Reproductive effects of pyrethroid use in beef cattle

Tyler Dohlman

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Reproductive effects of pyrethroid use in beef cattle

by

Tyler Dohlman

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Animal Physiology (Reproductive Physiology)

Program of Study Committee:
Patrick Gunn, Major Professor
James West
Jason Ross

Iowa State University
Ames, Iowa
2015

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DEDICATION

I dedicate my thesis to my wife, Amelia. Without her support and patience this would not have been possible. She allowed me the time away from home to accomplish this and supported me through this long journey.
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Finally, thanks to my wife (Amelia), friends, and family for their encouragement, patience, and support.
The effect of pyrethroid use in beef cattle on reproductive function and steroid biosynthesis was investigated. In the first study, BSEs were taken 5 d prior to and 14 d after treatment with label-dose of a commercial pyrethroid (permethrin)-based pour-on fly control product. Motility did not differ as a result of treatment. Although morphology improved across treatments between the first and second BSE, permethrin treated bulls had less improvement in percent of head sperm abnormalities and a tendency to have less improvement in midpiece sperm defects compared to control bulls, resulting in less improvement of primary abnormalities. Nonetheless, overall outcome for satisfactory breeder status was not impacted by treatment. In addition, there was no difference in the change of testosterone concentration as a result of treatment. Histopathological examination determined that testicular degeneration and tubule diameter did not differ as a result of treatment. However, it should be noted that regardless of treatment, degeneration score (higher score having more degeneration) was positively correlated with primary abnormalities and negatively correlated with normal sperm cells.

In the second study, superovulated beef heifers were flushed at 17 and 51 days after treatment with label-dose pour-on pyrethroid (permethrin) or saline control. No differences were noted in total embryos recovered regardless of treatment. However, total embryos did decrease in the second flush compared to the first. Embryo quality grade, total transferrable quality embryos (TQE), and overall flush success did not differ due to treatment. However, TQE did decrease in flush 2 compared to flush 1 regardless of treatment. Estradiol per ovulated
follicle and estradiol per total ovarian structure was greater in flush 2 but did not differ due to treatment. Furthermore, permethrin treated heifers tended to have reduced progesterone concentrations and lower progesterone per corpus luteum proportion at embryo recovery.

In summary, the data from the two studies indicate that permethrin administration at one-time label-dose in yearling bulls and superovulated beef heifers has minimal effects on reproductive physiology, but not to a degree that would indicate compromised reproductive function or fertility.
For over the past decade, United States Congress and the Environmental Protection Agency (EPA) have investigated chemicals that could potentially disrupt endocrine functions (US EPA, 2011a). Endocrine disruption is defined by any chemical that can derail normal endocrine function by interfere with the synthesis, secretion, transport, binding, action, or elimination of natural hormones. Most of the original work was focused on human exposures by environmental runoff and occupational hazards. The primary endocrine disrupting chemicals (EDC) of concern included the popular insecticide dichlorodiphenyltrichloroethane, also known as DDT, and diethylstibesterol (DES), a common drug used to prevent miscarriages in humans (Patisaul and Adewale, 2009). The disrupting agents can affect any part of the normal endocrine systems in the body; however, reproductive endocrine disruption is of high concern due to the potential harmful effects and the possibility of eliminating progeny and ultimately endangering species.

Insecticides, very similar to DDT, have been under intense scrutiny in the recent past due to abundant use in households, on vegetation and crops, and in livestock settings. The most common insecticides used today are pyrethroids, which are natural pyrethrin derivatives. The EPA claims that there are currently 3,500 registered pyrethroid products (US EPA, 2011a) with most of these products being used to combat insect burden on vegetation and crops. However, livestock producers utilize similar products (impregnated ear tags, topical pour-ons,
aerosolizing sprays, foggers, etc.) to eliminate or protect their animals from disease-transmitting insects.

Recent press publication has implied that use of pyrethroids can disrupt normal motility and morphology in bull semen (Volkmann, 2012). This in itself is concerning to beef cow-calf operations due to most of the industry relying on bull pasture breeding and the warranted use of such products to eliminate production losses in an economically tight-margin industry. Other research investigating similar reproductive affects in other male species have provided some validity to the observational findings presented by Volkmann (2012). However, case-controlled studies have been limited in providing a true understanding in the negative reproductive effects from pyrethroid exposure to breeding bulls, thus further evaluation is warranted.

In addition, female reproductive effects from pyrethroid exposure have not been fully evaluated. This is also of concern due to females being exposed to similar products during insect laden seasons. It is also crucial to understand the physiology of any detrimental effects due to pyrethroid exposure in females since they play a huge role in propagating offspring. Moreover, the lack of case-controlled studies warrants further investigation on pyrethroid exposure and its effects on reproduction.
CHAPTER 2
LITERATURE REVIEW

2.1 Introduction

This literature review covers and highlights the topics of male and female bovine reproductive physiology, insecticide and pyrethroid use, and the endocrine disruption of pyrethroids on reproduction in the male and female reproductive system. The functions of the Leydig and Sertoli cells and seminiferous tubules in spermatogenesis are initially addressed, followed by a review of bull puberty and testicular degeneration. The female reproductive endocrinology will follow with a review of the follicular and luteal phases. In addition, follicular dynamics and folliculogenesis will then be discussed. This is followed by a review of insecticide, specifically pyrethroids, use and their actions within mammalian bodies. Endocrine disruption and the associated chemicals will then be addressed followed by pyrethroid examples of endocrine disruption in male and female reproductive physiology. The review finishes with a general discussion and the rationale for the two research trials presented in the thesis.

2.2 Male Reproductive Physiology

2.2.1 Overview

The primary function of the bovine testicle is to produce male gametes (spermatozoa) and hormones that regulate reproductive function. This process is multiplexed and is controlled mainly through hormonal feedback mechanisms with the hypothalamus, pituitary, and gonads, also known as the hypothalamic-pituitary-gonadal axis (HPGA).
2.2.2 Leydig cell

The two main cells that control the function of the testes in a combined effort include the Leydig and Sertoli cells. The Leydig cells are clustered within the interstitial compartment of the testicle and provide meshwork between the seminiferous tubules, blood vasculature, and lymphatic system (Ewing and Zirkin, 1983). The Leydig cells are the main source of androgen production in the male bovine; even though the Leydig cells only occupy approximately 15 percent of the testicular volume (Hooker, 1994). These cells are considered to be steroidogenic even in fetal development (Attal, 1969) and throughout life allowing for hormonal signaling throughout the body and to androgen dependent organs, especially the testes (Ewing and Zirkin, 1983).

The production of testosterone is a complex cellular mechanism that involves conversion of precursor hormones utilizing enzymatic reactions. The conversions are carried out by hydroxylation, isomerization, side-chain cleavage, dehydrogenation, and aromatase action (Senger, 2004). Cholesterol is the essential precursor to androgen synthesis and is available through metabolic acquisition in plasma or lipid droplets. Conversion of cholesterol to pregnenolone is the rate limiting step to androgen synthesis and is controlled by hydroxylation with catalytic properties from enzymatic side-chain cleavage complex in the mitochondria, specifically cytochrome P450 side chain cleavage (cP450scc; (Hall, 1994, Kallen et al., 1998). Pregnenolone is further processed by enzymatic activity to progestin steroid precursors through two different pathways. Cleavage of the two-carbon side-chain allows for irreversible production of weak androgens that can be further processed to stronger androgens including
testosterone and the more biologically active dihydrotosterone (DHT) by the 5α-reductase enzyme (Senger, 2004).

Testosterone and other androgen production from the Leydig cells are controlled by the gonadotropins from the anterior pituitary, specifically luteinizing hormone (LH). Luteinizing hormone is synthesized and released from the anterior pituitary by the stimulation of gonadotropin-releasing hormone (GnRH) from the hypothalamus (Schanbacher, 1982). Luteinizing hormone and, consequently, testosterone elevate after GnRH release. This feedback mechanism has been proven through GnRH and human chorionic gonadotropin (HCG) stimulation tests (Falvo et al., 1975). The LH release and LH-receptivity provides episodic bursts of testosterone production via the Leydig cells (Amann and Schanbacher, 1983, Schanbacher, 1982, Bagu et al., 2006). Concentrations of testosterone and LH fluctuate daily in plasma, however, the fluctuations occur independently and is not similar between all individuals (Falvo et al., 1975). Testosterone is necessary for normal testicle function, and in addition, essential for androgen dependent organs including the epididymis and accessory sex glands to produce fructose and citric acid, which are vital sources of energy and a vehicle for sperm in the ejaculate (Setchell, 1978). Specifically, testosterone is primarily important for the Sertoli cell development and maturation.

### 2.2.3 Sertoli cell

The Sertoli cells lie adjacent to the seminiferous tubules and support the development and maturation of the germinal cells (spermatagonia). The population of active germ cells that produce spermatocytes and consequently control the capacity for daily sperm production is
solely dependent on the number of supporting Sertoli cells (Sofikitis et al., 2008, Johnson et al., 2008, Berndtson et al., 1987). Sertoli cells provide immunological support by structurally supplying tight junctions (blood-testis barrier) and by phagocytizing cytoplasm and surplus spermatozoa material (Berndtson et al., 1987). In addition, Sertoli cells also provide essential protein complexes to support differentiation, growth, and maturation to the developing spermatagonia and spermatocytes, specifically androgen binding protein (ABP), transferrin, activin, and inhibin (Suresh et al., 2011). The production of these products by the Sertoli cells is stimulated by follicle stimulating hormone (FSH) which is produced and secreted by the anterior pituitary. Sertoli cells have receptors for FSH and testosterone which allow for hormonal regulation for spermatogenesis (Bagu et al., 2006). Testosterone and DHT from the Leydig cells bind to ABP and cause the androgen steroids to become less lipophilic and more concentrated in the lumen of the seminiferous tubules to aid in spermatogenesis (Amann and Schanbacher, 1983). This process allows for cellular uptake of the strong androgens to target cells through nuclear signal transduction to regulate genetic translation of specific genes (Xiong et al., 2006). Transferrin is a major secretory product from the Sertoli cells and is postulated to transfer iron to spermatozoa to promote cell growth and maturation (Skinner et al., 1980). Inhibin and activin are also produced by the Sertoli cells and constantly regulate FSH by negative and positive feedback mechanism at the anterior pituitary level, respectively.

2.2.4 Seminiferous tubules

Seminiferous tubules lie within the testicular parenchyma and contain the Sertoli cells and the developing germinal cells. The tubules continue to grow in length and diameter
throughout the peripubertal period (Moura et al., 2011, Rawlings et al., 2008). Moura et al. (2011) found that 79% of the testicular parenchyma is occupied by the seminiferous tubules and they contain 72% round or elongated spermatids at 54 weeks of age in beef bulls. The growth is substantial in the developmental phase of the testes and is controlled by the feedback of the gonadotropins and the corresponding receptors on specific cells to produce hormones (specifically testosterone), to allow for spermatogenesis to occur (Moura et al., 2011, Evans et al., 1996).

2.2.5 Spermatogenesis

Spermatogenesis is the cyclic process of germinal spermatagonia stem cells to proliferate, transform, and mature into the final stages of a motile sperm cell, spermatid, which ends up in the ejaculate. There are three discrete stages of spermatogenesis: spermatocytogenesis (proliferation), meiosis, and spermiogenesis (differentiation). Spermatocytogenesis entails germ cells to proliferate by going through four distinct mitotic divisions creating sixteen primary spermatocytes from each active spermatogonia (spermatagonia B) which are interconnected by cytoplasm (Sofikitis et al., 2008). Primary spermatocytes undergo meiosis I to allow for diverse DNA replication to produce genetically unique cells (second spermatocytes). These same secondary spermatocytes undergo meiosis II to form the haploid round spermatids. Round spermatids transform and further mature into elongated, highly condensed spermatocytes with a flagella and is collectively called spermiogenesis (De Kretser and Kerr, 1994). Spermiogenesis is a complex series of events to make the sperm functionally ready to be released into the lumen of the seminiferous tubules.
and eventually funneled to the rete testis. There are four phases that constitute this differentiation phase: Golgi phase, cap phase, acrosomal phase, and the maturation phase (De Kretser and Kerr, 1994). The final period of spermiogenesis is referred to as spermiation which is defined by the release of the spermatids into the luminal area of the seminiferous tubules where migration can continue through the rete testis and the epididymis as the spermatid sheds its cytoplasm (Senger, 2004). In total, it takes 61 days to complete spermatogenesis and continues throughout the lifespan of the post-pubertal bull (Johnson et al., 2000). The bull starts to ejaculate normal motile sperm at puberty (typically 10-12 months of age in Bos Taurus breeds) and can produce 7-8 billion sperm cells daily (Aravindakshan et al., 2000).

### 2.2.6 Puberty

Puberty is both breed and hormone dependent (Lunstra et al., 1978). Lunstra et al. (2003) reported Angus-sired bulls to be 23-82 days younger at puberty than all other sire breeds except for Hereford. In addition, Brahman-sired bulls were older at puberty than all other sire breeds except Boran. Casas et al. (2014) also reported similar results with Wagyu and Swedish Red and White inheritance reaching puberty later than Angus-sired bulls. However, testis size at puberty is not different between breeds of bulls (Lunstra and Cundiff, 2003, Casas et al., 2007). Bulls with earlier peaks of gonadotropins in prepubescence stage and have higher concentration of testosterone between 7 and 13 months reach puberty quicker (Lunstra et al., 1978, Aravindakshan et al., 2000, Rawlings et al., 2008, Bagu et al., 2006, Evans et al., 1996). There is rapid growth of the testes that occurs after 25 weeks of age when serum
gonadotropins are low, most likely due to hypothalamic desensitization near sexual maturity (Bagu et al., 2006, Lunstra et al., 1978).

2.2.7 Testicular degeneration

Testosterone, FSH, and LH influence germ cell fate for spermatogenesis and their removal prompts spermatagonia apoptosis. Nandi et al. (1999) described those toxicants that inhibit normal production of hormones, specifically testosterone, damage and cause apoptosis of testicular stem cells by increasing Fas, a transmembrane receptor protein that belongs to the tumor necrosis factor (TNF) family (Nandi et al., 1999). Leydig cell death or damage reduces testosterone and increases testicular Fas content and ultimately causes germ cell apoptosis, in that order. Kim et al. (2001) investigated the role of caspase enzymes involved with germ cell apoptosis. Caspases are activated in cells undergoing apoptosis and play a crucial role in self-proteolysis. Reduction in intratesticular testosterone concentration, by means of endocrine disruption, is caspase activation dependent and allow nucleases to induce germ cell apoptosis (Kim et al., 2001). Even though this process explains DNA fragmentation of germ cells, spermatocytes and spermatids are also damaged or eliminated by endocrine imbalances (Odonnell et al., 1996).

Spermatogenic arrest and damage is a consequence of testicular degeneration and can have profound effects to fertility in the male. Reduction of testicle size or softening of testicular parenchyma is typical physical finding among infertile or subfertile males. Moura et al. (2001) showed that reduction of testicular size was not only a product of diminished germ cells but also due to reduced populations of Sertoli and Leydig cells in clinical spermatogenic arrested
bulls (Moura and Erickson, 2001). It was also concluded that deficiency in hormone secretion does not ultimately cause infertility and that potential sources of inadequate responses to gonadotropins, genetic defects, or other intratesticular factors may be potential initiators. In response to degeneration, some researchers have suggested stem cells are maintained in testis and are capable of self-renewal and differentiation to form new Leydig cells (Stanley et al., 2012). In addition, there is some postulated thoughts that Sertoli cells can re-enter mitotic division cycles to compensate for degeneration issues (Johnson et al., 2008). However, the regeneration model has only been shown in research using cellular transformation by integrating and overexpressing specific proteins in mice Sertoli cells.

2.3 Female Reproduction Physiology

2.3.1 Overview

The primary function of the ovary is to produce female gametes (oocytes) and to produce hormones that regulate the functions of the estrous cycle. Length of the bovine estrous cycle ranges from 18-24 days (average 21 days, day 0 being ovulation) and consists of two phases: follicular phase (4-6 days) and luteal phase (14-18 days; (Forde et al., 2011). Both phases are highly dependent on steroid production and function through the complex neuroendocrine feedback mechanisms via HPGA.

2.3.2 Endocrinology

The primary regulation of gonadotropin synthesis and release by the anterior pituitary is controlled by GnRH secretion into the hypothalamic-hypophyseal portal circulation system.
Gonadotropin releasing hormone is produced by the neurons in the tonic (ventromedial nucleus and arcuate nucleus) and surge (preoptic nucleus, anterior hypothalamic area, and suprachiasmatic nucleus) centers within the hypothalamus (Senger, 2004). The tonic center is responsible for episodic release of GnRH throughout the luteal phase of the estrous cycle. In contrast, the surge center provides pre-ovulatory surge of GnRH to initiate ovulation at the end of the follicular phase. Gonadotropin releasing hormone binds to its G-protein coupled receptor on the gonadotroph cells inducing signal transduction and releasing intracellular calcium which activates the mitogen activated protein kinases (MAPK) signal pathway (Weck et al., 1998). This stimulation of GnRH on the gonadotrophs in the anterior pituitary allows for synthesis and release of luteinizing hormone (LH) and follicle stimulating hormone (FSH). FSH is stored for short periods in anterior pituitary. Conversely, LH is stored for longer periods, specifically for the pre-ovulatory surge to induce ovulation (Farnworth, 1995). Both gonadotropins reach the ovaries by systemic circulation and allow for follicle development, maturation, and ovulation, with addition of stimulating ovarian synthesis of steroids and peptides. During follicular development, LH attaches to LH-receptors primarily on the theca cells which stimulates the production of testosterone via cholesterol-mediated conversion. Testosterone diffuses to the granulosa cell which contains FSH-receptors and once FSH binds, the granulosa cell can convert the testosterone to estradiol (Adams et al., 2008). Estradiol is released into follicular fluid or to systemic circulation providing effects on the HPGA and initiating positive and negative feedback to the hypothalamic centers. In addition, granulosa cells also contain LH-R in later follicular development, which allow for final maturation of the dominant follicle to ovulate after the pre-ovulatory surge of LH (Fortune et al., 2001, Ginther et al., 2001b).
2.3.3 Follicular phase

The follicular phase consists of proestrus (days 17-20) and estrus (days 21-1) periods of the estrous cycle and corresponds to the decreased concentrations of progesterone to basal levels via luteolysis of the corpus luteum (CL) by endogenous or exogenous prostaglandin F2α absorption. The decrease in progesterone removes the negative feedback that allows for LH secretion to decrease amplitude and increase pulse frequency to one pulse per hour (Adams et al., 2008, Hansel and Convey, 1983). Increase of LH pulse frequency allows the dominant follicle to increase concentrations of estradiol secretion and provide positive feedback to the surge center of the hypothalamus and initiate behavioral estrus once estradiol meets maximum concentration threshold, which varies based on age and individual. The estradiol positive feedback to the hypothalamus allows for advancement of dominate follicle maturation and ultimately provides the pre-ovulatory GnRH surge which initiates a rapid release of LH and FSH causing eminent ovulation (Ginther et al., 2001b). Ovulation from the dominant follicle occurs 10-14 hours after behavioral estrus ends (Forde et al., 2011).

2.3.4 Luteal phase

The luteal phase (14-18 days) consists of metestrus (days 2-4 days) and diestrus (5-17 days) which coincides with rising progesterone concentrations due to CL formation from the transformation of granulosa and theca cells into functional large and small luteal cells, respectively, after ovulation (Smith et al., 1994). On average, Bos taurus females have larger ovulatory follicles (16-20 mm) than in B. indicus breeds (10-13 mm; (Ginther et al., 1996,
Figueiredo et al., 1997) and ovulatory follicle size is often positively correlated with ensuing CL volume and progesterone secretion (Busch et al., 2008). Progesterone will continue to rise from ovulation until mid to late luteal phase. Progesterone by negative feedback, inhibits LH pulse frequency (one pulse every 4-6 hours) but with higher amplitude pulses. Progesterone initiates maternal recognition of pregnancy by down regulating progesterone receptors on the epithelial surface of the endometrium (Bazer et al., 2009). If pregnancy is not established by maternal recognition by interferon tau (INF-T), the endometrium will produce prostaglandin F2α (PGF2α) which will be transported to the ovary via the ovarian counter-current vasculature which will culminate lysis of the CL and eliminate progesterone biosynthesis (Bazer et al., 2009) and remove the negative feedback on LH (Ginther et al., 2001b).

2.3.5 Follicular dynamics/folliculogenesis

The ovary goes through predictable and dramatic changes through the relatively short period of the estrous cycle. Cattle fetal ovaries are estimated to contain $2.1 \times 10^6$ follicular germ cells and by birth it declines to 150,000 due to apoptosis (Erickson, 1966, Santos et al., 2013). This population of oocytes is limited and dramatically decreases especially after puberty, where heifers will initiate continuous repeated estrous cycles which deplete follicle population. Folliculogenesis is a sequential process relating to follicular development, growth, maturation, and ovulation. The entire period of ovarian folliculogenesis includes the period of maturation between primordial follicles and the developed, ovulating follicle (Baerwald, 2009). Primordial follicles, which are arrested in prophase I from embryonic development, are surrounded by flattened pre-granulosa cells. As primordial follicles sequentially proceeds to primary,
secondary, and to late tertiary (Griffian) follicles, there is a dramatic change in size,
morphology, and physiology. Primary follicles are characterized by the transformation of pre-
granulosa cells to cuboidal granulosa cells. This process is suggested to be independent of
gonadotropin stimulation (Findlay et al., 2002). As granulosa cells increase to 2-6 cell layers
around oocyte, secondary follicles are formed and become gonadotropin responsive. As the
follicle continues to grow and differentiate, theca cells surrounding the oocyte differentiate and
complete the formation of tertiary or antral follicle. The antral fluid that is produced within the
follicle continues to grow with gonadotropin stimulation and forms a Griffian or pre-ovulatory
follicle. In cattle, it has been estimated to take 160 days for primordial follicles to reach
Griffian stage (Russe, 1983), within that period it takes secondary follicles 42 days to reach
ovulatory size (Lussier et al., 1987).

Folliculogenesis is controlled by GnRH, gonadotropins (FSH and LH), and steroids
(estradiol and progesterone) by continuously contributing to the complex control of positive
and negative feedback mechanism of the HPGA. Follicles go through many processes in an
estrous cycle: recruitment (emergence), selection, dominance (deviation), and atresia or
ovulation. Follicular development in the bovine begins with a cohort (5-20) of small (3-5mm)
antral follicles that are gonadotropin dependent, also known as follicular recruitment or wave
emergence (Sunderland et al., 1994). *Bos taurus* and *Bos indicus* tend to have two or three
follicular waves (Figueiredo et al., 1997, Sartori et al., 2004) throughout the estrous cycle and
Figueiredo et al. (1997) showed evidence that 70% of females repeat the same wave pattern in
consecutive estrous cycles. However, the number of waves can change in an individual female
from one estrous cycle to the next, and ultimately cannot be predicted. The difference in
number of antral follicles associated with a follicular wave is speculated to be due to influence
of the IGF superfamily, which is thought to regulate ovarian follicular cell growth and

Emergence of a follicular wave coincides with an FSH surge recruiting all follicles
measuring ≤ 4mm in diameter (Adams et al., 1992). This initial growth rate differs between B
taurus and B. indicus breeds during recruitment/emergence phase, (1.1-2.0 mm/d vs. 0.9
mm/d) respectively (Figueiredo et al., 1997, Sartori et al., 2004). After wave emergence, the
follicular selection process allows 3-6 competent follicles to continue to mature and grow to
approximately 4-5 mm (Fortune et al., 2001). Subordinate follicles that do not reach selection
process proceed to regress and become atretic. Ginther (2001a) showed growth rate to differ at
16 hours prior to follicle deviation (selection phase) with the two largest follicles exhibiting
significantly more growth during this period when compared to other, subordinate follicles. FSH
concentrations are continually decreasing throughout the selection and dominant (deviation)
phases, with speculation that the largest follicles require less FSH as they continue to grow and
mature (Ginther et al., 2001a). Once follicles reach a diameter of 8.0-9.0 mm, follicular
deviation or dominance occurs (Ginther et al., 1996). This deviation is categorized by a
continued growth of the largest follicle and reduction in diameter of the yet viable subordinate
follicles. Follicular ablation experiments provided evidence that there is a hierarchy in follicle
development that allows one follicle within 2 days of wave emergence to be predetermined for
dominant stage (Ginther et al., 2001a). It was also evident that the dominant follicle inhibited
growth of the subordinate follicles and after ablation of the largest follicle, subordinate follicles
resumed growth and the next largest follicle would be selected for dominance (Ginther et al.,
There is also relevant evidence that inhibin production by the granulosa cells of the dominant follicle also regulates follicular growth by suppressing FSH supply to the subordinate follicle by directly modulating pituitary gonadotropin release (Guilbault et al., 1993).

Estradiol concentration increases in systemic circulation after dominate follicle formation (Hansel and Convey, 1983). In addition, Ginther et al (2001b) demonstrated an increase of estradiol concentrations and free insulin-like growth factor (IGF-1) in the follicular fluid of the dominant follicle (Ginther et al., 2001b). Progesterone treated heifers prevented the transient increase in LH concentrations at follicle deviation by restricting growth of the dominant follicle even though FSH increased post-deviation (Ginther et al., 2001b). These results indicate that LH is involved in follicular growth and estradiol production during follicle deviation and is consistent with negative feedback by circulating progesterone on LH pulse frequency.

### 2.4 Pyrethroids/Insecticides

#### 2.4.1 Overview

There are many natural and synthetic insecticides that have been or are being used to mitigate disease transmitting insects that are harmful to vegetation and various mammalian species. Currently, pyrethrin and pyrethroids are the main insecticides used globally with over 3,500 products registered due to the abandoning of more harmful insecticides such as organophosphates and carbamates (US EPA, 2011a). Natural pyrethrins, derived from the botanical *Chrysanthemum cinerariaefolium*, are potent insecticides that carry relatively low toxicity to mammals and agricultural crops. However, natural pyrethrins are degraded rapidly
via sunlight; therefore, the use of pyrethrins for crop and mammalian protection to control disease-carrying insects has been abandoned for synthetic analogues called pyrethroids (Anadon et al., 2009). Pyrethroids, when compared to pyrethrins, have an altered chemical structure, which allows them to be more stable in light and retain and mimic the insecticidal activity of the natural pyrethrins. Consequently, pyrethroids are widely used in agriculture including domestic and agricultural animal products for insect control and it is estimated that 400,000 pounds of permethrin, a specific pyrethroid, is used in agriculture annually in the United States (US EPA, 2011b).

2.4.2 Classifications of pyrethroids

The types of pyrethroids are classified by the presence or absence of an α-cyano group on the chemical structure, which enhances the insecticidal toxicity of the compound. Type I pyrethroids do not contain the α-cyano group and include compounds such as permethrin, allethrin, resmethrin, and the associated analogues. Type II obtain the α-cyano group and include compounds such as cypermethrin, deltamethrin, cyhalothrin, cyfluthrin, fenvalerate, and the included analogues (Anadon et al., 2009). These compounds typically exist as stereoisomers due to their acid moiety and two chiral carbon rings (Woodward and Woodward, 2013). Many of these pyrethroid compounds contain a synergist, such as piperonyl butoxide, to enhance the insecticidal activity and slow down chemical breakdown by inhibiting pyrethroid metabolism.
2.4.3 Mechanism of action

There is not a clear understanding of how these compounds actually work, however most research suggests that these compounds act by directly interfering with sodium-channel gated mechanisms and by potentially altering nerve function with chloride and calcium channel pathways (Palmquist et al., 2012). This proposed mechanism polarizes membranes and results in abnormal discharges in target neurons. Comparatively, Type I allow repetitive firing of channels and Type II continuously hold channels open. Furthermore, Type II delays inactivation of voltage-sensitive sodium channels substantially longer than Type I. Additionally, Type II, with the cyano group, potentially have some effect on the GABA receptor via an antagonistic pathway (Anadon et al., 2009).

2.4.4 Metabolism of permethrin

Major metabolites are created from cleavage of the ester bonds to yield hydroxyphenoxybenzoic acid derivatives, which are conjugated with sulfate. Phenoxybenzoic acid is also formed and converted to glucuronide and glycine conjugates in mammals. Metabolism of permethrin has been historically believed to be rapid and eliminated as metabolized form. It was discovered that orally treated rats rapidly metabolized permethrin via hydrolysis and hydroxylation (Elliott et al., 1976). The same group of researchers also presented evidence that \textit{trans}-permethrin is metabolized more rapidly and produce lower amounts of metabolites compared to \textit{cis}-permethrin.

Utilizing chromatography, lactating Jersey cows eliminated all C-labeled \textit{cis}- and \textit{trans}-pure (>99%) permethrin within 12-13 days after being orally treated (Gaughan et al., 1978).
Blood levels of either compound had a transient peak after dosages but had insignificant levels within 2-4 days post treatment. Gaughan et al. (1978) also tentatively identified 10 and 13 excreted metabolites of cis- and trans- permethrin, respectively. Excretion of metabolites was majorly through feces and urine. However, the chemical makeup of the compounds dictated excretion capacity and metabolic speed. Trans-permethrin had increased urine metabolites when compared to cis-permethrin, and comparatively, cis-permethrin had higher milk, liver, and fat residues and consisted mostly of un-metabolized compound, albeit in small amounts.

Permethrin metabolism was confirmed through oral treatment of cis- and trans-permethrin in lactating dairy goats (Ivie and Hunt, 1980). It was confirmed that trans-permethrin was highly excreted through urine and cis-permethrin being excreted in higher concentration in feces. They also identified and confirmed 26 metabolites of permethrin isomers with many oxidizing and/or conjugating with other compounds before excretion.

The basic metabolism of permethrin is somewhat understood but is still not conclusive. The rat, cow, and goat have similar metabolism of this insecticide, however the ultimate implications are not acknowledged, noting that there is a basal level retention of metabolites and/or pure compounds, especially on the skin (Gassner et al., 1997). The retention could be harmful due to the non-selective nature of these compounds and could perform transient endocrine disruption. With this understanding, new detection models, which are reliable and sensitive, have been utilized to investigate residues of synthetic pyrethroids with the use of high-performance liquid chromatography (HPLC) (Bissacot and Vassilieff, 1997).
2.5 Endocrine Disrupting Chemicals

2.5.1 Introduction

Endocrine disrupting chemicals (EDC) are defined by the EPA as chemicals (natural or synthetic) or mixtures that affect natural hormone mechanisms (US EPA, 2011a). Endocrine disruption, by definition, can occur in various ways due to the complexity of endocrine system in mammalian species. Some EDCs can manipulate the endocrine system by influencing changes to normal regulation of hormone concentrations that are vital to survival and/or homeostasis (Frye et al., 2012). The chemicals can change hormone availability which includes changes in secretion, binding, metabolism, cellular uptake, and overall control of neuroendocrine axis, such as the hypothalamic-pituitary-gonadal axis (HPGA) (Andrade et al., 2002a).

Endocrine alterations can exert more pronounced adverse consequences based on exposure in critical periods including intrauterine, perinatal, puberty, and adulthood (Frye et al., 2012). These critical period exposures can be profound in altering physiology, including reproductively, and can ultimately impact general populations (Patisaul and Adewale, 2009). Some EDCs have proposed strong estrogenic or anti-androgen characteristics which during puberty can play a huge role in aberrant organization on the HPGA and have profound and potentially permanent reproductive effects (Patisaul and Adewale, 2009, Frye et al., 2012). These potential EDCs effects are not limited and have been seen trans-generational or epigenetically (Patisaul and Adewale, 2009).
2.5.2 EPA regulations

Unlike other nations, the United States regulates all pesticides through the Environmental Protection Agency (EPA). Due to the concern of diethylstilbestrol (DES) and dichlorodiphenyltrichlorethane (DDT) being harmful estrogenic compounds, Congress passed the Food Quality Protection Act of 1996 (FQPA), which requires the EPA to consider information concerning effects on human health resulting from exposure to potential chemicals that have a relevant mechanism of toxicity as it relates to setting pesticide tolerances (Patisaul and Adewale, 2009). Due to the growing classes of pesticides, naturally-occurring pyrethrins and synthetic pyrethroids are included in the FQPA and are regulated to undergo specific recommendations under the Endocrine Disruptor Screening and testing Advisory Committee (EDSTAC) (Patisaul and Adewale, 2009).

2.5.3 Pyrethroid endocrine disruption in males

2.5.3.1 Human endocrine disruption

Pyrethrins have been associated as being a potential EDC through human and other mammalian studies. These studies vary in compounds, dosages, routes, exposure durations, and evaluated parameters. Male infertility clinic patients were evaluated for urine metabolites of pyrethrins and their exposure to such compounds through environmental or occupational hazards (Meeker et al., 2008, Meeker et al., 2009, Martenies and Perry, 2013). Meeker et al. (2008) compared amount and type of urine pyrethroid metabolites to specific sperm parameters. They reported that metabolite 3-phenooxybenzoic acid (3PBA), a common metabolite of pyrethrins, was increased in patients with lower sperm concentrations. In
addition, *trans*-3-(2,2-dichlorovinyl)-2,2-dimethlycyclopropane carboxylic acid (TDCCA), a specific permethrin metabolite, decreased sperm motility, sperm concentration, and normal morphology in a dose-dependent manner (Meeker et al., 2008). Furthermore, Meeker et al. (2008) reported *Cis*-3-(2,2-dichlorovinyl)-2,2-dimethlycyclopropane carboxylic acid (CDCCA), another specific permethrin metabolite, as well as 3PBA was also associated with sperm damage. The same research group also correlated hormone concentrations to pyrethroid exposure on male infertility patients. All metabolites were positively correlated with FSH and LH concentrations. The TDCCA and CDCCA metabolites were inversely correlated to Inhibin B concentrations with evidence of TDCCA having inverse association with 10% decline in testosterone (Meeker et al., 2009). These findings, even without a control group, uncovered associations with exposures of pyrethroids and the potential outcomes on male reproductive physiology based on chronic, low dose occupational or environmental exposure. However, the mechanism of action of indirect or direct effects is unknown. Other human research with pesticide endocrine disruption on male reproductive performance consistently focused on concentration, motility and morphology based on World Health Organization (WHO) fertility parameters (Martenies and Perry, 2013). Decrease sperm concentrations were most commonly reported with decrease motility being less frequent and morphology being less clear clinically (Martenies and Perry, 2013). All evaluated parameters in the human spermiogram are important but are a subjective evaluation on fertility.
2.5.3.2 Animal endocrine disruption

Multiple species in randomized control-based trials have been evaluated for alterations in reproduction via exposure to different type 2 pyrethroid compounds. These studies differed in dosages, routes, and exposure durations. Fenvalerate, a type II pyrethroid, given to rats by inhalation for (4 hours/day and 5 days a week) three months reduced absolute weight of testes, epididymal sperm counts, and sperm motility at 1/5 LC50 dosages (Mani et al., 2002). The inhalation of fenvalerate also reduced 17β-hydroxysteroid dehydrogenase (17β-HSD) and glucose-6-phosphate dehydrogenase (G6PDH) leading to subsequent decrease in serum testosterone concentration. However, fenvalerate (20 or 40 mg/kg) given orally for 30 days did not reduce testosterone concentrations (Arena et al., 2008). Nevertheless, both dosages of oral fenvalerate were toxic to the testes and epididymis by decreasing absolute weights and sperm counts (Arena et al., 2008). It was also observed that 40 mg/kg treated rats had elevated concentration of fenvalerate metabolites deposited in the epididymis, brain, testes, and liver, in that order. The metabolite concentrations allude to pyrethroids and/or their by-products potentially binding and infiltrating privileged sites in the body.

Similarly to fenvalerate, high oral dosages (30-60 mg/kg) of cypermethrin to adult male rats for 15 days did decrease daily sperm output (Li Yan et al., 2013). High dosages of cypermethrin also caused histological atrophy and distortion of seminiferous tubules including deformed and disordered arrangement of germ cells. Vacuolization of Sertoli cells and deforming of Leydig nuclei was also noted with subsequent decrease of serum testosterone concentration in oral cypermethrin treated rats. Remarkably, LH was not affected by the dosages of cypermethrin; however, rats treated with 60 mg/kg of cypermethrin increased
serum FSH levels (Li Yan et al., 2013). It should be noted that lower dosages of cypermethrin in the same study, did not show any significant changes compared to the controls. Comparatively, cypermethrin given orally for 12 weeks at lower dosages (13.15, 18.93, and 39.66 mg/kg) in rats had increased weights of preputial glands and decreased epididymal and testicular sperm counts at all treatment concentrations compared to the controls (Elbetieha et al., 2001). Elbetieha et al. (2001) also reported that the highest dosed male rats had decrease serum concentrations of testosterone, LH, and FSH. In addition, the male rats exposed to 18.93 and 39.66 mg/kg concentrations had lower fertility and implantation rates when allowed to breed unexposed females. The two higher doses also had significant increases in testes and seminal vesicle weights with a significant decrease in cell layers of seminiferous tubules and marked testicular hemorrhage. The increase in weight of organs is not well understood in this case but is speculated to be derived from accumulation of interstitial connective tissue or by a variety of other steroid hormones including circulating thyroid hormone. In relation to histopathology, Rodriguez et al. (2009) found intense increase of seminal gland epithelium height and cell proliferation by 24 hours after intraperitoneal injection of cypermethrin (1/5 LD$_{50}$) in adult mice but resolution was observed by day 9 of the study. However, mastocytes increased dramatically throughout the 34 day study (Rodriguez et al., 2009). Furthermore, mice with intraperitoneal injected dosages of cypermethrin (30, 60, 90 mg/kg for 5 consecutive days) increased number of sperm head abnormalities with toxic effects affecting mitotic activity by dosage (Kumar et al., 2004).

Deltamethrin is another type II pyrethroid that has shown aberrant effects on male reproduction. Subcutaneous treatments of different dosages and duration (2 ppm for 30 days,
20ppm for 45 days, and 200ppm for 60 days) of deltamethrin (DM) in male rats caused desquamated cells in the lumen of the seminiferous tubules accompanied by vacuolization with germ cells and tubules with apoptotic bodies (Issam et al., 2009). There was also interstitial tissue and Leydig cells that strongly regressed and spermatozoa were less present within the lumen of the seminiferous tubules. The increasing dosages for longer duration of DM decreased levels of FSH, LH, and testosterone. Oxidative stress was also noted by 30 days of treatment by increased malondialdehyde (MDA) (Issam et al., 2009). Similarly, oral treatment of 5/mg/kg deltamethrin for 4 weeks decreased testosterone, LH, and FSH concentrations (Ismail and Mohamed, 2012). The research group also observed up-regulation of mRNA for glutathione-s-transferase and heat-shock protein-70 (HSP-70), indicating testicular DNA damage. There was also down-regulation of steroidogenic acute regulatory (StAR) mRNA after oral deltamethrin exposure. In addition, in vitro incubation of rat epididymal sperm with different levels of deltamethrin (0, 10, 50, 100, 200 uM significantly declined sperm motility and viability and increased abnormal sperm morphology (Abdallah et al., 2010). Abdallah et al. (2010) also noted significant oxidative stress to sperm with deltamethrin in vitro by increases of superoxide dismutase (SOD), MDA, and catalase.

Even though there has been convincing results with the long-term exposures and in vitro studies, not all pyrethroid mammalian studies report steroid alterations, sperm abnormalities, or reproductive dysfunctions. Oral treatment of technical and formulated deltamethrin (2 and 4 mg/kg/d for 3 days) did not show any anti-androgenic activities using an in vivo Hershberger assay on male rats (Andrade et al., 2002b). In addition, there was no effect of fertility or sexual
competency with oral (63 and 100 mg/kg/day) treatment of lambda cyathrin (ICON®) given to mice for 7 consecutive days (Ratnasooriya et al., 2002).

Type I pyrethroids have also been shown to produce male endocrine disruption and reproductive abnormalities; however type I are less researched than the type II pyrethroids. Orally treated male mice with cis-permethrin (0, 35, and 70 mg/kg/d for 6 weeks) had lower caudal epididymal sperm counts and motility in a dose dependent manner (Zhang et al., 2007). Zhang et al. (2007) also noted decrease in plasma and testicular testosterone with lower LH concentrations. These findings was probably correlated to the mitochondrial membrane damage of Leydig cells seen on electron microscopy and the significant decrease in mitochondrial mRNA expression of StAR and cP450scc and their protein and enzyme expressions, respectively. The orally-treated permethrin mice did not show any reproductive organ weight differences, comparatively to type II pyrethroids, nor were there any differences in sperm morphology or FSH concentrations.

2.5.3.2.1 Ruminant endocrine disruption

In respect of species differences, male dwarf goats dipped twice (day 0 and 15) in different concentrations (0.1, 0.4, 0.8, 1.6%) of cypermethrin decreased ejaculate volume, progressive motility, sperm viability, and sperm concentration with addition of semen becoming more alkaline and more straw-colored by day 30 and gradually more pronounced by days 45-75 and by dose dependency (Ahmad et al., 2009). Sperm morphologic changes (tailless, bent tails, and coiled tails) in the high concentrate treated goats was seen at 45-60 days but most semen parameters improved by 75 days in the trial, eluding to potential lagging effect
with recovery due to treatment. Histological examination of the treatment groups showed decrease spermatogenesis with more immature spermatids and degenerative changes and loss of spermatagonia, spermatocytes, Sertoli cells, spermatids, and spermatozoa, which was also dose dependent (Ahmad et al., 2009). Male ruminants exposed to pyrethroids have also been clinically observed to have negative effects on reproduction. Observations by Volkmann (2012) implied that pyrethroid exposure to bulls and rams for a short duration could negatively impact sperm concentration, ejaculate volume, progressive sperm motility, and sperm morphology (including distal midpiece reflexes, distal cytoplasmic droplets, and detached heads) after a few days post-exposure. In addition, Volkmann (2012) claimed that pure pyrethroid, bifenthrin, compound was found in blood, urine, and semen in one of the observational cases. However, these observational studies were not conclusive of product chemical make-up nor was there a clear route of exposure or a true cause-effect relationship. Even though the observational studies had no controls, the hypothesis is warranted due to the previous literature supporting such a claim of male reproductive dysfunction with exposure to pyrethroids. Recently, Cain et al. (2014) showed no differences in sperm motility or morphology throughout an 84-day trial period with exposure to 150% label dose, given twice (day 0 and 14), of 1% permethrin dermally to purebred beef bulls. The results did not show similar results claimed by Volkmann even with overdosing (Cain et al., 2014). Similarly, French et al. (2015) did not see any effect on sperm parameters when crossbred bulls were exposed to cyfluthrin pour-on, cyfluthrin fly tags, and a combination of cyfluthrin pour-on and fly tags over a nine week study. In addition, there was no difference in serum testosterone concentrations between control and treated bulls (French et al., 2014). Likewise, crossbred bulls exposed to different combinations of pyrethroids
and pyrethrins (control – cyfluthrin pour-on and fly tags, treatment – cyfluthrin pour-on and fly
tags and pyrethrin premise spray fogger) showed no consistent change in sperm parameters or
testosterone in a nine week study (Stewart et al., 2015). However, there was a significant
decrease in overall sperm motility at week two and a significant decrease in serum testosterone
concentration at week one and a two-fold decrease at week nine. This study suggests that
combination exposure with pyrethrin, the natural insecticide compound, could have additive
and delayed negative effects on biosynthesis of testosterone.

2.5.4 Pyrethroid endocrine disruption in females

2.5.4.1 In Vitro studies

Due to the highly lipophilic nature of pyrethroids, follicular and oviductal fluids could
contain such pyrethroid or metabolite substances and thus alter follicular dynamics and have
negative effects on oocyte development and steroid biosynthesis (Hirshfield, 1997). Rat
preantral follicles cultured in vitro with fenvalerate (0, 1, 5, 25 μmol/ml), inhibited follicular
diameter growth within 24 hours; however, follicular survival rates remained unaffected (Fei et
al., 2010). There was also inhibition of concentrations of progesterone, testosterone, and
estradiol with decreases in StAR and cP450scc mRNA gene expression in a concentration-
related manner. Likewise, bifenthrin, a synthetic type I pyrethroid, used in vitro with rat ovarian
granulosa cells significantly decreased secretion of progesterone and prostaglandin E2 (PGE2),
exclusively by the 1S-cis-bifenthrin enantiomer (Liu et al., 2011). This research group also
discovered that 1S-cis-bifenthrin isomer reduced expression of cP450scc, StAR, cyclooxygenase-
2 (COX-2), and other progesterone biosynthesis genes, in addition to disrupting transcriptional
activation of StAR and COX-2 promoters. 1S-cis-bifenthrin also differentially inhibited the activity of protein kinase C (PKC), which mediates progesterone and prostaglandin biosynthesis. In addition, in vitro mature pig oocytes did not induce significant degeneration or have significant effects on oocyte viability cultured in different concentrations of cypermethrin, deltamethrin, or fenvalerate (Petr et al., 2013). However it was found that maturation was delayed in fully meiotic competent pig oocytes and induced maturation in partially competent oocytes. Interestingly, calcineurin non-inhibiting pyrethroids, allethrin or permethrin, did not affect oocyte maturation. The mechanism is unclear but could provide detrimental effects to fertilization and potential embryo development. Comparatively, cypermethrin used in vitro with bovine corpus luteal cells had decreased viable cell counts and produced significantly lower concentrations of progesterone due to the dramatic and severe degenerative and toxic effects to the luteal cells induced by differing dosages (10, 50, 100 ppm) and exposure times (Gill et al., 2011). The in vitro studies do not mimic the normal metabolism of mammalian species and therefore are not true indicators of physiological endocrine disruption. Nevertheless, in vitro studies do prompt for further investigation in animal models, especially in reproduction.

2.5.4.2 In Vivo Studies

According to some research, pyrethroids can inhibit proper function of steroids especially during puberty. Esfenvalerate, type II pyrethroid, delayed vaginal opening and decreased morning serum estrogen concentration after oral administration (1.0 and 5.0 mg/kg/day starting on postnatal day 22) to pre-pubertal female rats (Pine et al., 2008). Pine et al. (2008) also reported that rats treated with 1.0 mg/kg had a decrease in afternoon serum LH
concentration, which is vitally important for peripubertal development in the female rat. However, the decreased LH concentration could not be identified as a hypothalamic or pituitary responsive. In comparison, Arenas et al. (2008) showed no estrogenic effects via three day oral treatment (0.4, 1, 4, 8, 40 mg/kg) of fenvalerate to immature female rats utilizing an uterotrophic assay (Arena et al., 2008).

Pyrethroid exposure has also been shown to have negative impacts on conception and pregnancy rates. Lambda cyalothrin (ICON®) orally treated (6.3, 8.3, or 12.5 mg/kg/day) female rats in early gestation (days 1-7) showed detrimental pregnancy outcomes, mainly due to increased pre-implantation losses of developing embryos (Ratnasooriya et al., 2003). Ratnasooriya et al. (2003) also observed potential anti-progestogenic effects of lambda cyalothrin by resolving lower number of uterine implantations with treatment of exogenous progesterone. This may imply that some pyrethroids can cause de-synchronization of embryo implantation or that pyrethroids provide hostile environments for embryo survival. Similarly, sacrificed female albino rats had significant reduction of implantation sites after receiving oral dosages (1.0, 2.0, or 4.0 mg/kg) of deltamethrin immediately following confirmation of copulation through seven days of gestation (Lemos et al., 2011). Lemos et al. (2011) histologically described highly-dosed deltamethrin treated rats having obvious implantation abnormalities including vacuolated trophoblast cells, rare cytotrophoblasts, leukocyte infiltration, with increased sites of vascularization, including free blood in the uterine lumen. These results indicated compromising implantation process with alterations in the interaction between the embryos and endometrium due to pyrethroid treatment.
Exposure to pyrethroids during pregnancy can also have transgenerational or epigenetic effects to offspring. Moniz et al. (2005) showed dams intraperitoneally injected with fenvalerate (10 mg/kg) during gestation and in early lactation produced delayed sexual maturity and a reduced sexual behavior in female rat offspring without any steroid concentration differences (Moniz et al., 2005). Furthermore, Guerra et al. (2011) reported decreases in ovarian weight, pre-antral follicles, and corpus lutea in female rat offspring from dams orally treated with pure fenvalerate (40 mg/kg) during gestation and throughout lactation. In addition, female offspring showed increased early pregnancy resorptions when fertility tests were performed after first estrus (Guerra et al., 2011). In contrast, oral treatment of deltamethrin (1, 2, or 4 mg/kg) to pregnant and lactating female rats caused smaller seminiferous tubule diameter and lower testicular and epididymal absolute weights of male offspring only at the highest dose (Andrade et al., 2002a). Overall, trans-generational effects of oral deltamethrin seem to be minimal. However, pregnant rats exposed to oral treatment of low doses of deltamethrin (0.25, 0.5, 1.0 mg/kg) during gestation, produced dose-dependent changes in expression of cP450 in postnatal offspring brain and liver tissues (Johri et al., 2006). Even though this study did not elucidate on reproductive effects, this does show that pyrethroid exposure can affect the fetus through transplacental transfer and could have detrimental alterations to neuroendocrine function to offspring.

2.2.5 Conclusion

Endocrine disruption can be caused by many different pyrethroid compounds or mixtures on many different mammalian species. The physiological effects depend on
compound, dosage, route, and exposure duration. Direct effects can mimic steroids and bind to receptors as hormone ligands and affect the process of gene transcription or by apoptosis of germ cells. Indirect effects can interfere with normal neuroendocrine control and can produce harmful oxidative stress in the form of free radicals or other mediators. Endocrine disruption effect can be immediate or gradual and can be dose dependent. Some effects can be reversible, permanent, or transgenerational. Many pyrethroid compounds that are used are not pure compounds and are usually mixed with synergistic agents that have not been evaluated for potential antagonistic effects on reproductive performance.

2.6 Statement of the Problem

In the U.S. beef industry, cow-calf operations rely on bull pasture breeding as the primary means of reproduction. Because pasture breeding most often occurs during a time of insect pressure on the herd, insect control becomes an important issue to provide optimal health and well-being. To combat insect burden, insecticides have been utilized to eliminate the negative effects from flies, lice, and mites. Pyrethroids are the primary chemical used in veterinary products due to the dismissal of more ecological harmful insecticide compounds (organophosphates and carbamates). However, recent public press literature derived from observational findings, has stimulated thoughts and concerns for use of pyrethroids and the potential for reproductive failures, specifically in the bull. The hypothesis is that bulls exposed to pyrethroids will have decreased sperm motility, an increase in abnormal sperm morphology, and eventually diminished fertility. While the exact mechanisms have not been fully explained, previous research has linked possible mechanisms via endocrine disruption. However, as many
of these concerns and hypotheses have not been validated or well tested in a scientific setting, research to elucidate the effects of commercial pyrethroid-based veterinary products on reproductive function beef cattle is needed.

The overall goal of the experiments conducted in this thesis was to determine the impact of pyrethroid (permethrin) use in both male and female beef cattle reproductive function. Within this goal, the focus is to provide clarity to a highly debated beef production topic and provide a physiological basis by which commercial veterinary pyrethroid products may alter reproductive capacity in the yearling bulls and heifers. The emphasis was to validate or challenge the previously stated observation and research findings of pyrethroid use on bull reproductive parameters. Particularly given the amount of literature that suggests potential negative effects on female rats exposed to pyrethroids and females in other species, we elected to determine potential effects on steroid and reproductive alterations in superovulated beef heifers.
CHAPTER 3
EFFECTS OF PERMETHRIN USE IN YEARLING ANGUS BEEF BULLS ON REPRODUCTIVE
FUNCTION AND TESTICULAR HISTOPATHOLOGY

3.1 Abstract

Pyrethroid administration to a wide variety of laboratory animals has shown to cause
detrimental effects on male fertility, including sperm quality, by means of endocrine disruption.
The objective of this experiment was to study the effects of a commercial, pyrethroid-based
pour-on product, permethrin, on reproductive parameters and testicular histopathology of
yearling beef bulls. Black Angus bulls (n=60; 369 ± 17 d of age; 511 ± 33 kg; 6.2 ± 0.5 BCS) were
assigned to either 1) saline control (CON) or 2) permethrin pour-on administered at label dose
(PYR). All bulls had blood samples collected and were subjected to an industry standard
breeding soundness exam (BSE) via electroejaculation at both 5 d prior to and 14 d post-
treatment. Progressive motility and Eosin-Nigrosin stained morphology were analyzed using
high power magnification with phase contrast microscopy. Plasma testosterone concentrations
were analyzed via RIA. At 34 d post-treatment, bulls were slaughtered and one testicle per bull
was collected for histopathological examination. Categorical and continuous data were
analyzed with the GLIMMIX and MIXED procedures of SAS, respectively. Change in motility
between BSEs was not different due to treatment (P = 0.69). Although morphology improved
across treatments between BSEs, PYR bulls had less improvement in percent of head (P < 0.001)
sperm abnormalities compared to CON, resulting in less improvement of primary abnormalities
(P = 0.04). Nonetheless, morphological differences did not change the overall outcome for
satisfactory breeder status ($P = 0.82$). Change in testosterone concentration did not differ due to treatment ($P = 0.22$). Histopathological examination determined that testicular degeneration and tubule diameter did not differ as a result of treatment ($P \geq 0.19$). It should be noted, however, that degeneration score (higher score having more degeneration) was positively correlated with primary abnormalities ($P < 0.01; r = 0.35$) and negatively correlated with normal sperm cells ($P < 0.001; r = -0.43$). In summary, these data indicate that a single use of permethrin at label dose in yearling Angus bulls results in minimal detrimental effects on semen morphology, but not to a degree that impacts the ability of bulls to pass a standard BSE.

### 3.2 Introduction

For the past few decades, pyrethroids have been the leading global insecticide utilized in agriculture, including veterinary animal products, due to the phasing out of the more ecological harmful products such as organophosphates and carbamates (US EPA, 2011a). To improve productivity in cow-calf operations by eliminating potential insect-borne diseases, many producers and veterinarians utilize pyrethroid-based insecticide products. According to National Animal Health Monitoring System (NAHMS) by the United States Department of Agriculture, over one-half of beef operations used some type of insecticide fly control and over 70% of larger herds (> 50 head) used insecticides to control production losses due to disease transmitting pests (Usa et al., 2010). Cattle pyrethroid insecticides come in many application modalities such as: pour-ons, impregnated ear tags, dust bags, back rubbers, contact sprays, foggers, and residual/surface sprays. However, popular press literature has identified potential links between use of pyrethroids and its negative effects on beef bull reproductive health.
Consequently, this claim has stimulated uncertainties and fears for producers and veterinarians using pyrethroids on beef bulls during the breeding season.

Volkmann (2012) reported clinical observations of detrimental effects on bull sperm morphology and motility shortly after being exposed to pyrethroid compounds; however, compounds, route of exposure, dosages, and exposure duration were not noted. These observations do share similar findings in other clinical cases and laboratory studies using different pyrethroid chemicals with differing routes and dosages in a variety of species including humans, rat, mice, and goats (Meeker et al., 2008, Mani et al., 2002, Arena et al., 2008, Li Yan et al., 2013, Elbetieha et al., 2001, Ahmad et al., 2009, Kumar et al., 2004, Zhang et al., 2007). In addition, literature also supports potential endocrine disruption and its deleterious effects of male reproduction due to pyrethroid use with in vivo and in vitro trials (Meeker et al., 2009, Mani et al., 2002, Li Yan et al., 2013, Elbetieha et al., 2001, Issam et al., 2009, Zhang et al., 2007, Abdallah et al., 2010).

However, reports on the effect of pyrethroid administrations on bull fertility are unclear and inconsistent with the previous claim by Volkmann (2012). In a case controlled study, purebred beef bulls treated with 150% label dose of topical 1% Permethrin on d 0 and d 14 did not show any negative effects on normal sperm morphology throughout the 12 week study (Cain et al., 2014). In addition, French et al (2014) reported no difference between motility, morphology, or testosterone concentrations between beef bulls treated with beta-cyfluthrin pour-on or bulls exposed to a combination of pyrethroid pour-on and beta-cyfluthrin fly tag application. Similarly, Stewart et al (2013) reported no consistent difference in overall and progressive sperm motility or testosterone concentrations during a nine week trial using two
treated groups using cyfluthrin and pyrethrin spray products in combination with cyfluthrin pour-on and fly tags in crossbred beef bulls. However, peripubertal bulls, which potentially lack capacity to overcome reproductive disruption, have not been evaluated. Furthermore, administration of higher concentration products (5% permethrin, in current study) has not been assessed completely.

Effects from pyrethroid exposure have been assessed with standard BSE parameters and steroid (testosterone) concentrations; however, effect of pyrethroids on testicular histopathology has not been addressed in the bovine. The objective of this study was to measure reproductive parameters in peripubertal bulls using a commercially available pyrethroid-based pour-on. We hypothesized that use of a pyrethroid pour-on at label dose would have limited effects on semen and testicular characteristics even in peripubertal bulls.

### 3.3 Materials and Methods

#### 3.3.1 General

All protocols and procedures used were approved by the Iowa State University Institutional Animal Care and Use Committee. The project was conducted at the Armstrong Memorial Research and Demonstration Farm in Lewis, IA in April 2014. The project utilized bulls from the McNay Beef Research herd which are sourced from a single herd within the Iowa State University system. The products used in this study included: a synthetic, type I pyrethroid pour-on (permethrin; Ultra Boss®, Intervet/Merck Animal Health, Summit, NJ) and sterile saline (0.9% sodium chloride, Abbott Laboratories, North Chicago, IL). We evaluated permethrin pour-on (Ultra Boss®) at label dose due to its popular use based on sales from local distribution.
companies in the Midwest and having the highest concentration of pyrethroid substance in commercially available products.

3.3.2 Animals and treatments

Purebred Black Angus yearling beef bulls (n = 60; 369 ± 17 d of age; 511 ± 33 kg; 6.2 ± 0.5 BCS) were assigned to either 1) a saline control (CON; n=30) or 2) a permethrin pour-on (PYR; n=30) applied topically along the dorsal side of the bull per label directions. The PYR bulls received a label dose of permethrin (5% permethrin and 5% piperonyl butoxide; 3 mL per 45 kg body weight with maximum of 30 mL/animal) for lice and fly control. Because all bulls weighed more than 455 kg, all received the maximum label dose of 30 mL. The CON bulls received the same 30 mL volume of saline. As the treatments were applied topically, bulls were housed one pen per treatment to avoid cross-contamination. All bulls received the same environmental and nutritional influences before and after treatment.

The experimental design is illustrated in (Figure 3.1). Five days prior to treatment, initial body weight (BW) and body condition scores (BCS) were recorded. At that time, all bulls were subjected to an industry standard breeding soundness exam (BSE) following published guidelines established by the Society of Theriogenology (Chenoweth et al., 2010). The BSE consisted of a general physical exam, scrotal circumference (SC) measurement, external palpation of sex organs (scrotum, testes, and epididymis) and internal palpation of accessory sex glands, visual assessment of penis and prepuce, and collection of ejaculate for microscopic assessment. After initial BSE on d 0, bulls were treated respectively on d 5 and were subjected to duplicate and final BSE on d 19. A 14 day window between treatment and final BSE was
based on previous reports that indicate the largest morphological and motility alterations occur within two weeks after pyrethroid treatment (Volkmann, 2012).

Semen was collected via electroejaculation (Pulsator IV, Lane Manufacturing, Denver, CO) into a plastic collection bag and was immediately transferred to a warming plate (37°C). A small drop of ejaculate was placed on two warmed slides with one receiving a coverslip to assess overall sperm motility and one being stained with Eosin-Nigrosin for general morphology. A blinded, boarded theriogenologist analyzed overall percent motility by assessing multiple fields under light microscopy (40X). Morphology was analyzed using high power magnification (100X) and phase contrast modalities under oil immersion. One hundred sperm cells were assessed for morphological analysis. Morphological abnormalities were classified as primary or secondary and categorized by head, midpiece, proximal droplet, coiled tail, distal droplet, bent tail, and tail-less sperm defects.

At each BSE, blood was collected via coccygeal tail venipuncture into tubes containing potassium EDTA. Blood was placed on ice and was centrifuged (1750 x g for 25 minutes) within 8 h of collection. Plasma was transferred to polystyrene tubes and frozen (-20°C) for later analysis. Total testosterone concentration was analyzed via a commercially available solid-phase radioimmunoassay kit (Coat-A-Count®, Siemens Healthcare Diagnostics Inc., Los Angeles, CA). Across 2 assays, the average intra-assay CV was 2.5%, with the inter-assay CVs for pooled samples containing 6.69 ng/mL of testosterone was 4.1%. The average sensitivity across assays was 0.16 ng/mL (95% confidence interval).

After the final BSE, but prior to harvest, 1 bull was removed from the study due to temperament and 3 bulls were selected as replacements for the breeding herd. Thirty-nine
days after treatment, bulls were slaughtered (n=56) and 1 testicle was randomly selected for harvest. Within 1 hour of collection, testicles were processed (bread-loafed) and submerged in 10% neutral-buffered formalin. Histology samples from three standardized locations (proximal, middle, and distal) of the testicle were analyzed by a blinded, boarded pathologist. Testicular degeneration was scored on a 0-4 scale (0=normal, 1= rare, 2= mild, 3=moderate, and 4=severe degeneration). Seminiferous tubule diameter was also measured for 10 random tubules using computerized software (Olympus DP72 camera, cellSens® digital imaging software).

3.3.3 Statistical analysis

Differences between treatments for binomial data (BSE classification and testicular degeneration categories) were analyzed using GLIMMIX procedure of SAS 9.3 (SAS Institute Inc., Cary, NC). The remaining performance and reproductive continuous parameters were analyzed using the MIXED procedure of SAS. Because the study was conducted using yearling bulls that were likely peripubertal, days of age was used as a covariate in the model for all reproductive parameters. The final model included the fixed effect of treatment and individual bull served as the experimental unit. Statistical significance was acknowledged at $P \leq 0.05$, and $0.05 < P \leq 0.10$ was considered a tendency approaching significance.

3.4 Results

3.4.1 Growth and performance

Initial bull BW and BCS did not differ prior to treatments ($P = 0.60$, $P = 0.11$; respectively, Table 3.1). Furthermore, final bull BW and BCS did not differ when collected at d 14 post-
treatment \( (P = 0.60, P = 0.28; \text{respectively}) \). Thus, change in BW and BCS as well as average daily gain between BSEs did not differ as a result of treatment \( (P = 0.85, P = 0.78, \text{and} \ P = 0.85, \text{respectively}) \).

### 3.4.2 Reproductive parameters

Change in scrotal circumference between BSEs for PYR \( (1.0 \pm 1.4 \text{ cm}) \) and CON \( (1.3 \pm 1.4 \text{ cm}) \) did not differ due to treatment \( (P = 0.18; \text{Table 3.2}) \). Furthermore, change in testosterone between BSEs did not differ \( (P = 0.22) \) between PYR \( (0.08 \pm 0.8 \text{ ng/mL}) \) and CON \( (-1.22 \pm 0.8 \text{ ng/mL}) \). Initial overall sperm motility was not different \( (P = 0.27) \); however, motility in both the PYR and CON bulls numerically decreased post-treatment \( (12.9 \% \text{ and} \ 10.1 \%, \text{respectively}; \text{Table 3.2}) \). Nevertheless, change in motility between BSEs was not different due to treatment \( (P = 0.69) \). Sperm normal morphology numerically improved across both CON and PYR bulls between collections \( (8.9 \% \text{ and} \ 9.5 \%, \text{respectively}; \text{Table 3.3}) \); however, PYR bulls had less improvement in percent of sperm head abnormalities \( (P < 0.001) \), which resulted in overall reduced improvement of primary abnormalities \( (P = 0.04) \) when compared to CON. Also, CON bulls had less improvement in tailless sperm abnormalities \( (P = 0.05) \) compared to PYR bulls between collections. Comparatively, other primary (proximal droplets, and coiled tails) and secondary (midpiece, distal droplets, and bent tails) defects did not change between collections for CON and PYR bulls \( (P \geq 0.12, P \geq 0.57; \text{respectively}) \).

Prior to treatments, 63\% \( (19/30) \) CON bulls and 43.3\% \( (13/30) \) PYR bulls met the criteria for “satisfactory breeder” status through the Society of Theriogenology guidelines \( (P = 0.17; \text{Table 3.4}) \). At second collection, there was a numerical increase of “satisfactory breeders”
across CON and PYR bulls (66% and 70%, respectively); however there was no difference in bulls changing satisfactory status from collection one to collection two across treatments ($P = 0.82$).

### 3.4.3 Testicular histopathology

Histopathological examination determined that seminiferous tubule diameter did not differ as a result of treatment ($P = 0.99$; Table 3.5). In addition, testicular degeneration score did not differ across treatments ($P = 0.19$). However, it may be of note that 31.0% (9/29) PYR bulls had moderate or severe testicular degeneration compared to 14.8% (4/27) CON bulls ($P = 0.19$). In addition, 17.2% (5/29) PYR bulls had severe testicular degeneration compared to 3.7% (1/27) CON bulls ($P = 0.17$). Furthermore, degeneration score (higher score having more degeneration) was positively correlated with primary abnormalities ($P < 0.01$; $r = 0.35$) and negatively correlated with normal sperm cells ($P < 0.001$; $r = -0.43$).

### 3.5 Discussion

#### 3.5.1 General

Natural service breeding is the predominant practice for beef cattle operations in the United States (USDA, 2009) and the use of pyrethroids has been observed to minimize optimal bull fertility (Volkmann, 2012). The objective of this experiment was to study the effects of a concentrated pyrethroid (permethrin) pour-on on reproductive parameters and testicular histopathology of yearling beef bulls.

It is of value to reiterate that the current study utilized one-time label dose of permethrin pour-on at the concentration of 5% permethrin with mixture of cis- and trans-
isomers. This is in contrast to studies that have evaluated potential EDCs in which they overdosed bulls (Cain et al., 2014). While there is little doubt that over-dosing animals on any potential EDC likely elicits negative reproductive results, this study was designed to determine if a label-dose of 5% permethrin was safe for use or whether negative impacts on reproduction were noted that could compromise bull fertility.

The current study utilized peripubertal beef bulls which allowed evaluation of potential effects on developing bulls that may lack ability to overcome endocrine disruption and produce negative reproductive parameters. Moura et al. (2011) reported that changes in LH, FSH, and testosterone during maturation of the gonads is highly important for the Leydig and Sertoli cells to differentiate and proliferate for future production of spermatogenesis, as early as one to four months of age. Even though the current study did not allow bulls to complete an entire spermatogenic cycle post-treatment like other bull studies (Cain et al., 2014, French et al., 2014), the use of a large group of peripubertal bulls was thought to elucidate the negative effects better than aged bulls due to the potential of younger bulls being more susceptible to reproductive changes via pyrethroid exposure. Nevertheless, questions still remain if pyrethroid exposure in early gonadal development (prepuberty) will harm or delay future spermatogenesis by disrupting normal gonadal physiology early in life.

3.5.2 Growth and performance parameters

There were no differences noted in average daily gain or body condition scores due to treatment, which was expected due to the short duration of only 19 d between initial and final BW and BCS. In addition, as the study was conducted in a low ectoparasite season, combined
with bulls being fed a high-energy, ad-libitum diet, we did not expect to see any compromise in growth performance of CON bulls relative to PYR.

### 3.5.2 Reproductive parameters

Endocrine disruption has been proposed to be the mechanism of action by which pyrethroids alter the male reproductive system. Previous research has indicated that humans with increased occupational or environmental exposures to pyrethroids have high quantities of pyrethroid metabolites in their urine which corresponds to higher FSH and LH concentrations along with decreased Inhibin B concentrations (Meeker et al., 2008, Meeker et al., 2009). It was also shown that specific pyrethroid metabolites can induce a 10% decrease in testosterone which corresponded to decrease sperm motility, concentration, and normal morphology. In addition, rats that were exposed to fenvalerate by inhalation had reduced 17β-hydroxysteroid dehydrogenase and glucose-6-phosphate dehydrogenase leading to lower concentrations of testosterone and subsequently leading to reduced sperm motility and concentrations (Mani et al., 2002). The lack of difference in testosterone and other reproductive parameters is not surprising in our study, as Cain et al. (2014), French et al. (2014), and Stewart et al. (2015) also noted no consistent change in testosterone concentration and sperm parameters in bulls after exposure to similar pyrethroid products. However, Stewart et al. (2015) reported an inconsistent significant decrease in testosterone concentration in bulls at weeks 2 and 7 post-exposure to a combination of pyrethroid pour-on and fly tags, and a pyrethrin premise spray. It should be noted that our study only utilized 5% permethrin without the natural pyrethrin compound. Differences noted between Stewart et al. (2015) and the current study could be due
to the type of pyrethroid, combination, routes of administration, and duration of exposure, which has been shown to vary the degrees of anti-androgenic activity in other species (Arena et al., 2008, Mani et al., 2002).

Various species have been reported to display reduced motility after exposure to pyrethroids including rats, mice, and dwarf goats (Zhang et al., 2007, Ahmad et al., 2009, Abdallah et al., 2010). Similar to previous reports on pyrethroid exposed bulls (Cain et al., 2014, French et al., 2014), the current study did not present evidence for reduction of motility. However, post-treatment reduction in motility in this study was consistent across treatments. This is most likely attributed to a considerably lower ambient temperature at second collection and inability to adequately control thermoregulation of the semen sample obtained. Nonetheless, dwarf goats exposed to dermal application of cypermethrin, on days 0 and 15, showed a decrease in progressive motility initially at day 30; however, improvement of sperm motility was seen by day 75 of the study (Ahmad et al., 2009). These results indicate a potential transient negative effect on sperm motility but allude to non-permanent damage of sperm maturation and development at the testicular or epididymal level.

Morphology difference after dermal exposure to pyrethroids was not significantly different than controls in all previously published bull data (Cain et al., 2014, French et al., 2014), which concurs with the current study utilizing label-dose permethrin treatment. The PYR bulls did lack improvement in sperm head defects and subsequently had less improvement in primary abnormalities compared to CON bulls in the current study. However, the lack of improvement with sperm morphology did not change the overall outcome for satisfactory breeder status. Similar defects were noted in mice intraperitoneally injected with cypermethrin,
which showed toxic, mitotic active sperm head abnormalities (Kumar et al., 2004), and dwarf goats exposed to dermal application of cypermethrin showing an increase in tailless, bent and coiled tail defects (Ahmad et al., 2009). Both studies differ from Volkmann (2012) that reported an increase in distal midpiece reflexes, distal cytoplasmic droplets, and detached heads in bulls exposed to pyrethroid. However, the aforementioned mouse and goat studies may vary from the Volkmann (2012) observations due to the lack of information on dose, duration, and actual compound and/or mixture of pyrethroid(s) used in the reported clinical cases seen by Volkmann (2012). Data from the current study suggests that single administration of label-dose permethrin-exposed bulls do not experience the previously claimed negative reproductive effects with sperm motility and morphology. Given that a BSE is a “snap shot” evaluation of bull reproductive soundness; further research may be warranted to elucidate the long-term effects of chronic pyrethroid exposure. Although there was no consistent sperm morphologic or motility differences in the study, fertility and developmental competency of the permethrin treated bull sperm was not established and should be evaluated further potentially utilizing fluorescent spermatozoa stains and in vitro fertilization models.

3.5.3 Testicular histopathology

Perhaps the most intriguing observation in the current study was the weak trend for testicular degeneration to increase with PYR treated bulls after 34 days post-treatment. To the authors’ knowledge, this is the first study to assess testicular histopathology of bulls after exposure to pyrethroids. In comparison, histological examination of the treated male dwarf goats dipped twice (day 0 and 15) in different concentrations (0.1, 0.4, 0.8, 1.6%) of
Cypermethrin showed a dose-dependent decrease in spermatogenesis with more immature spermatids and degenerative changes and loss of spermatagonia, spermatocytes, Sertoli cells, spermatids, and spermatozoa (Ahmad et al., 2009). In addition, two studies with male rats treated with oral doses (30 and 60 mg/kg for 15 days; 18.93 and 39.66 mg/kg for 12 weeks) of cypermethrin had lesions consisting of atrophy and distortion of seminiferous tubules, deformed germ cells, and Sertoli and Leydig cell degenerative changes (Li Yan et al., 2013) and significant decrease in cell layers of seminiferous tubules and marked testicular hemorrhage, respectively (Elbetieha et al., 2001). The correlation between testicular degeneration and abnormal sperm morphology in the current study provides some valuable information about sperm morphologic parameters due to architecture of the testicles regardless of treatment. However, the correlation with testicular histopathology could allow for further investigation when evaluating endocrine disrupting compounds and the effects on reproductive parameters.

### 3.6 Conclusion

In conclusion, these data indicate that a single use of topical 5% permethrin at label dose in yearling Angus bulls results in minimal detrimental effects on semen morphology, but not to a degree that impacts the ability of those bulls to pass a standard BSE. Permethrin exposure did not elicit acute reproductive toxicity to the bulls; however, testicular histopathology warrants further investigation into long-term effects with different exposures to pyrethroids. Results of this study suggest the use of 5% permethrin pour-on on yearling bulls at label dose should be safe for commercial beef production settings combating or controlling insect burdens.
Figure 1. Experimental design of yearling Angus bulls treated with a pyrethroid pour-on.
Table 1. Performance and growth parameters of yearling Angus bulls treated with a pyrethroid pour-on

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Treatment</th>
<th>SEM</th>
<th>P-Value&lt;sup&gt;4&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CON</td>
<td>PYR</td>
<td></td>
</tr>
<tr>
<td>Body weight, kg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>512</td>
<td>508</td>
<td>6.1</td>
</tr>
<tr>
<td>Final</td>
<td>557</td>
<td>552</td>
<td>6.4</td>
</tr>
<tr>
<td>Change</td>
<td>43</td>
<td>42</td>
<td>2.2</td>
</tr>
<tr>
<td>Average daily gain,&lt;sup&gt;2&lt;/sup&gt; kg</td>
<td>2.24</td>
<td>2.21</td>
<td>0.117</td>
</tr>
<tr>
<td>Body condition score&lt;sup&gt;3&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>6.10</td>
<td>6.30</td>
<td>0.087</td>
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<tr>
<td>Final</td>
<td>6.02</td>
<td>6.20</td>
<td>0.115</td>
</tr>
<tr>
<td>Change</td>
<td>-0.07</td>
<td>-0.10</td>
<td>0.764</td>
</tr>
</tbody>
</table>

<sup>1</sup> Labeled dose and route of pour-on pyrethroid (PYR; Ultra Boss<sup>®</sup>); saline control (CON).

<sup>2</sup> Calculated by final weight minus initial weight and divided by 19 days of trial period.

<sup>3</sup> Based on industry standard (1-9) body condition scoring technique.

<sup>4</sup> Statistical significance was acknowledged at P ≤ 0.05, and 0.05 < P ≤ 0.10 was considered a tendency approaching significance.

Table 2. Reproductive parameters of yearling Angus bulls treated with a pyrethroid pour-on

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Treatment</th>
<th>SEM</th>
<th>P-Value&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CON</td>
<td>PYR</td>
<td></td>
</tr>
<tr>
<td>Scrotal circumference, cm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>35.5</td>
<td>35.5</td>
<td>0.42</td>
</tr>
<tr>
<td>Final</td>
<td>36.6</td>
<td>36.8</td>
<td>0.44</td>
</tr>
<tr>
<td>Change</td>
<td>1.0</td>
<td>1.3</td>
<td>0.14</td>
</tr>
<tr>
<td>Testosterone, ng/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>6.23</td>
<td>8.44</td>
<td>0.824</td>
</tr>
<tr>
<td>Final</td>
<td>6.47</td>
<td>7.21</td>
<td>0.699</td>
</tr>
<tr>
<td>Change</td>
<td>0.08</td>
<td>-1.22</td>
<td>0.816</td>
</tr>
<tr>
<td>Sperm motility, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>72.2</td>
<td>76.6</td>
<td>2.95</td>
</tr>
<tr>
<td>Final</td>
<td>56.6</td>
<td>66.8</td>
<td>4.15</td>
</tr>
<tr>
<td>Change</td>
<td>-12.9</td>
<td>-10.1</td>
<td>4.78</td>
</tr>
</tbody>
</table>

<sup>1</sup> Labeled dose and route of pour-on pyrethroid (PYR; Ultra Boss<sup>®</sup>); saline control (CON).

<sup>2</sup> Statistical significance was acknowledged at P ≤ 0.05, and 0.05 < P ≤ 0.10 was considered a tendency approaching significance.
Table 3. Semen morphology of yearling Angus bulls treated with a pyrethroid pour-on

<table>
<thead>
<tr>
<th>Semen morphology, %</th>
<th>Treatment¹</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CON</td>
<td>PYR</td>
<td>SEM</td>
<td>P-Value²</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>70.5</td>
<td>64.7</td>
<td>2.72</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>Final</td>
<td>79.0</td>
<td>73.7</td>
<td>2.43</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>Change</td>
<td>8.9</td>
<td>9.5</td>
<td>1.78</td>
<td>0.80</td>
<td></td>
</tr>
<tr>
<td>Abnormal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>29.5</td>
<td>35.3</td>
<td>2.72</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>Final</td>
<td>21.0</td>
<td>26.3</td>
<td>2.43</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>Change</td>
<td>-8.9</td>
<td>-9.5</td>
<td>1.78</td>
<td>0.80</td>
<td></td>
</tr>
<tr>
<td>Primary defects</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>15.8</td>
<td>15.4</td>
<td>2.63</td>
<td>0.91</td>
<td></td>
</tr>
<tr>
<td>Final</td>
<td>10.7</td>
<td>15.4</td>
<td>2.21</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>Change</td>
<td>-5.1</td>
<td>-0.08</td>
<td>1.71</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>Head defects</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>2.8 (10.1)</td>
<td>1.4 (4.4)</td>
<td>0.39 (0.13)</td>
<td>0.02 (0.003)</td>
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</tr>
<tr>
<td>Final</td>
<td>1.0 (5.9)</td>
<td>1.6 (6.6)</td>
<td>0.21 (0.01)</td>
<td>0.04 (0.42)</td>
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<tr>
<td>Change</td>
<td>-1.9</td>
<td>0.2 (2.4)</td>
<td>0.37</td>
<td>&lt; 0.001</td>
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<tr>
<td>Midpiece defects</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>4.7 (13.2)</td>
<td>3.8 (10.4)</td>
<td>1.21 (0.03)</td>
<td>0.63 (0.38)</td>
<td></td>
</tr>
<tr>
<td>Final</td>
<td>1.8 (10.8)</td>
<td>3.4 (13.0)</td>
<td>0.46 (0.02)</td>
<td>0.02 (0.50)</td>
<td></td>
</tr>
<tr>
<td>Change</td>
<td>-3.0</td>
<td>-0.4 (2.8)</td>
<td>1.14</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>Proximal droplet</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>3.7 (11.8)</td>
<td>6.1 (12.2)</td>
<td>1.62 (0.03)</td>
<td>0.31 (0.93)</td>
<td></td>
</tr>
<tr>
<td>Final</td>
<td>4.0 (14.9)</td>
<td>6.4 (17.6)</td>
<td>1.67 (0.03)</td>
<td>0.32 (0.49)</td>
<td></td>
</tr>
<tr>
<td>Change</td>
<td>0.3</td>
<td>0.3 (5.5)</td>
<td>0.98</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>Coiled tails</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>4.6 (13.5)</td>
<td>4.6 (12.8)</td>
<td>0.86 (0.02)</td>
<td>0.97 (0.69)</td>
<td></td>
</tr>
<tr>
<td>Final</td>
<td>4.0 (14.2)</td>
<td>4.0 (16.1)</td>
<td>0.78 (0.02)</td>
<td>0.98 (0.85)</td>
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</tr>
<tr>
<td>Change</td>
<td>-0.4</td>
<td>-1.0 (2.6)</td>
<td>0.64</td>
<td>0.47</td>
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</tr>
<tr>
<td>Secondary defects</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>14.3</td>
<td>18.6</td>
<td>1.5</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Final</td>
<td>10.7</td>
<td>10.9</td>
<td>1.09</td>
<td>0.90</td>
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<td>Change</td>
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<td>-7.7</td>
<td>1.75</td>
<td>0.13</td>
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<td>Distal droplets</td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Initial</td>
<td>1.5 (5.6)</td>
<td>0.8 (2.8)</td>
<td>0.37 (0.01)</td>
<td>0.24 (0.17)</td>
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</tr>
</tbody>
</table>
Table 3 continued

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<tbody>
<tr>
<td>Bent tails</td>
<td></td>
<td>5.9 (20.9)</td>
<td>5.8 (18.0)</td>
<td>0.70 (0.02)</td>
<td>0.89 (0.38)</td>
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<td></td>
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<td></td>
<td></td>
<td>-0.02</td>
<td>-0.7 (4.0)</td>
<td>0.83</td>
<td>0.57</td>
<td></td>
<td></td>
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<tr>
<td>Tailless sperm</td>
<td></td>
<td>6.9 (25.0)</td>
<td>12.6 (39.4)</td>
<td>1.20 (0.04)</td>
<td>0.002 (0.007)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.1 (15.9)</td>
<td>4.5 (18.6)</td>
<td>0.61 (0.03)</td>
<td>0.12 (0.64)</td>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>-4.4</td>
<td>-8.2 (-20.8)</td>
<td>1.32</td>
<td>0.05</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 Labeled dose and route of pour-on pyrethroid (PYR; Ultra Boss®); saline control (CON).
2 Statistical significance was acknowledged at P ≤ 0.05, and 0.05 < P ≤ 0.10 was considered a tendency approaching significance.
Table 4. Breeding Soundness Exam (BSE) classification results of yearling Angus bulls treated with a pyrethroid pour-on

<table>
<thead>
<tr>
<th>BSE classification</th>
<th>Treatment¹</th>
<th>CON</th>
<th>PYR</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Satisfactory classification, % (no./no.)²</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td></td>
<td>63.3 (19/30)</td>
<td>43.3(13/30)</td>
<td>0.17</td>
</tr>
<tr>
<td>Final</td>
<td></td>
<td>65.5 (19/29)</td>
<td>70.0(21/30)</td>
<td>0.72</td>
</tr>
<tr>
<td>Downgraded³</td>
<td></td>
<td>16.7 (3/18)</td>
<td>7.7 (1/13)</td>
<td>0.82</td>
</tr>
</tbody>
</table>

¹ Labeled dose and route of pour-on pyrethroid (PYR; Ultra Boss®); saline control (CON).
² Percent of bulls with satisfactory BSE standards (≥ 30% motility and 70% normal sperm morphology).
³ Percent of satisfactory at initial BSE that were classified as non-satisfactory on final BSE.

Table 5. Testicular histopathology of yearling Angus bulls treated with a pyrethroid pour-on (34 days post treatment)

<table>
<thead>
<tr>
<th>Histopathology parameter</th>
<th>Treatment¹</th>
<th>CON</th>
<th>PYR</th>
<th>SEM</th>
<th>P-Value⁹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tubular diameter², µm</td>
<td></td>
<td>153.1</td>
<td>153.2</td>
<td>2.18</td>
<td>0.99</td>
</tr>
<tr>
<td>Degeneration score³⁴</td>
<td></td>
<td>1.4</td>
<td>1.9</td>
<td>0.24</td>
<td>0.19</td>
</tr>
<tr>
<td>None/rare⁵ %</td>
<td></td>
<td>51.9(14/27)</td>
<td>48.3(14/29)</td>
<td>0.72</td>
<td></td>
</tr>
<tr>
<td>Mild or greater⁶ %</td>
<td></td>
<td>48.1(13/27)</td>
<td>51.7(15/29)</td>
<td>0.72</td>
<td></td>
</tr>
<tr>
<td>Moderate or greater⁷ %</td>
<td></td>
<td>14.8 (4/27)</td>
<td>31.0 (9/29)</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>Severe degeneration⁸ %</td>
<td></td>
<td>3.7 (1/27)</td>
<td>17.2 (5/29)</td>
<td>0.17</td>
<td></td>
</tr>
</tbody>
</table>

¹ Labeled dose and route of pour-on pyrethroid (PYR; Ultra Boss®); saline control (CON).
² Average of 10 random seminiferous tubules from each animal.
³ Degeneration scoring: (0) = normal or no degeneration, (1) = scattered and rare degeneration, (2) = mild degeneration, (3) = moderate degeneration, (4) = severe degeneration.
⁴ Three different sections (proximal, middle, and distal) of testicles used to average the degeneration score.
⁵ Includes degeneration scores (1) and (2).
⁶ Includes degeneration scores (2), (3), and (4).
⁷ Includes degeneration scores (3) and (4).
⁸ Includes degeneration score (4).
⁹ Statistical significance was acknowledged at P ≤ 0.05, and 0.05 < P ≤ 0.10 was considered a tendency approaching significance.
CHAPTER 4

EFFECTS OF LABEL-DOSE PERMETHRIN ADMINISTRATION ON REPRODUCTIVE FUNCTION IN SUPEROVULATED BEEF HEIFERS

4.1 Abstract

The objective was to study the effects of a commercial pyrethroid-based pour-on product, permethrin, on reproductive performance in superovulated beef heifers by assessing steroid biosynthesis and embryo quality. Nonpregnant, yearling beef heifers (n=10; 418 ± 33 kg; 5.5 ± 0.2 BCS) were assigned by BW and breed to either 1) saline control (CON) or 2) permethrin pour-on administered at label dose (PYR). Superovulation was achieved on all heifers utilizing a timed, 17-d, CIDR-based protocol with GnRH and PGF2α and decreasing total dosage of 240mg FSH administered twice daily for 4 days. Heifers were AI twice (at onset of estrus and 12 hrs later) by same technician with frozen semen from single bull collection. To determine short and long-term effects of permethrin on embryo quality and steroid biosynthesis, superovulation was initiated twice with collection of embryos occurring at 17 and 51 d post-treatment. Embryos were recovered 6.5 d after first AI via non-surgical flush and were evaluated by International Embryo Transfer Society standards. Blood was collected at standing estrus and d of embryo recovery. Estrogen (E2) and progesterone (P4) concentrations were analyzed via RIA. MIXED and GLIMMIX procedures of SAS were used to analyze continuous and categorical data, respectively. Heifer per flush was the experimental unit. Total embryos recovered did not differ due to treatment (P = 0.30), but did decrease in flush 2 compared to flush 1 (P = 0.02). Quality grade, total transferrable quality embryos (TQE), and overall flush success did not differ due to
treatment (P ≥ 0.16). However, TQE was decreased in flush 2 compared to flush 1 (P = 0.05). Total unfertilized oocytes was greater in CON (P = 0.04). The PYR heifers tended to have less total P4 (P = 0.15) and P4 per corpus luteum (P = 0.06) at recovery. E2 per ovulated follicle and E2 per total ovarian structure was greater in flush 2 (P ≤ 0.03) but did not differ due to treatment (P ≥ 0.23). In summary, these data indicate that permethrin administration at label dose in superovulated beef heifers has a tendency to reduce P4, but embryo quality is not affected.

4.2 Introduction

Pyrethroid exposure has been implicated to disrupt male reproductive and endocrine functions (Elbetieha et al., 2001, Mani et al., 2002, Kumar et al., 2004, Zhang et al., 2007, Arena et al., 2008, Meeker et al., 2008, Ahmad et al., 2009, Issam et al., 2009, Abdallah et al., 2010, Li Yan et al., 2013). In addition, endocrine disruption is speculated to influence the hypothalamic-pituitary-gonadal axis and impair the necessary feedback mechanisms of hormones that provide the required stability to regulate normal reproductive physiology. Previous observational findings have claimed that bulls exposed to pyrethroids have increase in abnormal sperm morphology (Volkmann, 2012). However, recent literature has refuted the claim by exposing bulls to multiple pyrethroid products and by different routes of administration (Cain et al., 2014, French et al., 2014, Stewart et al., 2015), including recent data from our lab on peripubertal bulls exposed to label-dose pour-on permethrin (Dohlman et al., 2015).
Endocrine disruption is not believed to be sex specific, and thus likely affect female reproductive physiology by inhibiting normal reproductive cyclicity and the ability to maintain pregnancy (Ratnasooriya et al., 2003). Previous research has indicated pyrethroids may inhibit progesterone concentrations by down-regulating expression of cP450scC and StAR (Fei et al., 2010, Liu et al., 2011). In addition, Pine et al. (2008) showed esfenvalerate, a type II pyrethroid, delayed vaginal opening and decreased morning serum estrogen concentration after oral administration (1.0 and 5.0 mg/kg/day starting on postnatal day 22) to pre-pubertal female rats. However, there are postulated thoughts that endocrine disruption chemicals (EDC) could also stimulate changes in the reproductive tract that impede sperm migration, sperm adhesion, capacitation, zona binding, acrosomal reaction, or penetration into the oocyte or the competency for maturation of a developing embryo. Pre-implantation losses with reduction of implantation sites has been reported with rats receiving lambda cyalothrin orally in early gestation, which could imply that exposure to pyrethroids could develop a hostile environment or cause abnormal synchronization of implantation. However, French et al. (2014) did not find similar results when exposing mature cows with different applications of pyrethroids.

The overall reproductive effects of pyrethroid exposure to female cattle have not been studied in as much detail as the bull. The objective of this study was to elucidate the effects of a commercial pyrethroid-based pour-on product, permethrin, on reproductive performance in superovulated beef heifers by assessing steroid synthesis and embryo quality. It was hypothesized that exposure to pyrethroid pour-on at label dose would cause minimal effects on reproductive parameters in the female bovine.
4.3 Materials and Methods

4.3.1 General

All protocols and procedures used were approved by the Iowa State University Institutional Animal Care and Use Committee. The project was conducted at the Iowa State University Zumwalt Station in Ames, IA in May-July 2014. The project utilized single-sourced heifers from the Iowa State University Beef Teaching herd. Products used in this study included: a synthetic type I pyrethroid pour-on (permethrin; Ultra Boss®, Intervet/Merck Animal Health, Summit, NJ) and sterile saline (0.9% sodium chloride, Abbott Laboratories, North Chicago, IL). We evaluated permethrin pour-on (Ultra Boss®) at label dose due to its popular use based on sales from local distribution companies in the Midwest and having the highest concentration of pyrethroid substance in commercially available products.

4.3.2 Animals and treatments

Non-pregnant, purebred Simmental and crossbred yearling beef heifers (n=10; 418 ± 33 kg; 5.5 ± 0.2 BCS) were used in this study. Prior to treatment, all heifers were subjected to a trans-rectal reproductive ultrasound to confirm normal ovarian activity and cyclicity. At that time, initial body weight (BW) and body conditioning scores (BCS) were recorded and heifers were blocked by breed and BW. Heifers were assigned to either 1) a saline control (CON; n=5) or 2) a permethrin pour-on treatment group (PYR; n=5). The PYR heifers received a one-time label dose of permethrin (5% permethrin and 5% piperonyl butoxide, 3 mL per 45kg BW up to a maximum of 30 mL) for lice and fly control. The CON group received the same volume of saline.
Both products were administered on the topline of the heifers. Treatment groups were housed one pen per treatment to avoid cross-contamination. All heifers received same environmental and nutritional treatment before and after treatment.

Treatments were initiated at the start of superovulation protocol. All heifers were subjected to superstimulation by utilizing a timed, 17-d, CIDR (EAZI-BREED™, Zoetis Inc., Kalamazoo, MI)-based protocol with GnRH (Cystorelin®, Merial LLC, Duluth, GA) and PGF2α (Estrumate®, Schering-Plough Animal Health Corp., Summit, NJ) with decreasing total dosage of 240mg FSH (Folltropin-V®, Bioniche Animal Health Canada Inc., Belleville, ON) administered twice daily for 4 days (Experimental Design, Figure 4.1). Heifers were artificially inseminated (AI) twice either at onset of estrus or by timed-AI and additionally 12 hrs later by same technician with one unit of frozen semen, at each insemination, from a single bull collection. A dose of GnRH (100 µg) was given at time of second breeding. At 6.5 d post-timed-AI, transrectal ultrasound was performed to assess corpus luteum (CL) number, number of unovulated follicles, and total ovarian structures (CL and unovulated follicles). Immediately following ultrasound, non-surgical embryo recovery was performed by horn flushing, with utilization of a closed gravity-flow flush system with 2 liters of commercial grade flush media. All recovered embryos were evaluated according to International Embryo Transfer Society standards by blinded American Embryo Transfer Association certified personnel.

To determine potential long-term effects of permethrin on embryo quality and steroid biosynthesis, an identical superstimulation protocol, as previously described, was initiated again 34 days post-treatment with embryo recovery performed 51 days post-treatment. On the second flush, 1 heifer had abnormal oviduct pathology and embryo data was not used.
Blood was collected via coccygeal tail vein at insertion of CIDR™, standing estrus, and day of embryo recovery to evaluate baseline (basal) estradiol-17β, peak estradiol-17β, and progesterone (P4) concentrations, respectively. Blood was put on ice and was centrifuged (1750 x g for 25 minutes) and plasma was removed and frozen (-20°C) for later analysis. Plasma samples collected on the day of CIDR insertion and at estrus were analyzed for circulating concentrations of estradiol-17β via RIA using methodology described by Perry and Perry (2008). Across 2 assays, the average intra-assay CV was 12.1% and the inter-assay CV for a pooled serum sample containing 7.65 pg/mL was 6.7%. The average sensitivity across assays was 0.76 pg/mL (95% confidence interval). Progesterone concentration at embryo recovery was also analyzed via a commercially available RIA kit (Coat-A-Count, Siemens Healthcare Diagnostics Inc., Los Angeles, CA). Intra-assay CV was 1.3% with a sensitivity of 0.30 ng/ml (95% confidence interval).

4.3.3 Statistical analysis

Data was analyzed using SAS 9.3 (SAS Institute Inc., Cary, NC) for a 2 x 2 factorial arrangement. Binary data were analyzed using the GLIMMIX procedure of SAS, while remaining, continuous reproductive and embryo parameters were analyzed using the MIXED procedure of SAS. The final model included the main effects of treatment and period (flush) and the appropriate interaction. Heifer per flush was the experimental unit and statistical significance was acknowledged at $P \leq 0.05$, and $0.05 < P \leq 0.10$ was considered a tendency approaching significance.
4.4 Results

4.4.1 Embryo quality

Total embryos recovered did not differ due to treatment ($P = 0.30$), but did decrease in flush 2 compared to flush 1 ($P = 0.02$). In addition, there was a treatment x flush interaction for total embryos recovered ($P = 0.02$; Table 6), as CON heifers had more embryos recovered in first flush and reduced embryos in the second flush compared to PYR heifers (Table 4.1). Embryo quality grades and flush success did not differ due to treatment ($P \geq 0.16$). However, CON heifers had an increase in unfertilized oocytes compared to PYR heifers ($P \leq 0.05$). Irrespective of treatment, quality grade 1 embryos and transferable quality embryos (TQE) decreased in flush two compared to flush one ($P \leq 0.05$). In addition, total unfertilized oocytes was greater in CON heifers than PYR heifers ($P = 0.04$). Due to greater unfertilized oocytes in CON heifers there was a subsequent treatment x flush interaction for quality Grade 4 embryos ($P \leq 0.02$).

4.4.2 Hormone analysis

Progesterone (P4) concentrations on the days of embryo collection were not different between treatments; however, PYR heifers had a weak decreasing trend in total P4 concentration ($P = 0.15$) and a tendency for reduced P4 per corpus luteum ($P = 0.06$; Table 4.2). Estradiol concentration per ovulated follicle (CL) and per total ovarian structure (CL and unovulated follicles), as determined by ultrasound at embryo recovery, was greater in flush two than flush one ($P \leq 0.02, P \leq 0.03$; respectively) but did not differ due to treatment ($P \geq 0.23$).
4.5 Discussion

Pyrethroid insecticide use and its reproductive effects on breeding bulls is debatable with inconsistent results from observational findings (Volkmann, 2012) and case-controlled studies (French et al., 2014, Cain et al., 2014). However, reproductive performance on female reproductive physiology in beef cattle has been limited and the reproductive effects are clearly unknown. The objective of the current study was to identify the reproductive effects of label-dose administration of pyrethroid (permethrin) on embryo quality and steroidogenesis in superovulated beef heifers.

4.5.1 Embryo quality

In vitro studies have shown inhibition of rat preantral follicular growth when incubated at different concentrations of fenvalerate, a synthetic type II pyrethroid; however, incubation with fenvalerate did not affect follicular survival rates (Fei et al., 2010). Similar results were shown with in vitro pig oocytes cultured in cypermethrin, deltamethrin, and fenvalerate concentrations (Petr et al., 2013). However, incubation with the different milieu of pyrethroid compounds did not induce any significant degeneration or compromise the viability to the porcine oocytes. Together, these results suggest that oocytes and embryos are not completely compromised and are fairly resistant to pyrethroid exposure even with direct contact with the compounds in vitro which support results from the current study. Interestingly, CON heifers did have higher UFO rates in both flushes compared to the PYR heifers. However, UFO and highly degenerate embryos often have a similar appearance, thus subjectivity in grading these 2
classes of embryos are the likely cause of the difference in UFO between treatments. Moreover, in a clinical setting, Grade 4 embryos are not typically evaluated in great depth and further categorized into either degenerate and UFO. Grade 4 embryos not differing as a result of treatment further reemphasized that subjectivity may likely have resulted in aforementioned UFO differences. While we acknowledge number of animals used was a limitation in this experiment, differences noted in embryo and hormone data, when comparing the two flushes, implies that sufficient power was obtained so that changes in reproductive function due to treatment would have been noted if indeed there was a treatment effect.

4.5.2 Steroid biosynthesis

Progesterone plays a key role in maternal recognition and maintenance of pregnancy in many species (Bazer et al., 2009). In previous in vitro studies, pyrethroid exposure has been reported to be endocrine disruptive by inhibiting progesterone concentrations. Gill et al. (2011) reported in vitro bovine corpus luteal cells decreased viability and subsequently had significantly lower progesterone concentrations when incubated with differing dosages and durations with cypermethrin. In addition, preantral follicles cultured in fenvalerate inhibited progesterone concentration with decrease gene expression for steroidogenic acute regulatory protein and cytochrome P450 side-chain cleavage enzyme, which are both important in cholesterol mediated conversion for progesterone synthesis (Fei et al., 2010). Down regulation of StAR and cP450scc and subsequent decrease in progesterone synthesis was also described by Liu et al. (2011) with in vitro rat granulosa cells incubated with bifenthrin. Remarkably, enantiomer 1S-cis-bifenthrin was the only bifenthrin derivative that caused such affect (Liu et
Currently, the results from in vitro studies show similar progesterone patterns with the same hypothesized mechanism of down-regulation of important cholesterol conversion factors.

Data from the current study showed PYR heifers trended to have lower P4 and P4 per corpus luteum and supports the findings in previous studies. Conversely, French et al. (2014) did not find any consistent difference in P4 concentration with Angus and crossbred cows treated with dose labeled cyfluthrin pour-on and fly tags. However, it was reported that there was a significant decrease in P4 concentrations 10 days post-Al in pyrethroid treated cows but not at 17 days post-Al; thus, progesterone differences did not correlate to differences in pregnancy rates (French et al., 2014). However, oral administration of lambda cyalothrin, a synthetic type II pyrethroid, to rats in early gestation showed detrimental pregnancy outcomes, mainly due to increased pre-implantation losses of developing embryos (Ratnasooriya et al., 2003). Ratnasooriya et al. (2003) also observed potential anti-progestogenic effects of cyalothrin by increasing uterine implantation sites with progesterone treatment. Similarly, Lemos et al. (2011) reported significant histological abnormalities with reduction of implantation sites in female albino rats receiving oral dosages of deltamethrin. According to these results, pyrethroids may have anti-progestogenic effects and may impact pregnancy outcomes; however, in the current study, implantation and developmental competency of the permethrin treated heifer-derived embryos was not established.

Esfenvalerate, a synthetic type II pyrethroid, has shown to decrease morning serum estrogen concentration and subsequent delay of puberty in pre-pubertal female rats exposed to oral administration at 1.0 and 5.0 mg/kg dosages starting on postnatal day 22 (Pine et al.,
Comparatively, Arenas et al. (2008) showed no estrogenic effect with three consecutive oral treatments of fenvalerate in immature rats. The effect of pyrethroids on estradiol concentrations has not fully been evaluated and explained. The current study showed greater estradiol concentration per ovulated follicle (CL) and per total ovarian structure in flush two than flush one; however, there was no difference in basal or peak estradiol concentrations across treatments. The lack of treatment difference may have been masked by utilizing pubertal heifers and superstimulation, both of which may be more resilient to pyrethroid exposure. Nevertheless, question still remains on prolonged or at significantly higher dosages of pyrethroid exposure disrupt estradiol secretion and affecting normal reproductive physiology. Investigation may be warranted in prepubertal heifers due to previous research alluding to immature females potentially being more susceptible to pyrethroid exposure which could harm or delay future oocyte maturation in early gonadal development.

4.6 Conclusion

In conclusion, these data indicate that a single use of topical 5% permethrin at label dose in superovulated heifers results in a weak tendency for reduced progesterone concentrations, but not to a degree that impacts embryo production or quality. Permethrin exposure did not elicit any reproductive toxicity on folliculogenesis or maturation. According to our results, the use of 5% permethrin pour-on on pubertal heifers at label dose should be safe for commercial beef production settings combating or controlling insect burdens.
<table>
<thead>
<tr>
<th>Day</th>
<th>Time</th>
<th>Administrations</th>
<th>Ultrasound/Blood Collection</th>
</tr>
</thead>
<tbody>
<tr>
<td>-33</td>
<td>am</td>
<td>.........</td>
<td>U/S, BW + BCS</td>
</tr>
<tr>
<td>0</td>
<td>am</td>
<td>CIDR™-insertion + 625 mcg PGF2α + Ultraboss® or Saline Treatment</td>
<td>Basal Estradiol</td>
</tr>
<tr>
<td>4</td>
<td>am</td>
<td>150 mcg GnRH</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>pm</td>
<td>40 mg FSH</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>am/pm</td>
<td>40 mg FSH, 30 mg FSH</td>
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<tr>
<td>7</td>
<td>am/pm</td>
<td>30 mg FSH, 30 mg FSH</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>am</td>
<td>30 mg FSH</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>pm</td>
<td>20 mg FSH + 625 mcg PGF2α</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>am</td>
<td>20 mg FSH + 625 mcg PGF2α + CIDR™-removal</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>pm</td>
<td>Al</td>
<td>Peak Estradiol</td>
</tr>
<tr>
<td>11</td>
<td>am</td>
<td>150 mcg GnRH + AI</td>
<td>Peak Estradiol</td>
</tr>
<tr>
<td>17</td>
<td>am</td>
<td>Embryo Recovery + 625 mcg PGF2α (post-flush)</td>
<td>Progesterone + U/S</td>
</tr>
<tr>
<td>34</td>
<td>am</td>
<td>Repeat d0 Protocol (Except pour-on treatments)</td>
<td>Basal Estradiol</td>
</tr>
<tr>
<td>51</td>
<td>am</td>
<td>Embryo Recovery + PGF2α (post-flush)</td>
<td>Progesterone + U/S</td>
</tr>
</tbody>
</table>

**Figure 1.** Experimental design of superovulated heifers treated with a pyrethroid pour-on
Table 6. Embryo flushing characteristics for yearling heifers after treatment with a pyrethroid pour-on

<table>
<thead>
<tr>
<th>Item</th>
<th>Treatment&lt;sup&gt;1&lt;/sup&gt;</th>
<th>CON</th>
<th>PYR</th>
<th>SEM&lt;sup&gt;2&lt;/sup&gt;</th>
<th>TRT</th>
<th>Flush</th>
<th>TxT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Item</td>
<td>F1</td>
<td>F2</td>
<td>F1</td>
<td>F2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Embryos</td>
<td>17.0</td>
<td>7.75</td>
<td>9.8</td>
<td>9.8</td>
<td>1.88</td>
<td>0.30</td>
<td>0.02</td>
</tr>
<tr>
<td>Embryo Quality Grade&lt;sup&gt;3&lt;/sup&gt;(%)&lt;sup&gt;4&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>4.0 (29.5)</td>
<td>2.25 (33.8)</td>
<td>5.4 (59.3)</td>
<td>3.0 (38.0)</td>
<td>0.83(0.12)</td>
<td>0.32(0.24)</td>
<td>0.04(0.15)</td>
</tr>
<tr>
<td>2</td>
<td>1.4 (12.5)</td>
<td>0.5 (5.8)</td>
<td>2.2 (17.3)</td>
<td>1.2 (15.4)</td>
<td>0.59(0.05)</td>
<td>0.38(0.33)</td>
<td>0.27(0.56)</td>
</tr>
<tr>
<td>TQE&lt;sup&gt;5&lt;/sup&gt;</td>
<td>5.4 (42.0)</td>
<td>2.75 (39.7)</td>
<td>7.6 (76.5)</td>
<td>4.2 (53.5)</td>
<td>1.32(0.14)</td>
<td>0.30(0.19)</td>
<td>0.05(0.08)</td>
</tr>
<tr>
<td>3</td>
<td>1.6 (7.3)</td>
<td>0.75 (11.3)</td>
<td>0.4 (4.0)</td>
<td>0.8 (6.3)</td>
<td>0.37(0.02)</td>
<td>0.29(0.22)</td>
<td>0.67(0.34)</td>
</tr>
<tr>
<td>4</td>
<td>10.0 (50.8)</td>
<td>4.25 (49.0)</td>
<td>1.6 (17.5)</td>
<td>4.8 (40.2)</td>
<td>2.12(0.12)</td>
<td>0.16(0.20)</td>
<td>0.67(0.14)</td>
</tr>
<tr>
<td>Degenerate&lt;sup&gt;6&lt;/sup&gt;</td>
<td>3.2 (16.7)</td>
<td>1.0 (11.7)</td>
<td>1.2 (13.5)</td>
<td>4.2 (34.2)</td>
<td>1.09(0.70)</td>
<td>0.72(0.35)</td>
<td>0.76(0.42)</td>
</tr>
<tr>
<td>UFO&lt;sup&gt;7&lt;/sup&gt;</td>
<td>6.8 (34.1)</td>
<td>3.0 (33.8)</td>
<td>0.4 (4.0)</td>
<td>0.6 (6.0)</td>
<td>1.40(0.08)</td>
<td>0.04(0.02)</td>
<td>0.14(0.71)</td>
</tr>
<tr>
<td>Flush Success&lt;sup&gt;8&lt;/sup&gt;</td>
<td>0.80</td>
<td>0.25</td>
<td>0.80</td>
<td>0.60</td>
<td>1.08</td>
<td>0.50</td>
<td>0.13</td>
</tr>
</tbody>
</table>

<sup>1</sup> CON = Saline Control; PYR= UltraBoss<sup>®</sup> at labeled recommendation, F1 = First embryo flushing (17 days after initial treatment), F2 = Second embryo flushing (51 days after initial treatment).

<sup>2</sup> P-values of main effects of treatment and flush and the treatment x flush interaction (P< 0.05; considered statistically significant).

<sup>3</sup> Average numbers of embryos graded by International Embryo Transfer Society (IETS) guidelines: 1 = Excellent or good, 2 = Fair, 3 = poor, 4 = dead or degenerate.

<sup>4</sup> Percentage of total embryos by quality grades.

<sup>5</sup> Transferable quality embryos: embryos that are of satisfactory quality to freeze and transfer. TQE = Grade 1 and Grade 2.

<sup>6</sup> Non-viable or dead embryos.

<sup>7</sup> Unfertilized oocytes.

<sup>8</sup> Percentage of animals that had ≥ 5 TQE (average of industry standards).
Table 7. Hormonal, ultrasound, and embryo flushing characteristics for yearling heifers after treatment with a pyrethroid pour-on

<table>
<thead>
<tr>
<th>Item</th>
<th>Treatment 1</th>
<th></th>
<th>Treatment 1</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CON</td>
<td>PYR</td>
<td>CON</td>
<td>PYR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>F1</td>
<td>F2</td>
<td>F1</td>
<td>F2</td>
<td>SEM</td>
<td>P-Value</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Structures</td>
<td>19.4</td>
<td>15.2</td>
<td>15.2</td>
<td>15.2</td>
<td>3.95</td>
<td>0.59</td>
<td>0.17</td>
<td>0.17</td>
</tr>
<tr>
<td>CL Total</td>
<td>18.0</td>
<td>12.8</td>
<td>13.8</td>
<td>12.8</td>
<td>2.47</td>
<td>0.56</td>
<td>0.12</td>
<td>0.27</td>
</tr>
<tr>
<td>Unovulated Total</td>
<td>1.4</td>
<td>2.4</td>
<td>1.4</td>
<td>2.4</td>
<td>0.58</td>
<td>1.00</td>
<td>0.21</td>
<td>1.00</td>
</tr>
<tr>
<td>Progesterone (ng/ml)</td>
<td>94.03</td>
<td>82.94</td>
<td>42.19</td>
<td>32.00</td>
<td>22.63</td>
<td>0.15</td>
<td>0.31</td>
<td>0.96</td>
</tr>
<tr>
<td>P4/CL Ratio</td>
<td>4.94</td>
<td>4.98</td>
<td>2.86</td>
<td>2.48</td>
<td>0.74</td>
<td>0.06</td>
<td>0.76</td>
<td>0.70</td>
</tr>
<tr>
<td>Resting Estradiol (pg/ml)</td>
<td>2.71</td>
<td>2.02</td>
<td>1.71</td>
<td>2.23</td>
<td>0.21</td>
<td>0.21</td>
<td>0.79</td>
<td>0.07</td>
</tr>
<tr>
<td>Peak Estradiol (pg/ml)</td>
<td>42.83</td>
<td>50.58</td>
<td>25.52</td>
<td>47.57</td>
<td>6.93</td>
<td>0.33</td>
<td>0.15</td>
<td>0.47</td>
</tr>
<tr>
<td>Estradiol/CL Ratio</td>
<td>2.41</td>
<td>5.07</td>
<td>1.88</td>
<td>3.66</td>
<td>0.57</td>
<td>0.26</td>
<td>0.02</td>
<td>0.60</td>
</tr>
<tr>
<td>Estradiol/Total Structure Ratio</td>
<td>2.22</td>
<td>3.94</td>
<td>1.70</td>
<td>2.96</td>
<td>0.41</td>
<td>0.23</td>
<td>0.03</td>
<td>0.71</td>
</tr>
<tr>
<td>Recovery Rate</td>
<td>96.0</td>
<td>89.0</td>
<td>74.0</td>
<td>77.0</td>
<td>0.12</td>
<td>0.35</td>
<td>0.87</td>
<td>0.69</td>
</tr>
</tbody>
</table>

1 CON = Saline Control; PYR = UltraBoss® at labeled recommendation, F1 = First embryo flushing (17 days after initial treatment), F2 = Second embryo flushing (51 days after initial treatment).
2 P-values of main effects of treatment and flush and the treatment x flush interaction (P ≤ 0.05; considered statistically significant).
3 Counted corpus lutea (CL) and unovulated structures from both ovaries via rectal ultrasound at embryo recovery.
4 Counted corpus lutea (CL) from both ovaries via rectal ultrasound at embryo recovery.
5 Counted unovulated structures from both ovaries via rectal ultrasound at embryo recovery.
6 Taken at embryo recovery by venipuncture of caudal tail vein.
7 Taken prior to initiation of CIDR protocol by venipuncture of caudal tail vein.
8 Taken during standing estrus or at timed artificial insemination (TAI) by venipuncture of caudal tail vein.
9 Number of total embryos divided by total number of corpus lutea (CL).
CHAPTER 5
GENERAL DISCUSSION

The experiments conducted in this thesis were initiated to give a physiological-based answer to a highly debated, producer-oriented question: Does commercial pyrethroid insecticide use affect reproduction in beef cattle? Previous observational findings would indicate that there is detrimental motility and morphological effects on bovine sperm when animals are exposed to pyrethroid or pyrethrin compounds which could ultimately cause infertility. However, these observational findings did not have comparative controls nor was the dose, duration, or route of the pyrethroid/pyrethrin compounds identified.

However, the observational findings seem plausible due to previous experiments in other mammalian species (rats, mice, and humans) indicating that pyrethroid exposure does negatively affect male reproduction in many ways. The reported affects included alterations of the endocrine system including production of natural hormones needed for normal physiological function of the gonads and their production of normal gametes. It was also reported that the normal tissue architecture of the gonads can have serious and sometimes permanent damage leading to reproductive failures. Nevertheless, it should be noted that most of the laboratory case-controlled studies typically utilized differing routes of administration that is typical in bovine, increased frequency, and overdosing of non-mammalian labeled pyrethroid products to find such affects. However, regardless of previous research, the use of pyrethroid insecticides in the beef cow-calf industry has initiated concern and fear for producers who routinely utilize pyrethroid products to combat production inefficiencies and insect-borne diseases due to ectoparasites.
Due to the highly-debated issue, three recent case-controlled studies have been performed to evaluate commercial pyrethroid products in beef bulls. Again, these studies utilized over-dosing of pyrethroids either by going off-label on dose or by utilizing additional applications of pyrethroid or pyrethrin compounds in the treatment groups. The two studies that evaluated sperm morphology found no changes due to pyrethroid treatment. In addition, all three studies showed no consistent change in motility or concentration of circulating testosterone. The results from the previous bull studies are very similar to our study in Chapter 3. Conversely, we treated according to label instructions to evaluate the affects which should be expected by producers utilizing labeled commercial products. Even though our study showed differences in change with sperm head abnormalities, the results were of negligible importance due to the changes not reflecting a difference in “satisfactory breeder” status with a typical BSE. In addition, a large group of peripubertal bulls were used in our study to potentially identify changes in developing and more susceptible males, which corresponded to previous research focusing on non-pubertal male mice and rats. However, one has to remember that changes in sperm morphology could easily be masked by the ability for bulls to produce 7 – 8 million sperm per day and that collection of semen could be representing sperm that was developed 60 days ago due to the spermatogenic cycle in the bull. In addition, none of the bull studies, including the Chapter 3 study, included any evaluation of sperm ability to fertilize and the potential for DNA disruption causing generational affects, which should be evaluated further by fluorescent morphology stains and in vitro models.

It should also be noted that most data in the literature that link declining testosterone concentrations to changes in motility and morphology has been collected in the differing
application route, over-dosing, increased frequency pyrethroid exposed laboratory models. The current bull studies that utilized dermal pyrethroid exposure did not show similar results with testosterone. Moreover, further investigation should evaluate breakpoint values of testosterone and its compromise to sperm cells and their ability to fertilize.

Insult to the testicle is rarely identified, with the exception to the highly documented dysregulation of testicular temperature. However, the laboratory studies utilizing mice and rats exposed to pyrethroid compounds, which were previously described, showed dramatic changes to the architecture of the testicles including the inability for the germ and stromal cells to produce normal healthy sperm cells. The bulls evaluated in Chapter 3 did not have significant changes to testicular histopathology due to treatment, but interestingly enough; bulls exposed to pyrethroid at label-dose did have a slight tendency to have a higher degeneration score and higher likelihood of moderate to severe testicular degeneration. The data was not statistically significant so inferences would be erroneous. However, one could postulate that pyrethroids could have the ability to affect the testicle without visual detection by normal evaluation methods of the ejaculate. Our current study only evaluated one collection 14 days post-treatment and the testicle harvest was 20 days subsequent to collection. It could be in part that our collection at that time did not correspond to the histopathology changes. To eliminate producer concern and potential affects, further evaluations should be directed toward histopathology changes and the changes seen in ejaculates after chronic exposures pyrethroids.

Pyrethroid exposure in male reproduction has been the main focus in bovine reproduction. However, female cattle are exposed to similar products and compounds throughout the breeding season and through gestation. Previous in vitro and in vivo studies
have elucidated to potential negative effects on the female reproductive system. It should be noted that previous literature has evaluated hormone concentrations and the developing gametes by in vitro models. These studies do not demonstrate the true physiological response of females that ultimately have to metabolize the compound, no matter the route, to potentially show negative effects on reproduction. Embryo quality did not change due to pyrethroid treated superovulated heifers in Chapter 4. These results were similar to pig oocytes that were inoculated in vitro with cypermethrin. Similar to the bull study, I contest that active pyrethroid compounds are able to reach reproductive organs in unmetabolized form to cause any damage to developing gametes. However, this has not been evaluated and clearly needs further investigation.

In addition, superovulated heifers treated with permethrin had a slight tendency to have less circulating progesterone at embryo recoveries. Due to the superovulation, progesterone per corpus luteum was also evaluated and showed similar tendency of reduction of progesterone due to pyrethroid treatment. Similar results with progesterone have been previously evaluated by in vitro studies utilizing bovine luteal cells. However, in vivo studies with rats have shown that high doses of pyrethroid exposure can cause decreased implantation rates causing decrease in pregnancy rates. Similar to our bull study, the superovulated heifers utilized label-dose of commercial product to evaluate the affects which should be expected by producers utilizing labeled commercial products. However, our study did not evaluate embryo competency nor pregnancy rates and the ability to maintain pregnancy. Further studies should evaluate the consequences of pyrethroid exposures and the effects on maternal recognition and pregnancy rates. In addition, previous studies have also shown detrimental effects on
offspring exposed in utero or early in postnatal life while fully developing reproductive competency.

However, due to the complexity of chemical compositions and application types of pyrethroid products and the differing exposure durations, dosages, and routes of administration, further experiments are warranted to elucidate specific reproductive effects including gamete and zygote development, fertilization capabilities, epigenetic and trans-generational effects, and reproductive organ alterations.

The understanding of endocrine disruption is continuously being evaluated and more routine products, such as dewormers, should be evaluated for reproductive effects. Reproductive inefficiency is a huge concern to beef cow-calf producers and the complete understanding of potential reproductive endocrine disruptors would be invaluable to the industry.
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Small Ruminant Advanced Reproductive Technology
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