of Neuroscience. 2010;30:3124-32.

[24] Clarkson J. Kisspeptin-GPR54 signaling is essential for preovulatory gonadotropin-releasing hormone neuron activation and the luteinizing hormone surge. The Journal of Neuroscience. 2008:8691-7.

[25] Tena-Sempere M. Kisspeptin signaling in the brain: Recent developments and future challenges. molecular and cellular endocrinology. 2010;314.

[26] Leonardi CEP. Effect of Kisspeptin-10 on plasma luteinizing hormone concentrations and follicular dynamics during the luteal phase in cattle. 2018.

[27] Abbara A. Kisspeptin receptor aagonist has therapeutic potential for female reproductive disorders. The Journal of Clinical Investigation. 2020;130:6739-53.

[28] Leonardi CEP, Dias FCF, Adams GP, Araujo ER, Singh J. Kisspeptin induces ovulation in heifers under low plasma progesterone concentrations. Theriogenology. 2020;141:26-34.

[29] Kadokawa H, Matsui M, Hayashi K, Matsunaga N, Kawashima C, Shimizu T, et al. Peripheral administration of kisspeptin-10 increases plasma concentrations of GH as well as LH in prepubertal Holstein heifers. Journal of Endocrinology. 2008;196:331-4.

[30] Caraty A, Smith JT, Lomet D, Ben Said S, Morrissey A, Cognie J, et al. Kisspeptin synchronizes Preovulatory surges in cyclical ewes and causes ovulation in seasonally acyclic ewes. Endocrinology. 2007;148:5258-67.

[31] Hu K. Kisspeptin/Kisspeptin Receptor System in the Ovary. Frontiers in Endocrinology. 2018;8.

[32] Castellano JM. Expression of KiSS-1 in Rat Ovary: Putative Local Regulator of Ovulation? The Endocrine Society. 2006;147.

[33] D'Occhio M. Peripheral action of kisspeptin at reproductive tissues—role in ovarian function and embryo implantation and relevance to assisted reproductive technology in livestock: a review. Biology of Reproduction. 2020;103.

[34] Ahmed AE, Saito H, Sawada T, Yaegashi T, Yamashita T, Hirata TI, et al. Characteristics of the Stimulatory Effect of Kisspeptin-10 on the Secretion of Luteinizing Hormone, Follicle-Stimulating Hormone and Growth Hormone in Prepubertal Male and Female Cattle. Journal of Reproduction and Development. 2009;55:650-4.

[35] Beltramo M, Decourt C. Towards new strategies to manage livestock reproduction using kisspeptin analogs. Theriogenology. 2018;112:2-10.

[36] Curtis AE, Cooke JH, Baxter JE, Parkinson JRC, Bataveljic A, Ghatei MA, et al. A kisspeptin-10 analog with greater in vivo bioactivity than kisspeptin-10. American Journal of Physiology-Endocrinology and Metabolism. 2010;298:E296-E303.

[37] d'Anglemont X. Mechanistic insights into the more potent effect of KP-54 compared to KP-10 in vivo. PlOS one. 2017;12.

[38] Millar R. Kisspeptin antagonists: Unraveling the role of kisspeptin in reproductive physiology. Brain Research. 2010:81-9.

[39] Whitlock BK, Daniel JA, Wilborn RR, Rodning SP, Maxwell HS, Steele BP, et al. Interaction of Estrogen and Progesterone on Kisspeptin-10-Stimulated Luteinizing Hormone and Growth Hormone in Ovariectomized Cows. Neuroendocrinology. 2008;88:212-5.

[40] Parker PA, Coffman EA, Pohler KG, Daniel JA, Aucagne V, Beltramo M, et al. Acute and subacute effects of a synthetic kisspeptin analog, C6, on serum concentrations of luteinizing hormone, follicle stimulating hormone, and testosterone in prepubertal bull calves. Theriogenology. 2019;130:111-9.

[41] Decourt C, Robert V, Anger K, Galibert M, Madinier JB, Liu X, et al. A synthetic kisspeptin analog that triggers ovulation and advances puberty. Scientific Reports. 2016;6.

[42] Shahed A. Differential Ovarian Expression of KiSS-1 and GPR-54 During the Estrous Cycle and Photoperiod Induced Recrudescence in Siberian Hamsters (Phodopus sungorus). Molecular Reproduction & Development. 2009;76:444-52.

[43] Vanholder T. Aetiology and pathogenesis of cystic ovarian follicles in dairy cattle: a review. EDP Sciences. 2006.

[44] U.S. Department of Agriculture NASS. Cattle. In: 1948-9099 I, editor. Washington, DC 2025.

[45] Messager S, Chatzidaki EE, Ma D, Hendrick AG, Zahn D, Dixon J, et al. Kisspeptin directly stimulates gonadotropin-releasing hormone release via G protein-coupled receptor 54. Proceedings of the National Academy of Sciences of the United States of America. 2005;102:1761-6.

[46] Navarro VM. Regulation of NKB Pathways and Their Roles in the Control of Kiss1 Neurons in the Arcuate Nucleus of the Male Mouse. endocrinology. 2011;152:4265-75.

[47] Ortega H. Molecular aspects of bovine cystic ovarian disease pathogenesis. Society for Reproduction and Fertility. 2015.

[48] d'Anglemont X. Kisspeptin Can Stimulate Gonadotropin-Releasing Hormone (GnRH) Release by a Direct Action at GnRH Nerve Terminals. The Endocrine Society. 2007;148:3926-32.

[49] Habeeb H. Effect of different dosages of PG-600 on ovulation and pregnancy rates in ewes during the breeding season. Department of Animal and Rangeland Sciences. 2018.

[50] Naylor D. Short communication: Characterizing ovine serum stress biomarkers during endotoxemia. Journal of Dairy Science. 2020;103:5501-8.

[51] Vonnahme K. Thyroid Hormones and Cortisol Concentrations in Offspring are Influenced by Maternal Supranutritional Selenium and Nutritional Plane in Sheep. Nutrition and Metabolic Insights. 2013;6:11-21.

[52] Zheng P. Effect of Hypoglycemic Drugs on Kisspeptin Expression in the Hypothalamic Arcuate Nucleus of PCOS Rats. Diabetes, Metabolic Syndrome and Obesity. 2023;16:2717-32.

[53] Mihm M. Follicle Wave Growth in Cattle. Reproduction of Domestic Animals. Blackwell Verlag2002.

[54] Ginther O. Selection of the Dominant Follicle in Cattle. Biology of Reproduction. 1996;55.

[55] Turino LN. Pharmacokinetics of progesterone in lactating dairy cows: Gaining some insights into the metabolism from kinetic modeling. American Dairy Science Association. 2009;93:988-99.

[56] Rodrigues RO. Effects of body weight loss on serum progesterone concentrations of non-lactating dairy cows. Theriogenology. 2010.

[57] Heber D. Pituitary desensitization and the regulation of pituitary gonadotropin-releasing hormone (GnRH) receptors following chronic administration of a superactive GnRH analog and testosterone: Pergamon Press; 1982.

[58] Regulations CoF. Title 21: Food andn Drugs, Part 511 - NEW ANIMAL DRUGS FOR INVESTIGATIONAL USE. Washington, D.C.: US Government Publishing Office; 2025.

[59] Jayasena CN, Nijher GMK, Abbara A, Murphy KG, Lim A, Patel D, et al. Twice-Weekly Administration of Kisspeptin-54 for 8 Weeks Stimulates Release of Reproductive Hormones in Women With Hypothalamic Amenorrhea. Clinical Pharmacology & Therapeutics. 2010;88:840-7.

APPENDIX

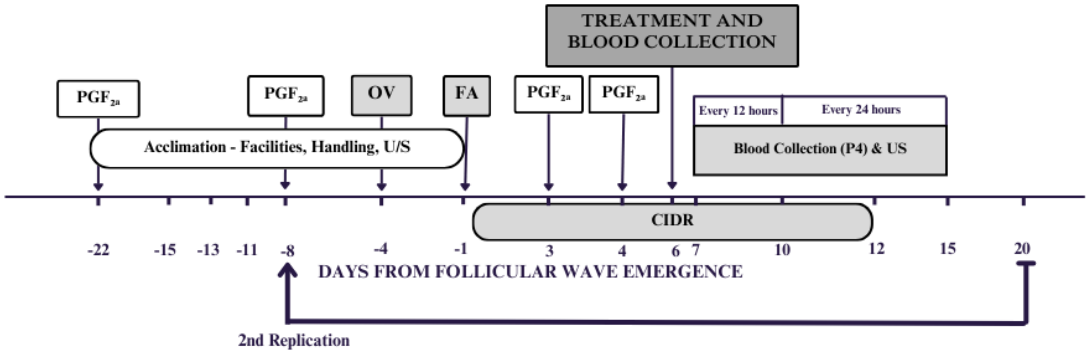

Figure 1: Summary of overall study timeline. Cows underwent two weeks of acclimation followed by synchronization of follicular wave emergence and CIDR insertion. Treatments were administered on Day 6 of the follicular wave (Study Day 1) and included pre-treatment blood sampling, transrectal ultrasonography, and hourly blood sampling. Blood sampling and ultrasonography continued every 12 hours for Study Days 7-10 and every 24 hours for Study Days 11-15. CIDRs were removed on Study Day 12. Replication 2 began on Study Day 20 with administration of prostaglandin $F_2\alpha$ ($PGF_2\alpha$) to take cows to Follicular Wave Emergence Day -8. CIDR = controlled internal drug release; OV = ovulation; FA = follicular ablation and/or administration of Cystorelin, $PGF_{2\alpha}$ = Prostaglandin$F_{2\alpha}$; US = Transrectal Ultrasonography

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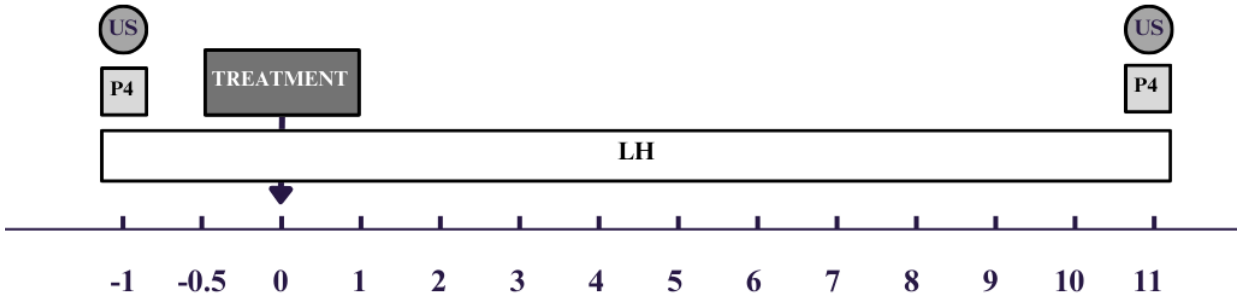

Figure 2: Summary of Study Day 1; Timeline of sampling events relative to treatment administration (Treatment; dark grey rectangle) on Study Day 1. Blood samples for determining LH concentrations (LH; white squares) were collected 1 and 0.5 hours prior to treatment administration.

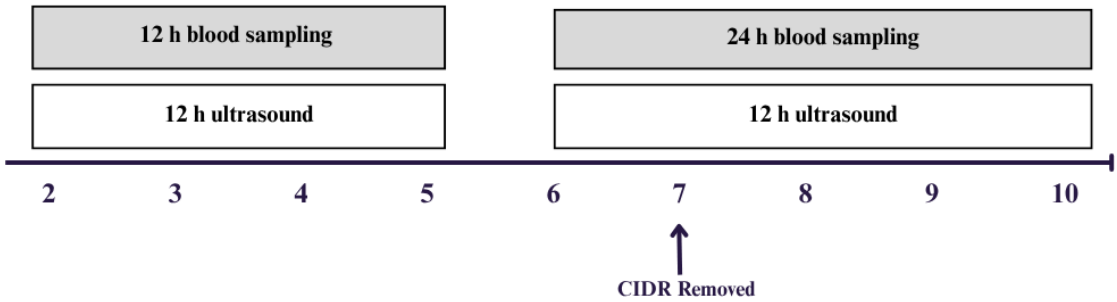

Figure 3: Timeline for blood sampling and transrectal ultrasonography for Study Days 2-10 following treatment administration on Study Day 1. A 3mL blood sample was collected (subsequently used to determine serum P4 concentrations) and a transrectal ultrasonography of ovaries was completed every 12 hours from Days 2-5, and once every 24 hours from Days 6-10. CIDR removal occurred on Study Day 7.

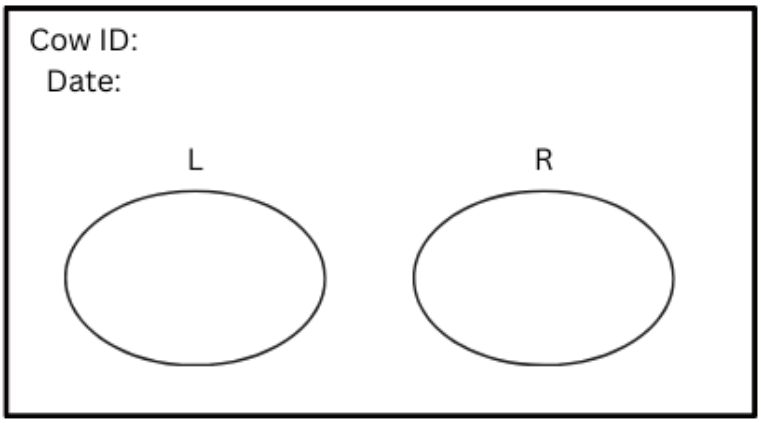

Figure 4: Ovarian follicular mapping template used in Experiment 1 to document dynamic changes in ovarian follicles. Circles represent the left (L) and right (R) ovary, with space for manual annotation of follicle number (MSF if multiple small follicles observed), size (mm), location, and notable structures (e.g., CL, CH, etc.). Cow ID (identification) and date and time were recorded to track individual progression over time.

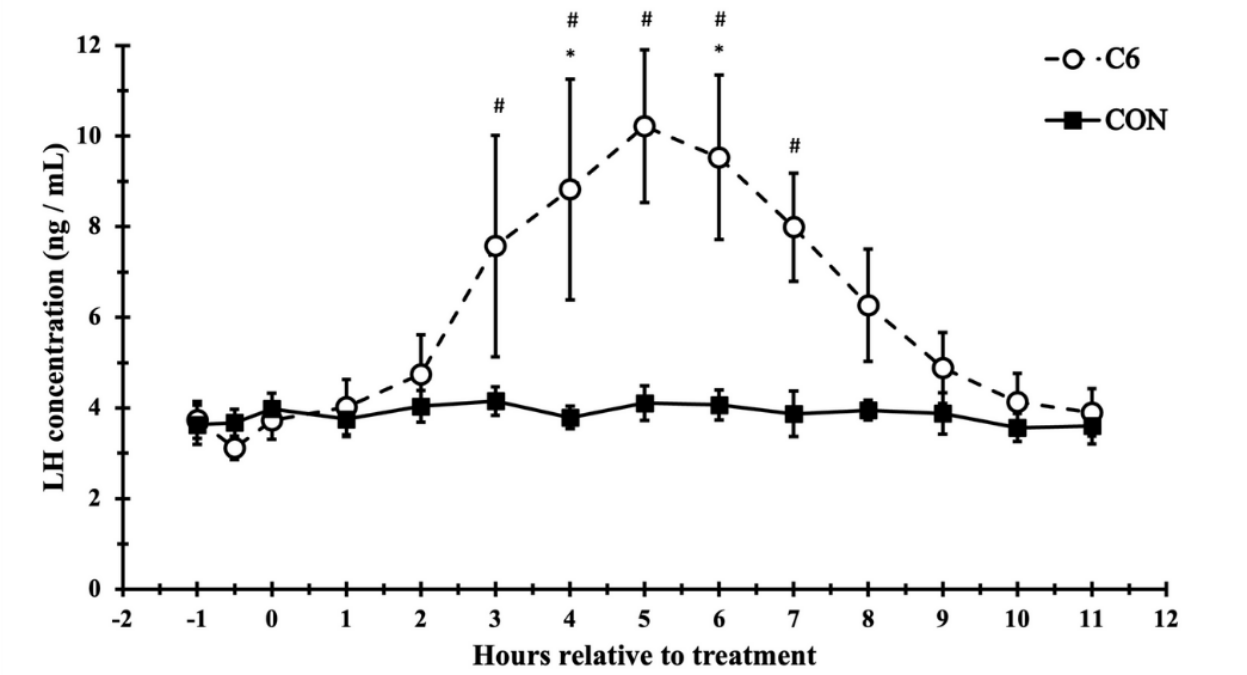

Figure 5: Plasma LH concentrations (ng/mL) in cows treated with Compound 6 (C6; n = 10; open circles) or saline control (CON; n = 10; solid squares). Treatment was administered at Study Hour 0. Plasma LH concentrations were measured hourly from 1 hour prior to 11 hours post-treatment. * Indicates time points at which LH concentrations in the C6 group were greater than the CON group for the same time points ($p < 0.05$; Hours 5 and Hour 7). # Indicate time points (Hours 3 through 8) at which LH concentrations in the C6 group were greater ($p < 0.05$) than baseline values within the same treatment (Hours -0.5 or 0). Error bars represent standard error of the mean (SEM).

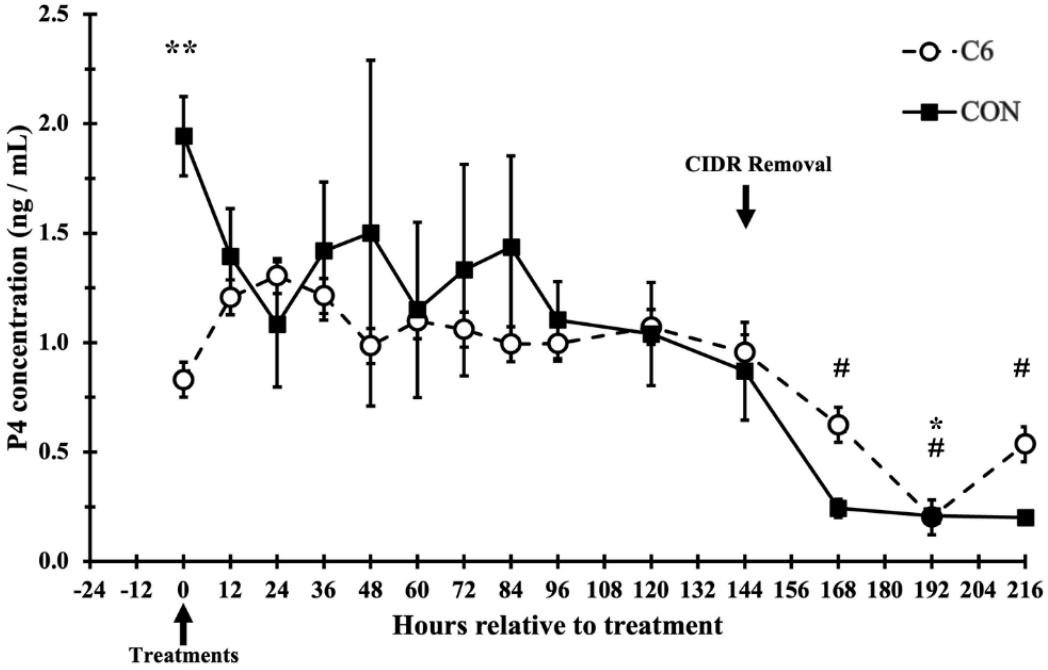

Figure 6: Serum progesterone (P4) concentrations (ng/mL) in cows treated with Compound 6 (C6; n = 6; open circles) or saline control (CON; n = 6; solid squares) in Experiment 2. Treatment was administered at Study Hour 0, and serum P4 concentrations were determined from Study Hour 0 through 216 hours post-treatment. CIDR removal occurred at Study Hour 144. Error bars represent standard error of the mean (SEM). * Indicate a significant increase ($p < 0.05$) from baseline (Study Hour 0) in the C6 group at Study Hour 192. #Iindicate a significant increase ($p < 0.05$) from baseline in the CON group at Study Hours 168, 192, and 216. ** Denote a significant difference ($p < 0.05$) between C6 and CON at baseline (Study Hour 0).

VITA

Emily Travis was born and raised in Knoxville, Tennessee. She earned her Bachelor of Science degree in Animal Science from the University of Tennessee, Knoxville in 2021, graduating Magna Cum Laude. During her undergraduate career, she was awarded multiple adult student scholarships and inducted into Gamma Sigma Delta, the honor society of agriculture.

Emily pursued a Master of Science degree in Comparative and Experimental Medicine at the University of Tennessee, which she is expected to complete in 2025. Her thesis, *"Compound 6, a synthetic kisspeptin analog, increases plasma luteinizing hormone concentrations in beef cows under low circulating progesterone concentrations",* explores novel approaches to enhancing reproductive efficiency in cattle.

She contributed to the peer-reviewed research article *"Chronic Endotoxin Administration Impairs Luteinizing Hormone Secretion and Kisspeptin Expression in Castrated, Male Sheep."* Emily is also certified in bovine artificial insemination through Select Sires and has a strong interest in large animal reproduction, research, and teaching.

In addition to her academic pursuits, Emily is a dedicated mother of two and is passionate about using her expertise to contribute to both scientific progress and future generations of students.