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THE EFFECTS OF FUNGAL-INFESTED FESCUE AND  
ZERANOL ON TESTICULAR DEVELOPMENT  
IN THE BULL

A Thesis

Presented for the

Master of Science

Degree

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## ABSTRACT

Two experiments were conducted to determine the effect of fescue infestation with the endophytic fungus, Acremonium coenophialum plus zeranol and endophyte-infested fescue alone on testicular development and hormonal secretions in the bull. Bulls were assigned to pastures infested with the fungus at control (0%), Low (50-70%), and High (80-84%) levels. In the first experiment, bulls were implanted at birth with zeranol and every 90 days thereafter.

Assessment of the effects of treatment on testicular development and the ability of the pituitary and testis to respond to GnRH challenges were determined. Gonadotropin releasing hormone (GnRH) challenges were effected when the bulls were 3, 5, and 8 months of age. In the second experiment bulls were removed from the pastures when they were 8 months old and injected with GnRH at 12 months of age to determine the late effects of the fungus. Jugular blood was collected at 0, 1, and 2 hours after GnRH injection and plasma was assayed for follicle-stimulating hormone (FSH), luteinizing hormone (LH), testosterone (T), androstenedione ( $\Delta 4A$ ) and prolactin (PRL). Bulls were castrated at 7 months of age in the first experiment, in the second experiment bulls were hemicastrated at either 3 or 8 months of age. Testis were weighed, measured, and prepared for histological evaluation. Cross-sections of semineferous tubules were evaluated for the number of Sertoli cells

(SC) and number of A-spermatogonia. In the 8 month-old testis, sections were evaluated for percentage of tubular cross sections occupied by the various generations of germ cells.

PRL secretions were significantly reduced ( $P < 0.05$ ) by fungal infestation in the zeranol and nonzeranol implanted bulls.

Serum LH levels were significantly reduced ( $P < .01$ ) by fungal infestation when the bulls were implanted with zeranol. FSH levels were also reduced by fungal infestation in the zeranol-treated bulls ( $P < 0.06$ ). Gonadotropins in the nonzeranol treated bulls may have been reduced by the fungus ( $P > 0.10$ ). Testosterone levels were not affected by fungal infestation except at 3 months of age, where they were significantly reduced ( $P < 0.01$ ) in the nonzeranol treated bulls. Androstenedione levels were not affected by the fungus at any age studied.

Testicular weights were significantly lower in bulls treated with zeranol ( $P < 0.07$ ) and a trend toward lowered testes weights was seen in the nonzeranol treated bulls ( $P > 0.10$ ). Sertoli cell number was significantly reduced in both the zeranol treated bulls ( $P < 0.01$ ) and nonzeranol treated bulls ( $P < 0.05$ ). Number of A-spermatogonia was not affected by the endophyte but percentage of cross sections with mature spermatids were significantly ( $P < 0.03$ ) higher in the control group.

It is concluded that feeding infested fescue significantly reduced PRL levels in the bull. Fungal infestation significantly affected gonadotropins

secretion in the zeranol treated bulls and they also reduced in the nonzeranol treated bulls but the difference was not significant. Testosterone secretion was reduced in the treated bull in the early prepubertal period in the nonzeranol treated bulls. Testicular weights and Sertoli cell number were decreased by fungal ingestion both with and without zeranol. Also, testicular development was delayed by fungus as evidenced by the histological evaluation. Decrements in number of Sertoli cells could lead to a permanent impairment of testicular function.

## CHAPTER I

## INTRODUCTION

Tall fescue (*Festuca arundinacea*) is one of the most widely grown and important forage species in the south and central regions of the United States. It occupies 35 million acres (Ball, 1984). The reasons for its popularity are its persistence in poor climatic conditions, adaptability, good forage yield and long growing season. Despite its desirable qualities, it causes problems in the beef industry. A fungal endophyte, *Acremonium coenophialum*, has been found in the fescue (Morgan-Jones and Grams, 1982), and has been associated with fescue toxicity (Schmidt et al., 1982). Symptoms associated with lowered performance include reduced weight gain, elevated rectal temperature, excessive salivation, rough hair coat, increased respiration rate and lower serum prolactin (Bond et al., 1984; Jackson et al., 1984; Porter et al., 1985; Read and Camp, 1986). The problems with animal performance are referred to as "fescue toxicity". It has been found that the toxic factors produced by the fungus are alkaloids (Bush et al., 1982; Beleskey et al., 1987).

Animals which ingest the infested fescue may exhibit lower fertility, abortion, and decreased conception rate (Bond et al., 1982; Daniels et al., 1983). In addition, feeding infested fescue reduces testicular parenchyma and daily sperm production in male rats (Zavos et al., 1986). These results indicate that the ingestion of fungal infected fescue reduces the reproductive

performance of both males and females. Zeranor is a nonsteroidal compound that has been used as an anabolic agent in beef cattle (Perry et al., 1970). Zeranor implant suppressed testicular growth and testosterone production when implanted in young bulls (Silcox et al., 1986). Furthermore, zeranor has been shown to abolish pulsatile release of LH and testosterone in response to exogenous GnRH (Renavi et al., 1988). These effects of zeranor on testicular function have been attributed to the estrogenic activity of zeranor (Katzenellebogen et al., 1979). The objectives of these studies were to determine the effects of fungal-infested fescue alone, and the effects of fungal-infested fescue plus zeranor on hormonal secretion and testicular development in the beef bull.

## CHAPTER II

## LITERATURE REVIEW

The Effects of Fungal-Infested  
Fescue on Animal Reproduction.

The performance of animals consuming fescue as grass or hay has been inconsistent (Jacobsen et al., 1970). Signs associated with lowered performance are rough hair coat, excessive salivation, rapid breathing, nervousness, elevated body temperature and desire to remain in the shade (Schmidt et al., 1982; Ball, 1984). Beleskey et al. (1987) identified a number of alkaloids in tall fescue and some were implicated in the occurrence of tall fescue toxicosis. They also found a strong correlation between loline derivative alkaloid concentration and fungal endophyte frequency. Lyons et al. (1986) demonstrated that ergopeptide alkaloids are present in all above ground parts of fungal infected fescue. The fungus occurs between the cell walls of fescue, especially stem, pith, leaf sheaths and seed (Bacon, 1983). Bush et al. (1982) reported that the presence of N-acetyl and N-formyl loline alkaloids in tall fescue were positively associated with the presence of the endophytic fungus and these alkaloids have been associated with summer toxicosis. High levels of Pyrrolizidic alkaloids were detected in mares grazing on infected tall fescue (Villahoz et al., 1984). Intra-peritoneal injection of ethanolic extracts of toxic fescue hay into calves caused symptoms similar to fescue toxicosis (Williams et

al., 1975). In addition, Daniels et al. (1981) reported that ether extracts of fescue when given orally to rats and rabbits during pregnancy resulted in abortion.

Heifers grazed on infected tall fescue G1-307 and Kentucky 31 (KY31) showed reductions in average daily gain and serum prolactin (PRL) when compared to heifers grazed on noninfected fescue (Bond and Bolt, 1986). They also reported that the initiation of cycling activity, as reflected by serum progesterone concentrations, was not delayed and there was no cessation of the estrous cycle. However, Danilson et al. (1986) noted that grazing heifers on fungal-infested tall fescue resulted in a reduction in weight at breeding time and a significant reduction in number pregnant. Garrett et al. (1980) surveyed 298 Missouri horse farms and reported problems with mares grazing tall fescue. These included agalactia, prolonged gestation, abortion, and thick placenta. Birth weight of calves that were born to dams that grazed fungus infected fescue was significantly lower than those from dams that grazed noninfested fescue (Bolt and Bond, 1986).

Jackson et al. (1986) conducted an experiment to determine the effect of feeding seed from endophyte-infested fescue on male rats. They reported that feeding a diet of either 25% or 50% infested fescue seed to male rats depressed serum PRL and feed intake without a dose response relationship. Zavos et al. (1987) reported that reproductive potential in both the male and female mice were negatively affected by infected fescue diet. Feeding a diet of 50% infected

tall fescue seed significantly decreased testicular weight and reproductive performance in the mice (Zavos et al., 1990). Zavos et al. (1988) also reported that feeding male rats non-infested tall fescue seed treated with methanolic extracts of infected seed resulted in reductions in feed intake, average daily gain, testicular weight and daily sperm production. These results suggest that the methanol extract contains the factors responsible for the lowered reproductive performance in male rats. Also, feeding 50% fungal infected fescue seed to male rats decreased daily sperm production, testicular parenchyma and epididymal weight (Zavos et al., 1986). However, feeding infected fescue hay to holstein bulls did not affect body weights, or reproductive development (Evans et al., 1988).

Toxic fescue depresses the PRL rise that follows milking in dairy cows and blocks the temperature-related increase in basal PRL secretion when fed to calves (Hurley et al., 1981; Jackson et al., 1982). Ewes grazing G1-307 fescue showed a dramatic decrease in serum PRL as compared to ewes grazing orchard grass, but no differences were found in the pituitary concentration of PRL (Bolt et al., 1982). This suggests that the reduction in plasma PRL involved impaired pituitary secretion but not pituitary synthesis of PRL. Ultrastructural studies to examine the effect of treatment of rat pituitary cultures with ergocornine demonstrated that neither PRL synthesis nor granule formation were affected. Exocytosis was, however, suppressed resulting in the intracellular destruction of excess secretory granules (Ectors et al., 1972). Prolactin secretion by bovine

pituitary cells in culture was inhibited when treated by ergocriptine (Smith et al., 1974).

Dopamine(DA) has been known to inhibit PRL secretion by the pituitary gland (Blask and Orstead, 1986). Ergot alkaloids compete for binding of DA receptors (Goldstein et al., 1980). Also, several synthetic ergopeptides that have in vivo and in vitro properties of DA agonists, stimulate central DA receptors and reduce DA turnover in several brain regions (Fuxe et al., 1978; Fluckiger, 1980; Porter et al., 1985). Treatment of wethers with spiperone (a DA antagonist) resulted in an elevation of serum PRL and a significant reduction in plasma DA (Henson et al., 1987). Spiperone increased PRL by binding to DA receptors and blocking the inhibiting effects of DA. Injection of heifers grazing G1-307 tall fescue with DA antagonists domperidone or spiperone increased serum PRL and improved grazing behavior (Bolt et al., 1983). This suggests that toxic fescue contains alkaloids that possess dopaminergic activity.

#### The Role of Prolactin in Reproduction in Male Mammals.

A considerable amount of evidence indicates that PRL is involved in the regulation of the function of the mammalian testis, but its role in male reproduction is not clearly established. Most of the evidence that PRL may interact with testicular tissue comes from investigations in rodents. Suggestions

that PRL can act directly on the male reproductive system received strong support from the demonstration that specific PRL receptors are present in the interstitial compartment of the testis and in the accessory reproductive glands (Aragona et al., 1977; Charreau et al., 1977b). PRL binds specifically to membrane receptors localized in the interstitial tissue of the rat testis.

Autoradiographic studies demonstrated the presence of the PRL binding sites to the Leydig cells and the secretory epithelium of the prostate (Costlow and McGuire, 1977).

Prolactin is essential for maintenance of LH receptors in the Leydig cells of rats. Aragona et al. (1977) demonstrated that in the immature male rat inhibition of PRL release by 2  $\alpha$ -bromoergocriptine resulted in a reduction in testicular LH receptors. Prolactin deficiency in hereditary dwarf mice is associated with reduction in the ability of the testis to bind LH (Bohnet and Friesen, 1976), and a decrease in PRL levels in hamsters exposed to a short photoperiod is also accompanied by a decrease in the number of testicular LH receptors (Bex and Bartke, 1977). In addition, PRL treatment increases LH receptors in the testes of dwarf mice, in atrophic testes of light deprived hamsters and in rat luteal cells (Holt et al., 1976; Bohnet and Friesen, 1976; Bex et al., 1978). Treatment of hypophysectomized rats with PRL increases the concentration of LH receptors which declined as a result of hypophysectomy (Zipf et al., 1978). Barenton (1981) reported that injection of PRL into two-year old rams which had regressed testis did not influence the testicular content

of gonadotropin receptors. This suggests that PRL is not directly involved in gonadotropin receptors regulation in the ram. Furthermore, serum PRL increased either by photo stimulation or decreased by bromocriptine treatment in the ram had no effects on LH receptors, but there was a delay in the beginning of testicular growth in rams treated with bromocriptine (Barenton and Pelletier, 1980).

Hereditary dwarf mice, where PRL and growth hormone are both deficient, are hypogonadal and sterile. The treatment of dwarf mice with PRL stimulates growth of the testis and accessory glands, increases T synthesis and induces fertility (Bartke, 1977). In hypophysectomized rats and mice treatment with PRL significantly potentiates the effect of LH on the restoration of T synthesis and spermatogenesis (Bartke, 1971a; Hafiez et al., 1972). Injection of hypophysectomized mice with PRL decreased testicular involution and increased testicular weight and spermatogenesis (Bartke, 1971a). Furthermore, a combined treatment of PRL and LH promoted spermatogenesis and androgen production significantly more than treatment with LH alone. Testicular regression induced by light deprivation in the hamster could be restored by PRL injection (Bex and Bartke, 1977). Prolactin treatment of the hamster results in stimulation of testicular growth, restoration of T production and an increase in LH receptors (Bartke et al., 1975; Bex et al., 1978).

A considerable number of reports suggest that PRL has a direct effect on steroidogenesis. In immature rats, treatment with bromocriptine is associated

with a significant reduction in testicular steroidogenesis without any change in LH secretion (Suescun et al., 1985). In addition, active immunization against PRL has been shown to reduce the stimulatory effects of LH on serum T levels in adult male rats (Chandrashekar and Bartke, 1986; Chandrashekar and Bartke, 1988). Fung et al. (1989) reported that PRL inhibited LH-stimulated T secretion from perfused rat testis at low doses. Studies on goats and rams indicated that summer elevation in peripheral PRL concentrations coincides with the increase in Plasma T levels which precedes the onset of the breeding season (Racey et al., 1975; Ravault, 1976). Moreover, increase of T concentrations have been shown to follow experimentally induced elevations of plasma PRL levels in men (Rubin et al., 1978).

A mechanism by which PRL may affect steroidogenesis is suggested by the observation that treatment of hereditary dwarf mice with PRL increases the concentration of esterified cholesterol in the testis (Bartke, 1971b). In hypophysectomized mice treated with PRL and LH, the concentration of esterified cholesterol was higher than in mice treated with LH alone (Bartke, 1971a). In addition, PRL alone or in combination with LH can maintain normal levels of esterified cholesterol in the corpus luteum of hypophysectomized rats (Behrman, 1970). Also, PRL enhances LH induced T secretion by increasing the conversion of cholesterol to pregnenolone and  $3\beta$ -hydroxysteroid dehydrogenase activity in the testis of dwarf mice (Hafeez et al., 1971; Hafeez et al., 1972). Thus, it was concluded that PRL increases the

esterified cholesterol in the testis and these stores provide a pool of precursors for steroidogenesis and consequently increase the ability of the testis to produce T in response to LH.

Receptors for PRL have been identified in the prostate, seminal vesicle and epididymis of a variety of species (Aragona and Friesen, 1975; Barkey et al., 1977; Charreau et al., 1977a); indicating that PRL could interact with these organs and be of significance in their regulation. Prolactin has been shown to have a marked stimulatory effect on the growth and secretory capability of the prostate, an action which appears to involve synergism with androgens (Thomas and Mandhar, 1975). Prolactin secreted from a single pituitary graft under the kidney of a 30-day old rat stimulated the accessory glands in intact, castrated, castrated-adrenalectomized, or hypophysectomized rats (Negro-vilar et al., 1977). Chronic deficiency of serum PRL caused by repeated injection of an antiserum against PRL to 3-day old rats for 58 days or by injection of bromocriptine to 30-day old mice significantly reduced prostatic and seminal vesicular weights (Bartke, 1974; Hostetter and Piacsek, 1977). Prolactin may affect sex accessory glands by increasing the nuclear uptake of  $5\alpha$ -dihydrotestosterone, suggesting that PRL may increase the number of androgen receptors (Baker et al., 1977). Negro-vilar et al. (1977) proposed that PRL may affect the accessory glands by three mechanisms, promotion of growth by acting directly at the organ level, by synergizing with androgens, or increasing the output of androgens from the testis.

Some studies have demonstrated the ability of chronically elevated PRL to suppress basal gonadotropins secretion in the male rats (Lu et al., 1976; Greeley and Kizer, 1979), and in women with amenorrhea-galactorrhea syndrome (Asfour et al., 1977).

The treatment of rams with bromocriptine suppressed serum PRL without any changes in LH secretion (Ravault et al., 1977). In adult male rats, hyperprolactinemia experimentally induced by ectopic pituitary grafts, however, resulted in a reduction in circulating levels of gonadotropins (McNeilly et al., 1978). Also, treatment of castrate and intact male rabbits (Larsen and Odell, 1987) and castrate male rats (Celotti, et al., 1978) with PRL resulted in a reduction in LH secretion. These data suggest that PRL can act on the pituitary and the hypothalamus to influence LH secretion.

Hyperprolactinemia also interferes with gonadal function. Fang et al (1974) reported that testicular atrophy in rats bearing PRL secreting tumors. In these animals, T levels were significantly depressed but LH levels were elevated. Furthermore, Bixler et al. (1977) demonstrated that Thioridazine, a PRL stimulant, lowered sleep-related increase in T concentration in men.

#### Effect of Zeranol on Testicular Function.

Zeranol is a nonsteroidal compound that elicits many of the same biochemical and biological effects as estradiol (Katzenellenbogen et al., 1979).

It has been used as an anabolic agent in beef cattle (Perry et al., 1970).

Testicular growth was inhibited when the bulls were implanted with Zeranol within 3 months of age (Juniewicz et al., 1985). However, when bulls were implanted after weaning or as yearlings it did not affect testicular development (Staigmiller et al., 1985). Zeranol inhibited T production and spermatogenesis (Silcox et al., 1986). These researchers also found that GnRH-stimulated T secretion was inhibited by zeranol. In the zeranol-implanted bulls, the amount of smooth endoplasmic reticulum in the Leydig cells was reduced. Bull calves, implanted with zeranol from birth through 6 months of age, exhibited suppressed GnRH-induced release of LH, but not FSH (Deschamps et al., 1987).

#### Function and Control of Gonadotropins in the Adult Male.

The endocrine and gametogenic functions of the testis are under the control of the central nervous system. The main regulatory component of this system is GnRH which is a decapeptide produced by neurons located in the hypothalamic-preoptic region (Everett, 1988). GnRH is released into the region of the median eminence and transported through the hypophyseal portal circulation into the anterior pituitary gland.

Gonadotropin releasing hormone activates its receptors on the gonadotrops resulting in the synthesis and release of gonadotropins (Eskay et al., 1977). It has been shown that GnRH is released in a pulsatile manner

resulting in the pulsatile release FSH and LH by the anterior pituitary (Knobil, 1981). The pulsatile release of the GnRH is the consequence of synchronous discharge of GnRH-containing neurons. Thus, the frequency of LH pulses in the blood is a direct reflection of the frequency of episodic release of GnRH (Levine et al., 1982). Previous evidence presented by Knobil (1980) indicated that pulsatile GnRH release is essential for maintaining the sensitivity of the anterior pituitary to GnRH. Continuous infusion of GnRH fails to release LH in Rhesus Monkeys, but pulsatile infusion releases LH (Knobil, 1980). However, in the rat continuous infusion of GnRH stimulates LH release (Blake, 1976).

Both gonadotropins consist of two peptide chains ( $\alpha$  and  $\beta$ ). One subunit ( $\alpha$ ) is common to both hormones and each hormone is formed by the association of an  $\alpha$  subunit with a hormone specific  $\beta$  subunit (Hadley, 1984).

Although GnRH can stimulate the release of both LH and FSH from the pituitary, there are a number of instances in which LH and FSH secretion are divergent. Stimulation of dorsal anterior hypothalamic area and a region extending ventrally and caudally toward the posterior median eminence can stimulate selective FSH secretion (McCann et al., 1984). Active immunization against GnRH suppress LH secretion without any immediate effect on FSH in the boar (Awoniyi et al., 1988). Also, passive immunization of specific receptors blocked with GnRH antagonists had no effects on pulsatile release of FSH (Culler and Negro-vilar, 1986). This suggests that there might be a releasing factor for FSH independent of GnRH.

Gonadal steroids are the major regulatory substances controlling gonadotropins release. Removal of the gonads results in an increase in the frequency and amplitude of GnRH and LH pulses (Steiner et al., 1982; Plant and Dubey, 1984). Specific receptors which bind androgens and estrogens are located in the areas of the male brain known to be associated with gonadotropins secretion (MacLusky and Clark, 1980).

In men, T infusion decreased LH pulse frequency, but estradiol ( $E_2$ ) infusion decreased pulse amplitude (Santen, 1977). This suggests that T acts at the hypothalamus and  $E_2$  at the level of the pituitary. Also, T has been shown to increase the magnitude of the LH response to GnRH in 6-month old castrated bulls (Lacroix and Pelletier, 1979a). It has been suggested that some of the effects of T on the regulation of LH secretion are due to conversion of T to  $E_2$  either centrally or/and perpherally (Worgul et al., 1981). Inhibition of central nervous system aromatase caused a significant increase in plasma LH in the presence of normal T concentration in dogs (Awoniyi et al., 1986).

In the bull, negative feedback by  $E_2$  on LH is mediated primarily at the level of the hypothalamus by blocking the pulsatile release of GnRH (D'Occhio et al., 1982). Active immunization against  $E_2$  resulted in an increase in gonadotropins and T production in the ram (Schanbacher et al., 1987b). This indicates that  $E_2$  is a potent regulator of the gonadotropins in the ram. Pulsatile GnRH administration overcomes the inhibitory effects of  $E_2$  implants on testicular development and gonadotropins secretion in the bull (Schanbacher,

1981). This suggests that  $E_2$  acts at the level of the hypothalamus to block GnRH release in the bull. Implanting of prepuberal bulls with  $E_2$  retarded testicular development and prevented pulsatile secretion of LH. Testosterone production was also reduced (Schanbacher, 1981). Peripheral  $E_2$  can affect Leydig-cells function indirectly via its feedback action on LH secretion (de Jang et al., 1975) or via its effect on testicular steroids synthesis (Kalla et al., 1980). Estradiol also has the ability to reduce LH binding sites in the testis of the bulls implanted with  $E_2$  (Schanbacher, et al., 1987b). This suggests that an inhibitory effect of  $E_2$  might be imposed directly on Leydig cells.

Despite its negative effects,  $E_2$  has been shown to have positive effects on gonadotropins secretion. Treatment with  $E_2$  enhances the effects of GnRH stimulated LH in castrated bulls (D'Occhio et al., 1982).

Inhibin is a gonadal peptide produced by the Sertoli cells and has been defined as an agent that suppresses the release of FSH (diZerega and Sherins, 1981). The primary site of action of inhibin is at the pituitary where it suppresses the synthesis of FSH by the gonadotropes (Scott and Burger, 1981). Inhibin has been reported to suppress the synthesis and release of FSH in vitro, and it also suppresses GnRH stimulated FSH synthesis and release in a dose-dependent way (Davies et al., 1979; Martin et al., 1986; Fukuda et al. 1987).

The inhibitory effects of gonadal steroids on GnRH secretion is mediated in part by interactions of these steroids with a number of central neurotransmitters. Epinephrine, dopamine and serotonin have been shown to

be implicated in the regulation of GnRH and LH release, and the activities of these neurotransmitters are modulated by gonadal steroids (Negro-Vilar and Ojeda, 1981).

Endogenous opiate peptides are important modulators of different hypothalamic-peptidergic regions that regulate pituitary function. Morphine and  $\beta$ -endorphine have been shown to suppress basal and GnRH-stimulated LH release (Cacicedo and Franco, 1985; Blank et al., 1986). The opiate-receptor-antagonist, naloxone, stimulates GnRH release in vitro, and when infused in the proximity of GnRH neurons in the hypothalamus, LH release was also increased (Kalra, 1981; Kalra et al., 1987). This suggests that endogenous opiate peptides in the hypothalamus suppress GnRH release.

The dopaminergic antagonists, Fluphenazine and Domperidone have been shown to increase LH pulse frequency in the ewe (Dailey et al., 1987). Luteinizing hormone release, after the administration of GnRH, was diminished by infusion of noradrenaline in the intact ewes. Furthermore, intravenous infusion of ewes and heifers with norepinephrine has been shown to inhibit the secretion of LH (Deaver and Dailey, 1982; Hardin and Randel, 1983). However, Negro-vilar et al. (1979) reported that noradrenaline and dopamine have stimulatory effects on GnRH release in vitro in the male rat. Injections of norepinephrine or epinephrine into the third ventricle of ovariectomized, steroid-primed ewes leads to a release of LH (Vijayan and McCann, 1978). In the bull, concentrations of norepinephrine and dopamine within the stalk

median eminence increased with age and were positively correlated with plasma T (Deaver and Peters, 1988).

Luteinizing hormone receptors have been located in the Leydig cells of the rats (de Krester et al., 1971). Luteinizing hormone has a trophic action on the Leydig cells, and it is the primary regulator of T production (de Kretser, 1984). Also, LH is required for the maintenance, differentiation and function of Leydig cells (Keeney et al., 1988). Hypophysectomy or neuralization of LH activity results in a diminution of the Leydig cells and cessation of T production (de Kretser, 1984). Also, LH stimulation increases the quantity of mitochondria and smooth endoplasmic reticulum in Leydig cells. Luteinizing hormone has an important role in spermatogenesis through its stimulation of T production. High levels of T are required for the maintenance of spermatogenesis (diZerega and Sherins, 1981). A positive relationship was found between LH concentration and testicular diameter in the ram (Courrot and Ortavart, 1981). Active immunization against LH resulted in an inhibition of Sertoli cells differentiation and function (Chemes et al., 1979).

Follicle stimulating hormone receptors are present on the Sertoli cells (Orth and Christenen, 1977). It has been reported that FSH exerts a number of effects on Sertoli cells. These include increase in size, stimulation of protein synthesis and production and secretion of fluids (Courrot and Courtens, 1982; Culler and Negro-Vilar, 1986). Also, FSH stimulates the mitosis of Sertoli cells and influences the microenvironment of spermatogenic cells by stimulating the

production and secretion of a number of substances by the Sertoli cells (Jutte et al., 1983; Orth, 1984). There have been some reports that indicate that FSH influences the maturation of Leydig cells, and enhances LH stimulation of T secretion (diZerega and Sherins, 1981; Keer and Sharpe, 1985). Serum FSH has been shown to increase after hemicastration in the bull (Schanbacher et al., 1987a). Results of some studies on the regulation of testicular function in adult animals indicate that FSH plays a minor role in the maintenance of spermatogenesis (Cunningham and Huckins, 1979). In hypophysectomized rats, spermatogenesis could be maintained with high doses of T alone (de Kretser, 1984). In monkeys, a positive correlation has been found between FSH concentrations and the number of germinal cells in the seminiferous tubules (van Alphen et al., 1988). Also, the neutralization of FSH by passive immunization against FSH in the male monkey caused a slight impairment in spermatogenesis and fertility (Murty et al., 1979).

#### Prepuberal Testicular Development.

During the infantile period of development in the bull, there is almost an absence of episodic secretion of LH (Deaver, 1985). The transition from an infantile to prepubertal state is characterized by an increase in pulsatile secretion of LH (Amann, 1983; Wise et al., 1987). This increase in LH pulses occurs when the bull is 8-12 weeks of age (Curtis and Amann, 1981). Rodriguez and Wise (1989) suggested that maturational changes in the

hypothalamic release of GnRH drives the transition from the infantile to prepubertal period by increasing pulsatile LH release. Furthermore, the sensitivity of the anterior pituitary gland to hypothalamic stimuli increases simultaneously with the onset of pulsatile LH secretion (Amann et al., 1986). This sensitivity is associated with an increase in GnRH receptors and the availability of releasable LH. The anterior-pituitary response to GnRH continues to increase until a maximum is reached at puberty in the bull (Lacroix and Pelletier, 1979a).

The testis can be divided functionally into an interstitium and seminiferous tubules, which are responsible for T production and spermatogenesis respectively (Setchell, 1977). The interstitial compartment is composed of Leydig cells, macrophages, blood vessels and lymphatic vessels. The seminiferous tubules are composed of a peritubular layer of myoid cells, Sertoli cells and germ cells (Setchell, 1977).

### Leydig Cells

The primary targets of LH in the testis are the Leydig-cells. There is much evidence to suggest that the increase in Leydig-cell number during puberty is achieved primarily by differentiation from a primitive cell type and interstitial cell mitosis (Hooker, 1970; Gondos et al., 1979).

In the bull, Leydig cells start to differentiate at 12 to 16 weeks of age (Hooker, 1970). At this time, the Leydig cells, undergo some changes in

nuclear form, increase in cytoplasmic volume and develop cytoplasmic organelles. Changes in organelles include extensive proliferation of smooth endoplasmic reticulum, appearance of large round mitochondria with tubular cristae and development of a prominent Golgi apparatus. All of these morphological changes are under the control of LH (de Kretser, 1984). In the rat, the number of Leydig cells increases from 20 to 50 days postnatal without any change in T Production (Knorr et al., 1970; Purvis et al., 1978). However, androsterone and  $5\alpha$ -androstane- $3\alpha$ ,  $7\beta$ -diol are the main steroids produced during this time of development. Studies on rats demonstrated that changes in  $5\alpha$ -reductase activity occurred during testicular development. At 20-25 days postnatal, a decrease in T synthesis occurs at the same time that  $5\alpha$ -reductase activity increases and results in the synthesis of  $5\alpha$ -reduced androgens (such as  $5\alpha$ -androstene- $3$ ,  $17\beta$ -diol) (Cochran et al., 1979). In the bull, the ratio of production of androstenedione to testosterone is much greater in the immature bull than in the adult (Abdel Malak and Thibier, 1979; McCarthy et al., 1979b). In addition, before 4 months of age, LH pulses are not followed by a release of T (Lacroix and Pelletier, 1979b; Amann, 1983). In older animals, on the other hand, significant amounts of T are secreted following each LH pulse. It has been suggested that this could be due to an increase in Leydig cells number and a decline in the steroid-metabolizing enzyme  $5\alpha$ - steroid reductase and change in the steroidogenic pathway (Knorr et al., 1970; Tsujimura et al., 1975).

### Seminiferous Tubules.

Morphometric analysis of the adult rat testis indicates that the seminiferous tubules comprises over 80% of the testicular volume (Mori and Christensen, 1980). Thus, the more important contribution to the determination of adult testis size are the Sertoli cells and germ cells. Factors affecting Sertoli cell proliferation have a large potential for influencing testicular function in the adult animal (Erickson and Blend, 1976). The germ cell population in the adult animal is dependent on the magnitude of the Sertoli-cell population (Erickson and Blend, 1976; Kluin et al., 1984; Orth, 1985). Sertoli cells are in intimate contact with germ cells during all phases of spermatogenesis. Therefore the fundamental function of Sertoli cells is to create a site suitable for the proliferation and maturation of germ cells, and to supply the nutrients needed by the germ cells (Foster, 1988).

Most Sertoli-cell mitoses occur during fetal life in rats (Orth, 1982). After birth, Sertoli cells are mitotically active for only a short time. Sertoli-cell mitosis is dependent on FSH, since FSH increases DNA synthesis and promotes mitotic activity of Sertoli cells (Griswold et al., 1977). Mitotic figures of bovine Sertoli cells are numerous between birth and 80 days, but few mitotic figures are seen thereafter (Erickson, 1963). The number of Sertoli cells is believed to be fixed at about 5 months of age in the bull (Attal and Courot, 1963). Sertoli cells undergo extensive morphological and biochemical changes at the end of the prepubertal phase. The differentiation of the Sertoli cell

appears to be controlled to a large extent by FSH and T (Chemes et al., 1979). In the bull, differentiation of Sertoli cells is completed between 20 and 28 weeks postnatal, changes include cell shape, organelles, and the nucleus (Sinowatz and Amselgruber, 1986). Junctional complexes between neighboring Sertoli cells developed at 24 weeks of age, and these result in the blood-testis barrier which divides the tubular epithelium into basal and adluminal compartments (Setchell, 1977).

Androgen receptors have been located on the Sertoli cell (Sanborn et al., 1977) and in the absence of T, growth and morphological differentiation of Sertoli cell are inhibited (Dym and Raj, 1977; Chemes et al., 1979). Marked increase of intratesticular concentration of T around 18 weeks of age corresponds to the initiation of Sertoli cell differentiation in the bull (McCarthy et al., 1979a). Collectively, these data suggest that the T has an important role in Sertoli-cell differentiation.

### Germ Cells.

At one month of age in the bull the only germ cell present in the seminiferous tubules is the gonocyte (Curtis and Amann, 1981). The gonocytes proliferate and give rise to stem and differentiating spermatogonia. Six spermatogonial divisions have been observed in the bull (Berndston and Desjardins, 1974). These are three type-A generations, one intermediate generation and two generations of type B-spermatogonia. The first type A-

spermatogonia appears around 9 weeks of age in the bull (Erickson, 1963).

However, they are not seen in a significant number until 12 weeks of age. The primary spermatocytes, products of mitosis of the last generation of spermatogonia, are first seen at 24 weeks of age. The secondary spermatocytes then divide and give rise to spermatids. Round and elongated spermatids are evident at 28 and 32 weeks of age, respectively and mature spermatids can be seen at 7 months of age (Ortavant et al., 1977; Curtis and Amann, 1981). The initiation of spermatogenesis is more dependent on the development of the animal than on its age (Courot, 1962; Ortavant et al., 1977).

## CHAPTER III

## MATERIALS AND METHODS

Experimental Design.

This study consisted of two parts: 1) an assessment of the effects of fungal-infested fescue plus zeranol on testicular development and hormonal secretions in the beef bull, and 2) an assessment of the effects of fungal-infested fescue only on the same criteria. Studies were performed on crossbred bull calves at the Highland Rim Experiment Station.

Calves resulted from Angus-Hereford cows mated to Simmental bulls and cows and calves were grazed on tall fescue (*Festuca arundinacea* Schreb.) pastures infested to varying degrees with the fungal endophyte Acremonium Coenophialum. Levels of infestation was based on average ELISA determination for individual pasture. Treatments consisted of 1) control (0%) infestation, 2) low fungal infestation (50-70%) and 3) high fungal infestation (80-84%). Each of 8 pastures was grazed by five cows and their calves.

## Study 1.

In 1986, 21 bull calves were implanted with 36 mg of zeranol at birth and then every 3 months thereafter. Assessments of the effects of treatment (fungal endophyte and zeranol) on the ability of the pituitary and testis to

respond to GnRH challenges were made. The first assessment was performed when the average age of the bull calves was 3 months. A Gonadotropin-releasing hormone (GnRH) analog, des-Gly, [D-Ala]-GnRH ethylamide (10 ug) was injected and a 12 ml blood sample was collected prior to and 1 and 2 hours after injection. Another GnRH injection (10 ug) was given when the average age of the calves was 5 months and the same procedure was followed. Two 12 ml blood samples were collected at 60 minute intervals when the bulls were 7 months of age. Immediately following the last bleeding, the bulls were castrated and the excised testes were weighed and measured. Transverse sections of 4 mm in thickness were excised from the center and poles of each testis and fixed in Bouin's fluid. The fixed and paraffin embedded tissue was sectioned at 5 um and stained with hematoxylin and orange G. Segments were evaluated for the total population of Sertoli cells and number of A-spermatogonia. At blood collection time, samples were put on ice and centrifuged within 12 hours at 3000 round per minute for 20 minutes. Plasma was removed and stored at -20°C until assayed for gonadotropins and PRL.

## Study 2.

Bull calves from two successive years (1987 and 1988) were used to quantify the effects of fungal endophyte on testicular development. In the 1987 study, bulls were bled at 3 and 6 months of age for basal assessment of hormone secretion. Calves were removed from pastures when they were 8

months of age and a 10 ug injection of GnRH was given at 12 months to determine the late effects of the fungus. Bulls were unilaterally castrated at 3 months of age, and the remaining testis was removed immediately after the last bleeding at 12 months of age. The testes were prepared for histological evaluation.

In the 1988 study, bulls were injected with a GnRH (10 ug) at 3 and 8 months of age, and bled as indicated earlier. Bulls were castrated when 8 months of age and the testes were prepared for histological analysis. The testes were evaluated for total number of Sertoli cells, number of A-spermatogonia and percentage of cross sections possessing various germ-cell associations. Plasma was collected and handled as indicated earlier and assayed for gonadotropins, PRL, T, and  $\Delta 4A$ .

#### Radioimmunoassay Techniques.

Luteinizing Hormone. Plasma LH was assayed using the method of Niswender et al. (1969) as modified by Bolt (unpublished). LH antiserum (#15 anti-ovine LH) was provided by Dr. Gordon Niswender, Colorado State University. The antibody did not significantly cross-react with FSH, TSH or growth hormone (Niswender et al., 1969). The reference curve (31-4000 pg) was constructed using triplicate samples of the LH standard supplied by Dr. L. L. Reichert (LER 1056). An antibody dilution of 1:40,000 and a tracer activity of 18,000 cpm LH-I<sup>125</sup> was used to get an average total binding of 40%.

Plasma levels of LH were determined by comparing the unknowns to the standard curve. Nonspecific binding was <3.5%. Intra- and interassay coefficient of variation was determined by the measure of a standard sample of pooled plasma from steers in each assay. Intra-and interassay variation were  $4.8 \pm 2.7$  and  $8.2 \pm 2.0$  respectively. Details of the RIA Protocol are included in Appendix 2.

Follicle Stimulating Hormone. Values of FSH were determined using the RIA developed by Bolt and Rollins (1983) with modification. FSH reference standard (USDA-bFSH-B1) and FSH antibody (USDA-5-0122) were supplied by Dr. D. J. Bolt. Cross reactivity of the antibody with LH, growth hormone and PRL was <0.09% , and with TSH was 0.05% (Bolt and Rollins, 1983). A reference curve (0.25 - 32ng) was formed using triplicate samples of the standard. The antibody dilution was 1:10,000 and the tracer activity was 18,000 cpm of FSH-I<sup>125</sup> to obtain an average binding capacity of 20%. Intraassay variation was  $1.4 \pm 0.5$  and inter assay variation was  $5.1 \pm 0.8$ . Non specific binding was <2.3%. Details for the RIA Protocol are in Appendix 2.

Prolactin. Prolactin was measured using RIA method described by Forrest et al.(1980). Prolactin antibody (DJB 7-0330) and PRL reference standard (USDA-bPRL-B-1) were supplied by Dr. D. J. Bolt. A reference curve (0.05-25.6, ng) was constructed using triplicate samples. An antibody dilution of 1:200,000 and a tracer activity of 18,000 cpm PRL-I<sup>125</sup> was used to get an

average total binding capacity of 50%. Intra assay variation was  $2.5 \pm 0.8$ . Nonspecific binding was  $<3.0\%$ . Details of the RIA protocol for PRL are in Appendix 2.

Testosterone. Plasma T was determined by using the method of Gordon and Sherwood (1978) with modification. The antibody (#250 anti-T-11-BSA serum) was provided by Dr. Gordon Niswender. A reference curve (10-1000 pg) was constructed using duplicate samples of the standard. The antibody dilution was 1:14,000 and a tracer activity of 15,000 cpm [1, 2, 6, 7  $^3\text{H}$ ]-T (Amersham Co.) were used to provide an average total binding capacity of 30%. Intra assay variation was  $1.9 \pm 0.6$  and interassay variation was  $8.3 \pm 2.6$ . Nonspecific binding was  $<2.17$ . Details of the T RIA Protocol is included in Appendix 2.

Androstenedione. Androstenedione levels were measured by using the method of Rao et al.(1982). Androstenedione antibody (anti-Androstene-3, 17-Dione-9- $\alpha$ -BSA) was used(supplied by Dr. Rao). Cross reactivity of the antibody with  $5\alpha$  , and  $5\beta$ -androstan-3, 17-dione were 30.4 and 5.9% respectively, cross reactivity with testosterone and progesterone were 2.5 and .5% respectively (Rao et al., 1982). A reference curve (5-500 pg) was constructed using duplicate samples of the standard. The antibody dilution was 1:16,000 and a tracer activity of 15,000 cpm [1, 2, 6, 7  $^3\text{H}$ ]-Androst-4-ene-3, 17-Dione was

used to get an average total binding of 30%. Intra assay variation was  $3.3 \pm 1.5$  and interassay variation was  $8.2 \pm 2.1$ . Nonspecific binding was  $<3.16$ .

To validate all assays in our lab, varying concentrations of the hormones were added to a previously characterized plasma. Ninety-five (95) to 102% of the hormones were recovered and the dose-response curves were parallel to the standard curve. Details of RIA protocol is included in Appendix 2.

### Histological Evaluation.

Estimation of the total population of Sertoli cells was accomplished by using the procedure described by Erickson and Blend (1976): 1) Testicular volume was determined by applying the formula  $V = \pi R^2 h$  to measurements of testicular length and width. 2) Portion of testicular volume occupied by the seminiferous tubules was estimated by the method of Chalkly (1943). 3) Volume occupied by a unit of 1000 tubule cross sections was estimated by applying the formula  $V = \pi R^2 h$  to section thickness and the tubular diameter. 4) The number of Sertoli cells per tubular cross section was estimated by counting only the nuclei of Sertoli cells that had a visible nucleolus. 5) Calculation of total population of Sertoli cells was estimated as follows: volume occupied by seminiferous tubules was divided by the volume occupied by 1000 cross sections and this was multiplied by the number of Sertoli cells per 1000 cross sections.

The number of gonocytes and A-spermatogonia were enumerated and expressed as number per 100 Sertoli cells. An average of 300 cross-sections per testis was examined and tubules were classified according to the type of germ cells they contained and each classification was expressed as a percentage of the total cross-sections of seminiferous tubules evaluated.

#### Statistical Analysis.

The effects of treatments on hormone levels and testicular data were tested for significance using a one-way analysis of variance and the student-Newman-Keuls (SNK) test (Sokal and Rohlf, 1981). Effects of treatment and differences between the treatments were tested using orthogonal contrasts. Two-way analysis of variance was performed to determine the interactions between treatment and age. Correlations (Pearson's product-moment correlation coefficient) were made between prolactin and the other hormones as well as between testicular histology and hormone levels.

Means, standard deviations, standard errors and correlations were calculated using the Statistical Analysis Systems (SAS, 1985a), and analysis of variance, SNK, and orthogonal contrast were calculated according to SAS (1985b).

## CHAPTER IV

## RESULTS

Study 1. The Effects of Fungal-infested Fescue and Zeranor on Testicular Development in the Bull.

Effects of a GnRH Challenge in the Bull at 3 Months of Age. Means and standard errors of gonadotropins and PRL after a GnRH challenge at 3 months of age are shown in Table I. Gonadotropins increased in response to GnRH stimulation ( $P < 0.01$ ). Grazing on fungus infested fescue did not significantly affect either basal or stimulated FSH secretion ( $P > 0.10$ ). Basal LH levels in the control group were significantly higher ( $p < 0.01$ ) than LH levels in the low and high groups. Basal LH showed no dose response relationship. Like the basal levels, stimulated LH levels in the control group were significantly ( $P < 0.06$ ) higher than that of low and high groups (control vs. low and high groups  $P < 0.02$ ). There was a trend toward a relationship between the level of fungal infestation and GnRH-stimulated LH.

Prolactin levels were not affected by the GnRH challenge, therefore PRL levels (3 bleedings) were averaged and used in the evaluation of treatment effect. Prolactin levels in the control group were significantly higher ( $P < 0.01$ ) than PRL levels in the low and high infested groups. There was no dose related response in PRL levels. Body weights in the low and high infestation groups were significantly lower ( $P < 0.01$ ) than that of the control group.

Table I. Effect of Fungal-infested Fescue on PRL and Basal and GnRH-induced Gonadotropins Secretion and Body Weight in Zeranol Treated Bulls of 3 Months of Age.\*

| Levels of Infestation | Number of Bulls | FSH (ng/ml) <sup>□</sup> |            | LH (ng/ml)              |                         | PRL (ng/ml)*              | Body Weight (Kg)*       |
|-----------------------|-----------------|--------------------------|------------|-------------------------|-------------------------|---------------------------|-------------------------|
|                       |                 | Basal                    | Stimulated | Basal*                  | Stimulated**            |                           |                         |
| Control (0%)          | 5               | 1.3 ± 0.1                | 8.5 ± 2.6  | 0.9 ± 0.10 <sup>a</sup> | 39.8 ± 3.2 <sup>a</sup> | 182.6 ± 10.7 <sup>a</sup> | 153 ± 12.7 <sup>a</sup> |
| Low (50-70%)          | 9               | 1.4 ± 0.1                | 8.9 ± 1.6  | 0.6 ± 0.03 <sup>b</sup> | 30.0 ± 1.9 <sup>b</sup> | 34.8 ± 12.3 <sup>b</sup>  | 107 ± 5.1 <sup>b</sup>  |
| High (80-84%)         | 8               | 1.3 ± 0.1                | 8.9 ± 1.6  | 0.6 ± 0.02 <sup>b</sup> | 28.2 ± 3.5 <sup>b</sup> | 41.7 ± 3.4 <sup>b</sup>   | 108 ± 4.8 <sup>b</sup>  |

\*Mean ± Standard Error

<sup>□</sup>No significant difference between treatment groups (P>0.10)

\*a,b Means in the same column with different letters are significantly different (P<0.01)

\*\*a,b Means in the same column with different letters are significantly different (P<0.06)

Prolactin was positively correlated with basal and stimulated LH ( $r = .50$  and  $.57$ ,  $P < 0.01$ ). Also PRL was positively correlated with body weight ( $r = .71$ ;  $P < 0.01$ ).

Effects of a GnRH Challenge in the Bull at 5 Months of Age. Effects of a GnRH challenge in bulls at 5 months of age are shown in Table II. Basal FSH levels did not significantly differ between treatment groups ( $P > 0.10$ ). However, FSH levels in the low and high infestation groups were slightly reduced indicating a possible fungal endophyte effect. FSH levels after GnRH injection in the control group were significantly higher ( $P < 0.06$ ) than that of bulls in low and high infestation groups. Basal FSH levels were significantly higher ( $P < 0.01$ ) than those of 3-month old bulls (Figure 1).

Treatment did not significantly affect serum PRL levels ( $P > 0.10$ ). However, PRL levels in the low group were lower than that of control and high groups yet the difference was not significant ( $P > 0.10$ ). Body weights (BW) in the low and high infestation groups were significantly lower than the control group ( $P < 0.01$ ). PRL was positively correlated with GnRH-stimulated FSH ( $r = .41$ ;  $P < 0.06$ ).

Basal Hormonal Profiles in the Bull at 7 Months of Age. Basal concentrations of gonadotropins, PRL and BW in the 7-month old bulls are listed in Table III. There was no significant treatment effect on basal FSH levels, but FSH levels were reduced in the treated groups ( $P > 0.10$ ). This might

Table II. Effect of Fungal-infested Fescue on Body Weight, PRL, and Basal and GnRH-induced FSH Secretion in Bulls of 5 Months of Age\*

| Levels of Infestation | Number of Bulls | FSH (ng/ml)        |                          | PRL (ng/ml) <sup>□</sup> | Body Weight (Kg) <sup>*</sup> |
|-----------------------|-----------------|--------------------|--------------------------|--------------------------|-------------------------------|
|                       |                 | Basal <sup>□</sup> | Stimulated <sup>**</sup> |                          |                               |
| Control (0%)          | 5               | 2.2 ± 0.2          | 11.1 ± 0.3               | 120.0 ± 25.6             | 229 ± 7.4 <sup>a</sup>        |
| Low (50-70%)          | 9               | 1.9 ± 0.1          | 8.3 ± 0.1                | 70.7 ± 11.4              | 192 ± 7.1 <sup>b</sup>        |
| High (80-84%)         | 8               | 2.0 ± 0.2          | 9.2 ± 0.8                | 110.5 ± 23.9             | 191 ± 7.3 <sup>b</sup>        |

\*Mean ± Standard Error

□No significant difference between treatment groups (P>0.10)

\*\*Control group significantly higher than the treated population (P<0.06)

\* a,b Means in the same column with different letters are significantly different (P<0.01)

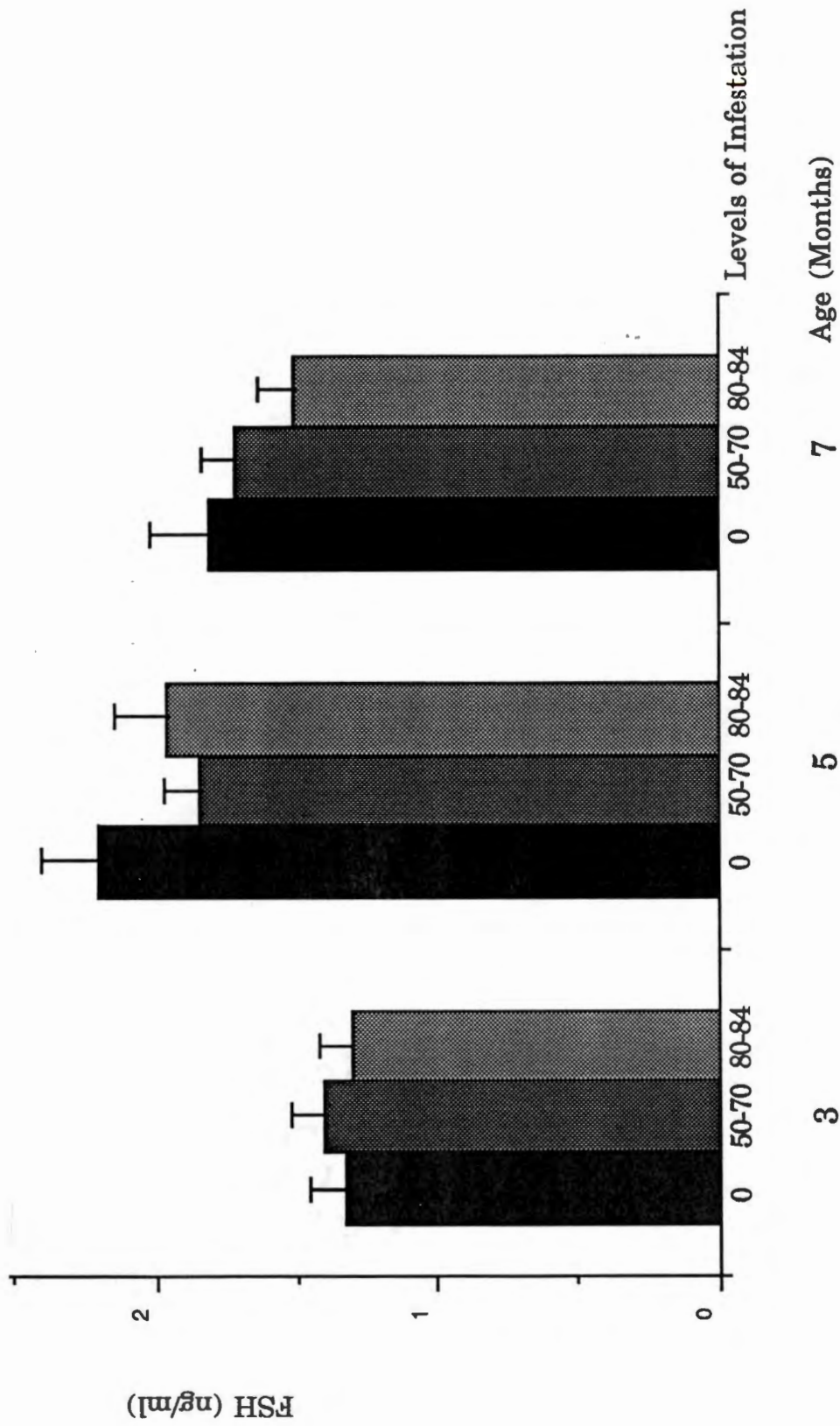


FIGURE 1. Effects of Fungal-Infested Fescue on FSH Levels at Different Ages in Zeranol Treated Bulls

Table III. Effect of Fungal-infested Fescue on Basal Gonadotropins, PRL and Body Weight in Zeranol Treated Bulls of 7 Months of Age\*

| Levels of Infestation | Number of Bulls | FSH (ng/ml) <sup>□</sup> | LH (ng/ml) <sup>***</sup> | PRL (ng/ml) <sup>**</sup>   | Body Weight (Kg) <sup>*</sup> |
|-----------------------|-----------------|--------------------------|---------------------------|-----------------------------|-------------------------------|
| Control (0%)          | 5               | 1.81 ± 0.2               | 0.30 ± 0.10 <sup>a</sup>  | 59.20 ± 18.7 <sup>a</sup>   | 270 ± 9.8 <sup>a</sup>        |
| Low (50-70%)          | 9               | 1.71 ± 0.1               | 0.14 ± 0.03 <sup>b</sup>  | 12.51 ± 3.3 <sup>b</sup>    | 218 ± 8.0 <sup>b</sup>        |
| High (80-84%)         | 8               | 1.51 ± 0.1               | 0.13 ± 0.01 <sup>b</sup>  | 26.47 ± 12.3 <sup>a,b</sup> | 219 ± 9.1 <sup>b</sup>        |

\*Mean ± Standard Error

□No significant difference between treatment groups (P>0.10)

\*\*\*<sup>a,b</sup>Means in the same column with different letters are significantly different (P<0.08)

\*\*<sup>a,b</sup>Means in the same column with different letters are significantly different (P<0.03)

\*<sup>a,b</sup>Means in the same column with different letters are significantly different (P<0.01)

suggest a possible fungal endophyte effect on FSH secretion. Basal FSH levels showed a modest dose-dependent response. Basal FSH levels in 7-month old bulls were significantly lower ( $P<0.01$ ) than that of 5-month old and higher ( $P<0.01$ ) than that of 3-month old bulls (Figure 1).

Basal LH levels in the control group were significantly higher ( $P<0.08$ ) than the treated groups. When low and high groups combined they differed from the control group at the 0.03 level of probability. Prolactin levels in the control were significantly greater ( $P<0.03$ ) than those of the low and high populations. Prolactin secretions were influenced ( $P<0.01$ ) by the age x treatment interaction (Figure 2). Body weights were also significantly reduced ( $P<0.01$ ) in the low and high groups by 16%. Gonadotropins were not affected by the age x treatment interaction.

Testicular Development in the Bull at 7 Months of Age. Testicular data are shown in Table IV. Testicular weights (TW) in the control group were significantly higher ( $P<0.07$ ) when compared to the low and high groups. Total population of Sertoli cells (SC) was significantly ( $P<0.01$ ) reduced in the low and high infestation groups as compared to the control. Number of type A-spermatogonia per 100 Sertoli cells was not significantly reduced in the fungal infestation groups ( $P>0.10$ ). However, an apparent dose-response relationship existed. PRL was positively correlated with TW ( $r = .44$ ;  $P<0.04$ ) and with SC ( $r = .49$ ;  $P<0.02$ ). SC was correlated with TW ( $r = .82$ ;  $P<0.01$ ) and with

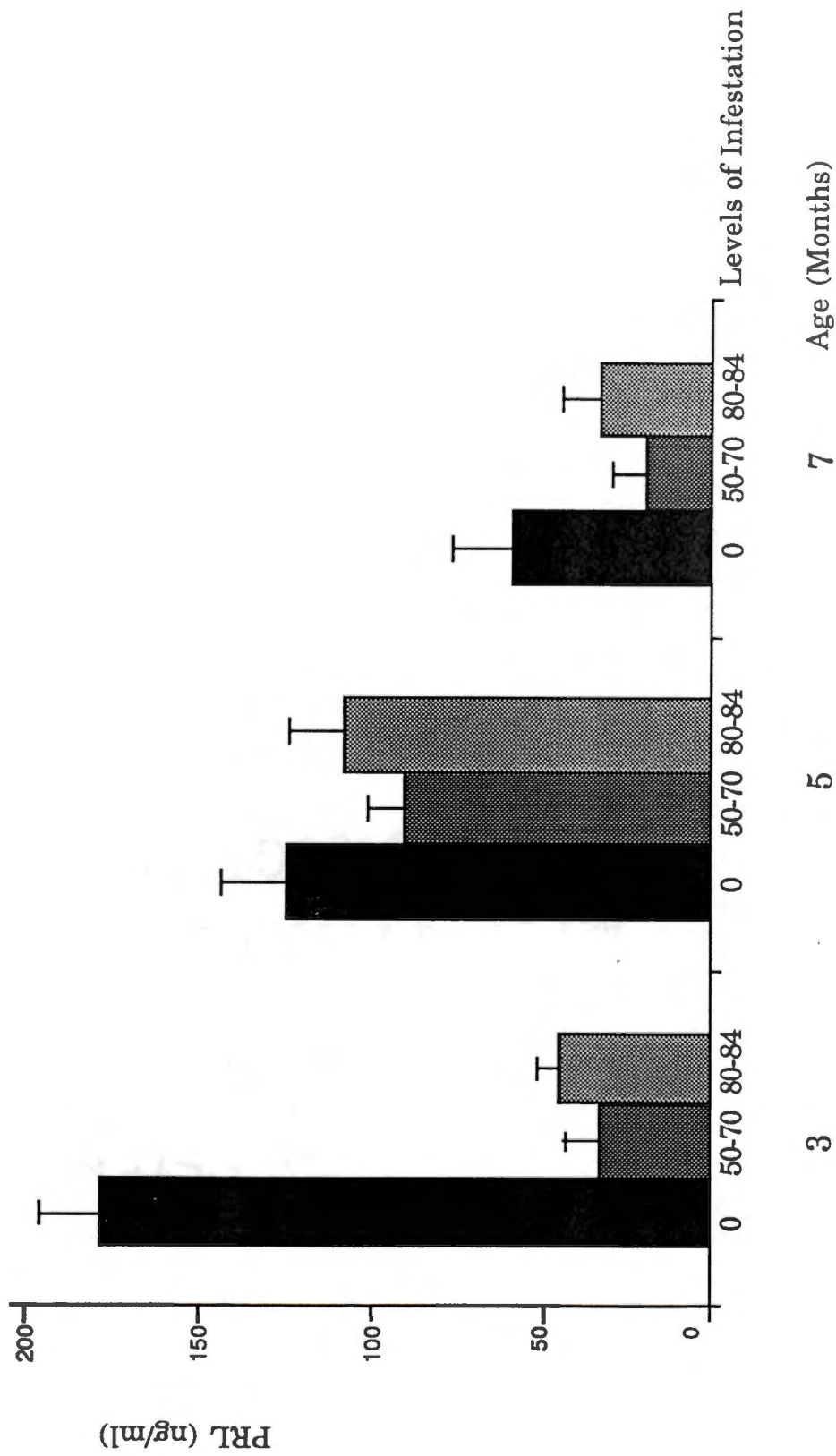


FIGURE 2. Effects of Fungal-Infested Fescue on PRL Levels at Different Ages in Zeranol Treated Bulls

Table IV. Effect of Fungal-infested Fescue on Testicular Weight and Development and Body Weight in Zeranol treated Bulls of 7 Months of Age\*

| Levels of Infestation | Number of Bulls | Testicular Weight (g)** | Total Populations of Sertoli Cells (x 10 <sup>9</sup> ) * | Type A-Spermatogonia/ 100 Sertoli Cells <sup>‡</sup> |
|-----------------------|-----------------|-------------------------|-----------------------------------------------------------|------------------------------------------------------|
| Control (0%)          | 5               | 19.9 ± 1.8              | 29.0 ± 1.9 <sup>a</sup>                                   | 5.3 ± 0.9                                            |
| Low (50-70%)          | 9               | 15.8 ± 0.8              | 20.4 ± 1.3 <sup>b</sup>                                   | 4.3 ± 1.0                                            |
| High (80-84%)         | 8               | 16.3 ± 1.7              | 19.8 ± 1.3 <sup>b</sup>                                   | 3.1 ± 0.6                                            |

\*Mean ± Standard Error

<sup>a,b</sup>Means in the same column with different letters are significantly different (P<0.01)

\*\*Control group was significantly different from the Low and high population (P<0.07)

<sup>‡</sup>No significant different between treatment groups (P>0.10)

BW ( $r = .52$ ;  $P < 0.01$ ). There were no correlations between gonadotropins and testicular data.

Study 2: Effects of Fungal-Infested Fescue Only on Testicular Development in the Bull.

Effects of a GnRH Challenge in Bulls at 3 Months of Age. Data from 1987 and 1988 bulls at 3-months of age were combined and analyzed for the basal hormonal secretions, but for the GnRH-stimulated hormones only 1988 bulls were included because the 1987 bulls were not injected with GnRH. Gonadotropins, PRL and androgens values were not significantly affected by the year ( $P > 0.10$ ). Gonadotropins, PRL and BW are listed in Table V.

Levels of FSH increased significantly in response to a GnRH challenge ( $P < 0.01$ ). Ingestion of fungal-infested fescue did not significantly affect either basal or stimulated FSH levels ( $P > 0.10$ ).

Luteinizing hormone release also increased after the GnRH challenge ( $P < 0.01$ ). There was no treatment effect on LH secretion ( $P > 0.10$ ). Combined PRL levels in low and high infestation groups were significantly lower ( $P < 0.07$ ) than that of the control group. The high infestation group had a significantly lower ( $P < 0.03$ ) PRL levels than the control group. PRL levels decreased as infestation levels increased.

Ingestion of fungal endophyte had no significant effects on body weight ( $P > 0.10$ ), but body weight was reduced by 10% in the treated population.

Table V. Effect of Fungal-infested Fescue on Basal and GnRH-induced Gonadotropins Secretions and PRL in Bulls of 3 Months of Age\*

| Levels<br>or<br>Infestation | Number<br>of<br>Bulls <sup>†</sup> | FSH (ng/ml) <sup>‡</sup> |                         | LH (ng/ml) <sup>‡</sup> |                         | PRL<br>(ng/ml) <sup>***</sup> | Body<br>Weight<br>(Kg) <sup>‡</sup> |
|-----------------------------|------------------------------------|--------------------------|-------------------------|-------------------------|-------------------------|-------------------------------|-------------------------------------|
|                             |                                    | Basal                    | Stimulated <sup>x</sup> | Basal                   | Stimulated <sup>x</sup> |                               |                                     |
| Control<br>(0%)             | 6                                  | 2.2 ± 0.2                | 3.7 ± 0.3               | 0.9 ± 0.1               | 12.2 ± 0.6              | 128.5 ± 15.6                  | 137 ± 11.9                          |
| Low<br>(50-70%)             | 19                                 | 2.8 ± 0.3                | 4.2 ± 0.3               | 0.8 ± 0.2               | 13.0 ± 2.2              | 99.8 ± 8.3                    | 123 ± 6.2                           |
| High<br>(80-84%)            | 12                                 | 2.4 ± 0.3                | 4.1 ± 0.3               | 0.9 ± 0.2               | 12.4 ± 2.6              | 78.9 ± 14.8                   | 122 ± 7.1                           |

\*Mean ± Standard Error

<sup>†</sup>1987 and 1988 Bulls

<sup>‡</sup>No Significant difference between treatment groups (P>0.10)

<sup>x</sup>1988 Bulls Only

<sup>\*\*\*</sup>Control group significantly greater than low and high population (P<0.07)

Testosterone and androstenedione data after a GnRH challenge at 3-months of age are listed in Table VI. The basal levels of T were similar ( $P>0.10$ ) among the three treatment groups. GnRH stimulated T levels in the control group were significantly higher ( $P<0.01$ ) than those of low and high groups. Treatment did not affect either basal or GnRH-stimulated  $\Delta 4A$  ( $P>0.10$ ).

Testicular Development in the Bull at 3 Months of Age. Testicular data for the 3-month old bulls are shown in Table VII. Testicular weight (TW) was not affected by the treatment ( $P>0.10$ ). A combination of control and low groups when contrasted against the high group was also not significantly different ( $P>0.10$ ), but TW in the high group was reduced by 39%. Total population of Sertoli cells was unaffected by treatment ( $P>0.10$ ). There was, however, a clear dose-related trend between SC and infestation levels. Sertoli-cell counts were reduced by 38% in the high infestation group. Number of type A- spermatogonia was not affected by the fungus ( $P>0.10$ ).

Prolactin was not correlated with either androgen. Prolactin was positively correlated with SC ( $r = .51$ ;  $P<0.02$ ). Testicular weight was positively correlated with SC ( $r = .90$ ;  $P<0.01$ ) and with basal T ( $r = .81$ ;  $P<0.01$ ).

Table VI. Effect of Fungal-infested Fescue on Basal and GnRH-induced Androgens Secretion in Bulls of 3 Months of Age\*

| Levels of Infestation | Number of Bulls <sup>†</sup> | T(ng/ml)           |                             | $\Delta$ 4A (ng/ml) <sup>‡</sup> |                         |
|-----------------------|------------------------------|--------------------|-----------------------------|----------------------------------|-------------------------|
|                       |                              | Basal <sup>‡</sup> | Stimulated <sup>*x</sup>    | Basal                            | Stimulated <sup>x</sup> |
| Control (0%)          | 6                            | 0.77 $\pm$ 0.5     | 4.13 $\pm$ 0.7 <sup>a</sup> | 0.34 $\pm$ 0.02                  | 0.56 $\pm$ 0.10         |
| Low (50-70%)          | 19                           | 0.72 $\pm$ 0.1     | 1.45 $\pm$ 0.5 <sup>b</sup> | 0.36 $\pm$ 0.04                  | 0.51 $\pm$ 0.07         |
| High (80-84%)         | 17                           | 0.65 $\pm$ 0.2     | 1.20 $\pm$ 0.3 <sup>b</sup> | 0.35 $\pm$ 0.02                  | 0.56 $\pm$ 0.06         |

\*Mean  $\pm$  Standard Error

<sup>†</sup>1987 and 1988 Bulls

<sup>‡</sup>No significant difference between treatment groups (P<0.10)

<sup>a,b</sup>Means in the same column with different letters are significantly different (P<0.01)

<sup>x</sup>1988 Bulls Only

Table VII. Effect of Fungal-infested Fescue on Testicular Weight and Development in Bulls of 3 Months of Age\*

| Levels of Infestation | Number of Bulls <sup>♦</sup> | Testicular Weight (g) <sup>■</sup> | Total Population of Sertoli (x 10 <sup>9</sup> ) <sup>■</sup> | Type A-Spermatogonia /100 Sertoli Cells <sup>■</sup> |
|-----------------------|------------------------------|------------------------------------|---------------------------------------------------------------|------------------------------------------------------|
| Control (0%)          | 3                            | 15.51 ± 6.52                       | 25.18 ± 7.8                                                   | 3.60 ± 0.9                                           |
| Low (50-70%)          | 11                           | 15.32 ± 3.90                       | 23.85 ± 5.6                                                   | 3.56 ± 0.8                                           |
| High (80-84%)         | 8                            | 9.28 ± 4.01                        | 15.56 ± 2.8                                                   | 4.19 ± 0.9                                           |

\*Mean ± Standard Error

♦1987 Bulls only

■No significant difference between groups (P>0.10)

Effects of Fungal-infested Fescue on Basal Hormonal Secretions at 6-

Months of Age. Basal hormonal profiles and BW are listed in Table VIII.

Values of the hormones are the means of two samples taken at an interval of 3 hours. There was no treatment effect on basal FSH concentrations ( $P>0.10$ ). Basal levels of FSH were not significantly different ( $P>0.10$ ) from those of the 3-month old bulls (Figure 3).

Basal LH levels were not affected by the treatment ( $P>0.10$ ). Luteinizing hormone levels of 6-month old bulls were significantly greater ( $P<0.01$ ) than those of the 3-month old bulls (Figure 4).

Prolactin levels in the control group were significantly ( $P<0.06$ ) higher than in low and high groups, but there was no dose-response relationship. Although nonsignificant, PRL levels of the 6-month old bulls were ( $P>0.10$ ) lower than those of 3-month old bulls (Figure 5).

Basal T levels were not affected by the treatment. Unlike the 3-month old levels, T levels in the control group were not elevated compared to the treated groups. There was no dose-response relationship. Basal T levels in the 6-month old bulls were significantly higher ( $P<0.01$ ) than those of 3-month old bulls (Figure 6). The  $\Delta 4A$  levels were not affected by the treatment ( $P>0.10$ ). The  $\Delta 4A$  levels in the 6-month old bulls were not significantly different ( $P>0.10$ ) from those of 3-month old bulls (Figure 7). Body weights were not affected by the fungus at this age.

Table VIII. Effect of Fungal-infested Fescue on Basal Hormonal Profiles and Body Weight in Bulls of 6 Months of Age \*

| Levels of Infestation | Number of Bulls <sup>♦</sup> | FSH (ng/ml) <sup>▢</sup> | LH (ng/ml) <sup>▢</sup> | PRL (ng/ml) <sup>***</sup> | T (ng/ml) <sup>▢</sup> | Δ4A (ng/ml) <sup>▢</sup> | Body Weight (Kg) <sup>▢</sup> |
|-----------------------|------------------------------|--------------------------|-------------------------|----------------------------|------------------------|--------------------------|-------------------------------|
| Control (0%)          | 3                            | 2.0 ± 0.6                | 1.1 ± 0.4               | 150.0 ± 83.0 <sup>a</sup>  | 1.1 ± 0.1              | 0.33 ± 0.1               | 212 ± 24.4                    |
| Low (50-70%)          | 11                           | 2.1 ± 0.2                | 1.2 ± 0.3               | 53.2 ± 12.6 <sup>b</sup>   | 1.3 ± 0.4              | 0.32 ± 0.1               | 216 ± 10.1                    |
| High (80-84%)         | 8                            | 2.5 ± 0.2                | 1.3 ± 0.3               | 54.8 ± 13.6 <sup>b</sup>   | 1.1 ± 0.3              | 0.27 ± 0.1               | 212 ± 12.1                    |

\*Mean ± Standard Error

♦1987 Bulls Only

▢No significant difference between treatment groups (P>0.10)

\*\*\*a,b Means in the same column with different letters are significantly different (P<0.06)

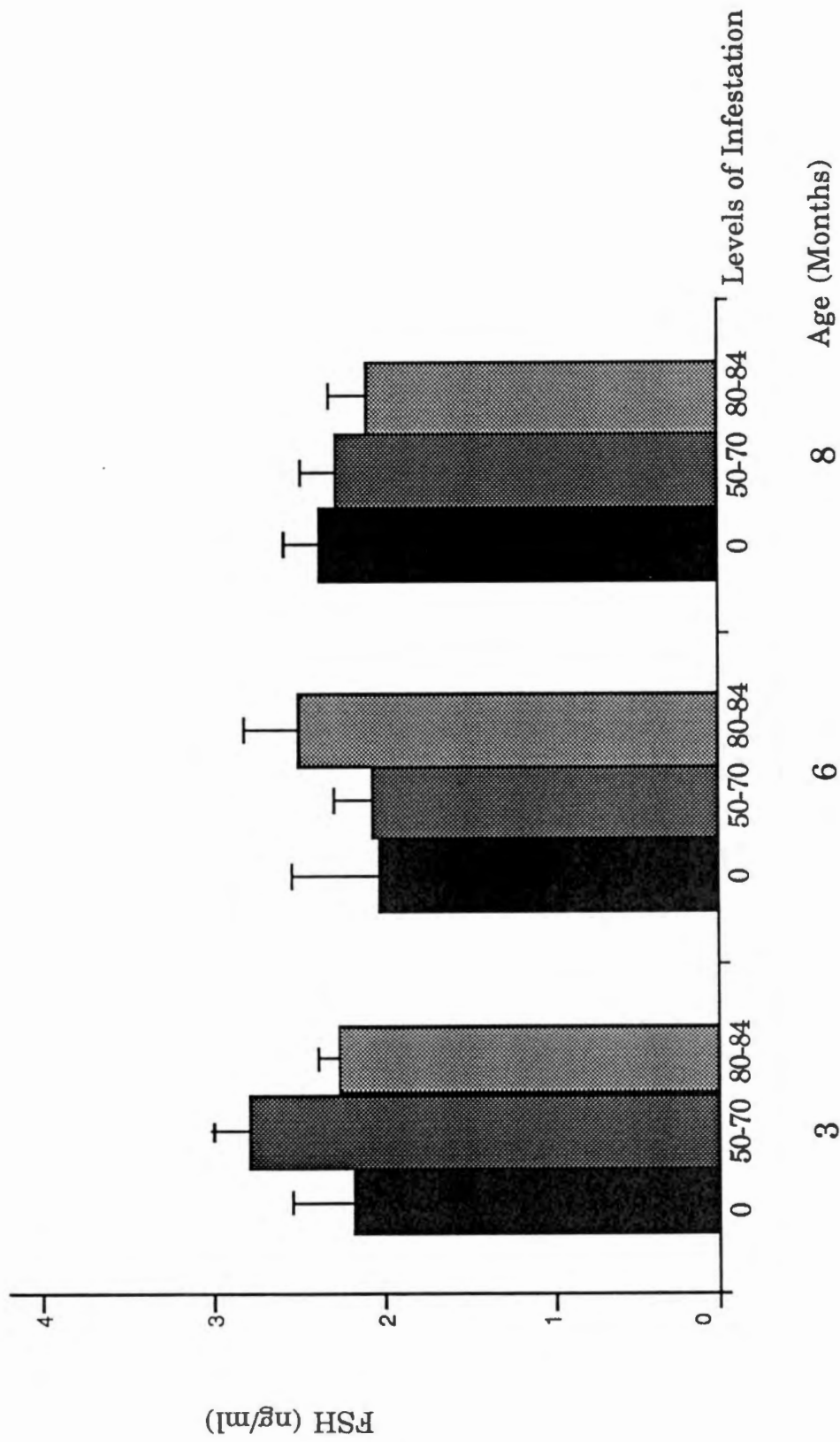


FIGURE 3. Effects of Fungal-Infested Fescue on Basal FSH Levels at Different Ages in the Bull

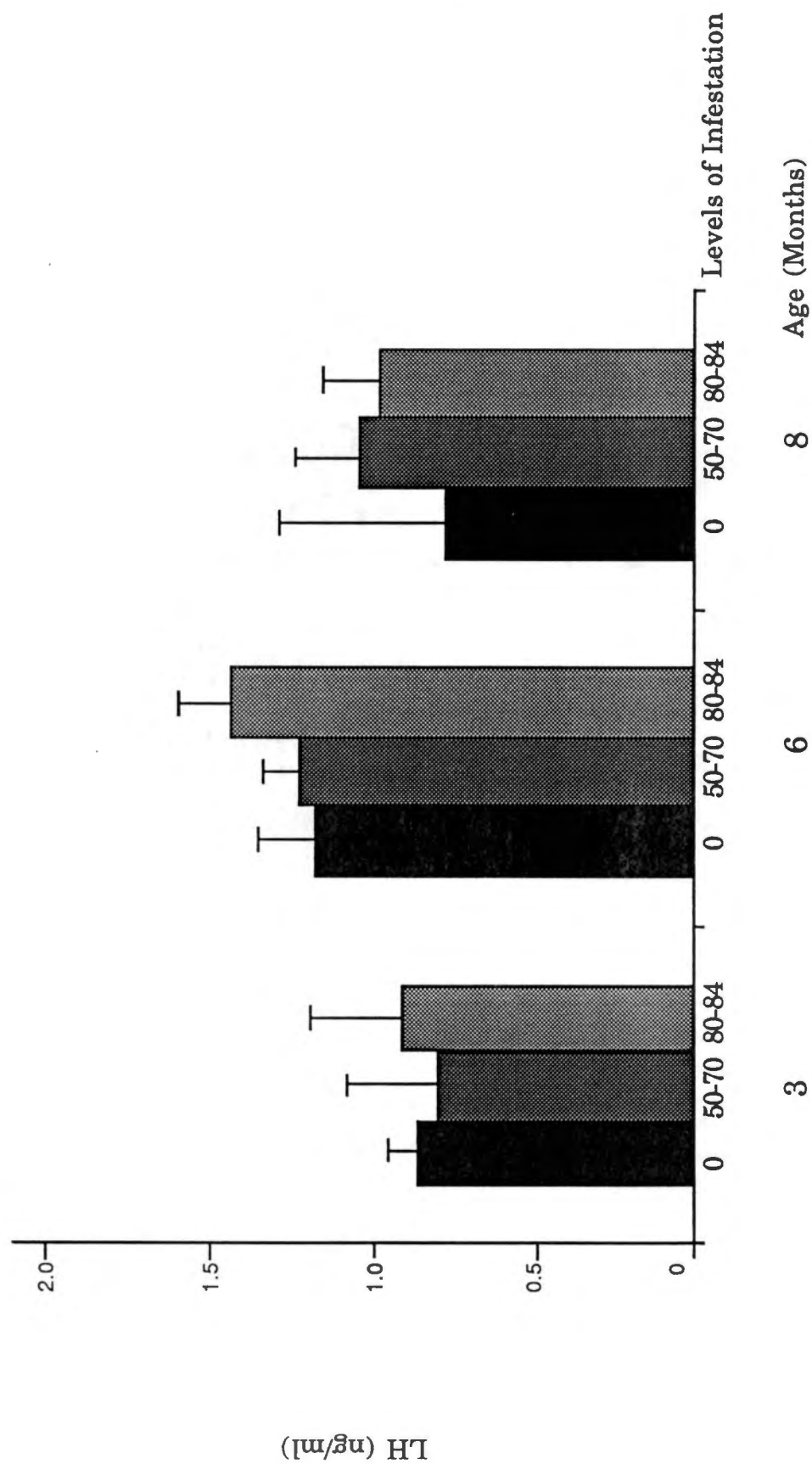


FIGURE 4. Effects of Fungal-Infested Fescue on Basal LH Levels at Different Ages in the Bull

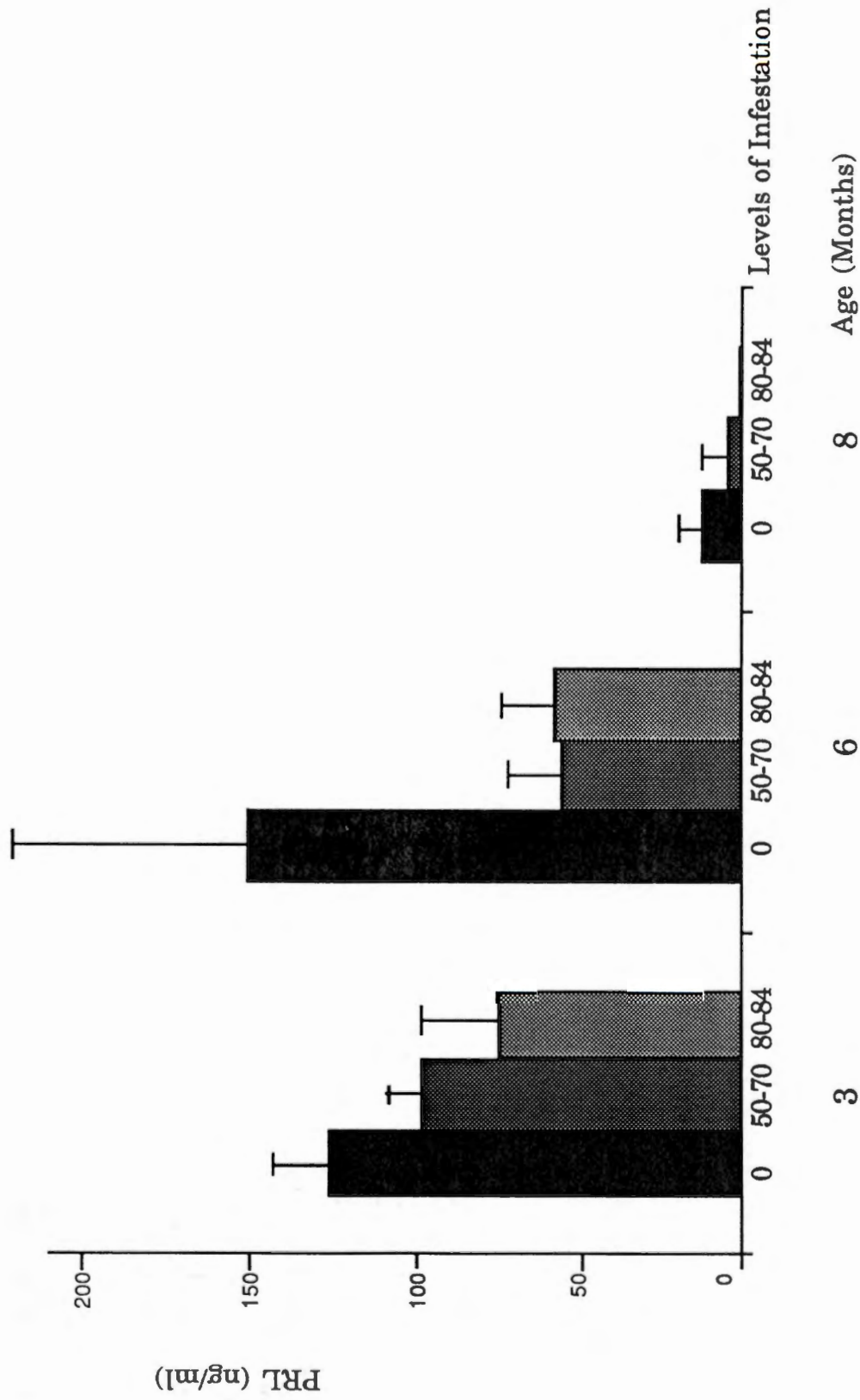


FIGURE 5. Effects of Fungal-Infested Fescue on PRL Levels at Different Ages in Bulls of Varying Ages

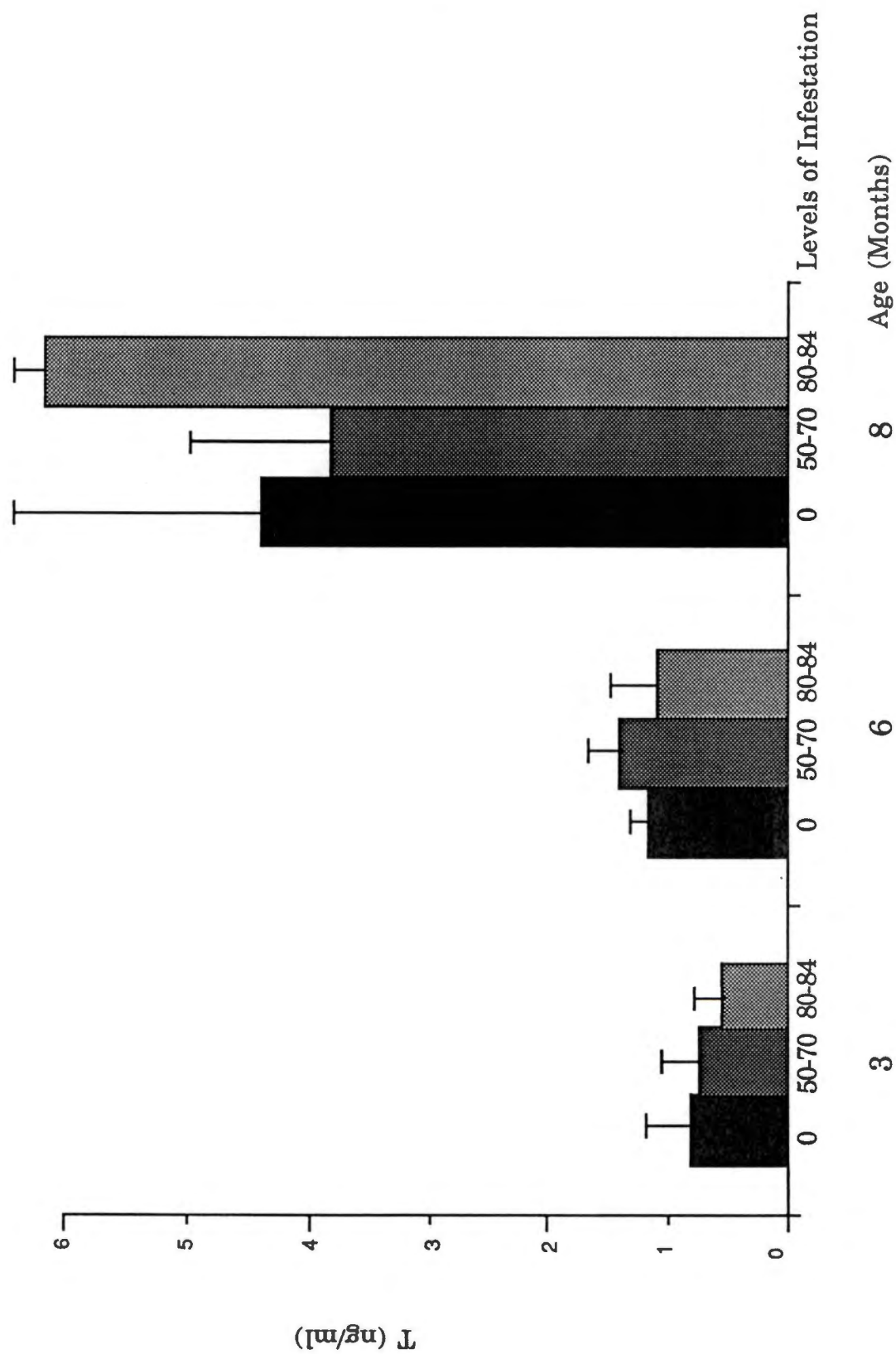


FIGURE 6. Effects of Fungal-Infested Fescue on Testosterone Secretion at Different Ages in the Bull

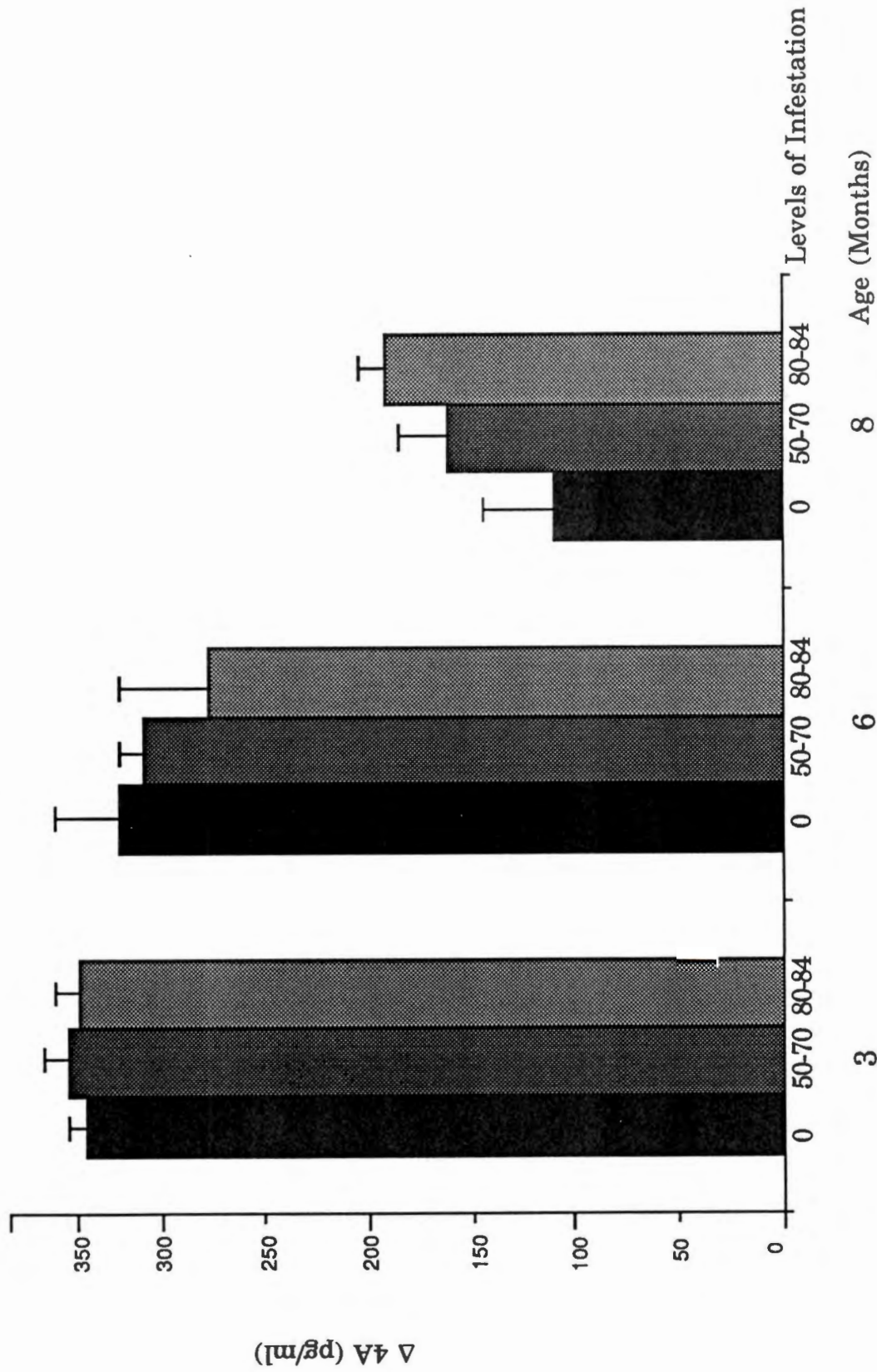


FIGURE 7. Effects of Fungal-Infested Fescue on Androstendione Levels at Different Ages in the Bull

Effects of a GnRH Challenge in the Bull at 8 Months of Age. A GnRH injection (10 ug) resulted in a significant increase in basal gonadotropin and androgens ( $P<0.01$ ). Gonadotropins and PRL levels are listed in Table IX. There was no significant treatment effect ( $P>0.10$ ) on basal or stimulated FSH levels. However, basal FSH levels decreased as infestation levels increased but the differences were not significant ( $P>0.10$ ). GnRH-stimulated FSH levels in the high infestation group were nonsignificantly lower than those of control and low groups.

Treatment also did not affect either basal or stimulated LH ( $P>0.10$ ). Both basal and stimulated LH levels in the high group were lower ( $P>0.10$ ) than that of the low group, indicating a possible dose-response relationship. LH levels were not significantly different from that of 3-month old bulls ( $P>0.10$ ), but they were significantly lower ( $P<0.05$ ) than that of 6-month old bulls (Figure 4).

Prolactin levels in both low and high infested groups were significantly lower ( $P<0.02$ ) than that of the control group. PRL levels decreased as infestation levels increased. In the 8-month old bulls, PRL levels were significantly lower ( $P<0.01$ ) than PRL levels in either the 3- or 6-month bulls (Figure 5).

Table X lists the means and standard errors of T and  $\Delta 4A$ . Fungal infestation did not significantly affect either basal or stimulated T ( $P>0.10$ ).

Table IX. Effect of Fungal-infested Fescue on PRL and Basal and GnRH-induced Gonadotropins Secretion in Bulls of 8 Months of Age \*

| Levels of Infestation | Number of Bulls <sup>♦</sup> | FSH (ng/ml) <sup>□</sup> |            | LH (ng/ml) <sup>□</sup> |            | PRL (ng/ml)**            |
|-----------------------|------------------------------|--------------------------|------------|-------------------------|------------|--------------------------|
|                       |                              | Basal                    | Stimulated | Basal                   | Stimulated |                          |
| Control (0%)          | 3                            | 2.5 ± 0.1                | 4.5 ± 1.4  | 0.8 ± 0.3               | 24.1 ± 6.8 | 5.57 ± 3.10 <sup>a</sup> |
| Low (50-70%)          | 8                            | 2.4 ± 0.1                | 4.6 ± 0.5  | 0.9 ± 0.1               | 30.6 ± 1.9 | 1.69 ± 1.02 <sup>b</sup> |
| High (80-84%)         | 9                            | 2.3 ± 0.1                | 4.3 ± 0.3  | 0.9 ± 0.1               | 23.4 ± 4.7 | 0.13 ± 0.06 <sup>b</sup> |

\*Mean ± Standard Error

♦1988 Bulls only

□No significant difference between treatment groups (P>0.10)

\*\*<sup>a,b</sup>Means in the same column with different letters are significantly different (P<0.02)

Table X. Effect of Fungal-infested Fescue on Basal and GnRH-induced Androgens Secretion in Bulls of 8 Months of Age\*

| Level of Infestation | Number of Bulls <sup>†</sup> | T(ng/ml) <sup>‡</sup> |                | $\Delta$ 4A (ng/ml) <sup>‡</sup> |                 |
|----------------------|------------------------------|-----------------------|----------------|----------------------------------|-----------------|
|                      |                              | Basal                 | Stimulated     | Basal                            | Stimulated      |
| Control (0%)         | 3                            | 4.4 $\pm$ 2.8         | 10.3 $\pm$ 2.3 | 0.12 $\pm$ 0.04                  | 0.22 $\pm$ 0.04 |
| Low (50-70%)         | 8                            | 3.7 $\pm$ 1.2         | 10.9 $\pm$ 2.3 | 0.16 $\pm$ 0.05                  | 0.28 $\pm$ 0.03 |
| High (80-84%)        | 9                            | 6.1 $\pm$ 1.0         | 11.7 $\pm$ 0.6 | 0.19 $\pm$ 0.03                  | 0.29 $\pm$ 0.02 |

\*Mean  $\pm$  Standard Error

<sup>†</sup>1988 Bulls

<sup>‡</sup>No significant difference between treatment groups (P>0.10)

High infestation group had the highest basal and stimulated T concentrations and the low group had the lowest basal levels. Basal T in the 8-month old bulls were significantly higher ( $P < 0.01$ ) than those of 3- and 6-month old animals (Figure 6). The  $\Delta 4A$  levels were not affected by the treatment ( $P > 0.10$ ). Again  $\Delta 4A$  levels in the high infestation group were the highest among treatment groups yet the difference was not significant ( $P > 0.10$ ). Basal  $\Delta 4A$  levels in the 8-month old bulls were significantly lower ( $P < 0.01$ ) than that of 6-month old and of 3-month old bulls (Figure 7). Hormonal profiles were not affected by the age x treatment interaction.

Basal LH levels were positively correlated with basal T ( $r = .59$ ;  $P < 0.01$ ) and with basal  $\Delta 4A$  ( $r = .47$ ;  $P < 0.03$ ).

Testicular Development in the Bull at 8 Months of Age. Body weights in the control group were significantly higher ( $P < 0.02$ ) than the treated population groups (Table XI). Within the endophyte infested groups, the high infestation group was heavier ( $P < 0.05$ ) than the low infestation group. Testicular weights were also reduced in the low and high infestation groups, but the difference was not statistically significant ( $P > 0.10$ ).

Number of A-spermatogonia was not affected by fungal infestation ( $P > 0.10$ ). Total population of Sertoli cells in the control group was significantly higher ( $P < 0.05$ ) than that of the low group, but not from that of

Table XI. Effect of Fungal-infested Fescue on Body Weight, Testicular Weight, No. of A-Spermatogonia and Sertoli Cells in Bulls of 8 Months of Age\*

| Levels of Infestation | Number of Bulls♦ | Body Weight (Kg)*        | Testicular Weight (g)♠ | No of A-Spermatogonia / 100 Sertoli Cells♠ | Total Population of Sertoli Cells (x 10 <sup>9</sup> )** |
|-----------------------|------------------|--------------------------|------------------------|--------------------------------------------|----------------------------------------------------------|
| Control (0%)          | 3                | 299 ± 25.3 <sup>a</sup>  | 163 ± 6.3              | 6.70 ± 1.9                                 | 32.01 ± 1.5                                              |
| Low (50-70%)          | 8                | 227 ± 13.3 <sup>b</sup>  | 118 ± 18.6             | 5.57 ± 1.1                                 | 23.03 ± 2.5                                              |
| High (80-84%)         | 9                | 262 ± 9.2 <sup>a,b</sup> | 135 ± 13.7             | 6.50 ± 0.8                                 | 26.26 ± 1.9                                              |

\*Mean ± Standard Error

♦1988 Bulls

\*<sup>a,b</sup>Means in the same column with different letters are significantly different (P<0.01)

♠No significant difference between treatment groups (P>0.10)

\*\*Control group was significantly higher than the treated population (P<0.05)

high group ( $P>0.10$ ). However, when combined the low and high populations were significantly lower than the control group ( $P<0.05$ ).

Histological evaluation of the seminiferous tubules are shown in Table XII. Controls had the lowest percentage of cross sections without germ cells, with spermatogonia only and where spermatocytes were the most advanced germ cells. However, the difference was not significant. When these three criteria were pooled, the difference was significant ( $P<0.04$ ). Round and elongated spermatids in the control were higher than the treated population ( $P>0.10$ ), but when they were pooled they were significantly higher ( $P<0.06$ ) than that of the treated groups. The control group had higher ( $P<0.03$ ) cross-sections with mature spermatids than did the other groups.

Sertoli cell numbers were positively correlated with basal T ( $r = .42$ ;  $P<0.07$ ), with  $\Delta 4A$  ( $r = .45$ ;  $P<0.05$ ) and with testicular weight ( $r = .65$ ;  $P<0.01$ ). Testicular weight was also correlated with basal T ( $r = .64$ ;  $P<0.01$ ) and stimulated T ( $r = .49$ ;  $P<0.02$ ), and with basal  $\Delta 4A$  ( $r = .66$ ;  $P<0.01$ ). Basal FSH was positively correlated with the presence of mature spermatids ( $r = .53$ ;  $P<0.01$ ).

Hormonal Secretions After GnRH Challenge at 12-Months of Age in the Bull. Basal and stimulated FSH were not affected ( $P>0.10$ ) by fungal infestation when the animals were removed from respective treatment pastures

Table XII. Effect of Fungal-infested Fescue on Testicular Development in Bulls of 8 Months of Age \*

| % Cross Sections With: |                              |                            |                                 |                                              |                               |                                   |                                 |
|------------------------|------------------------------|----------------------------|---------------------------------|----------------------------------------------|-------------------------------|-----------------------------------|---------------------------------|
| Levels of Infestation  | Number of Bulls <sup>†</sup> | No Germ Cells <sup>‡</sup> | Spermatogonia Only <sup>‡</sup> | Spermatogonia and Spermatocytes <sup>‡</sup> | Round Spermatids <sup>‡</sup> | Elongated Spermatids <sup>‡</sup> | Mature Spermatids <sup>**</sup> |
|                        |                              | 1                          | 2                               | 3                                            | 4                             | 5                                 | 6                               |
| Control (0%)           | 3                            | 0.4 ± 0.4                  | 1.3 ± 0.6                       | 6.9 ± 5.9                                    | 34.8 ± 4.1                    | 38.9 ± 3.3                        | 17.7 ± 9.6 <sup>a</sup>         |
| Low (50-70%)           | 8                            | 14.8 ± 5.4                 | 13.2 ± 3.6                      | 20.3 ± 5.3                                   | 27.3 ± 4.8                    | 21.2 ± 5.3                        | 3.8 ± 1.2 <sup>b</sup>          |
| High (80-84%)          | 9                            | 5.8 ± 4.7                  | 12.3 ± 2.2                      | 25.6 ± 7.3                                   | 26.1 ± 4.5                    | 25.5 ± 6.4                        | 4.8 ± 1.8 <sup>b</sup>          |

<sup>†</sup>Mean ± Standard Error

<sup>‡</sup>1988 Bulls

<sup>‡</sup>No significant difference between treatment groups (P>0.10)

<sup>\*\*</sup>Means in the same column with different letters are significantly different (P<0.03).

1, 2, 3 when pooled, the control was significantly lower than the treated groups (P<0.04). 4 and 5 when pooled the control was significantly higher than the treated groups (P<0.06).

at 8 months of age (Table XIII). Basal FSH levels in 12-month old bulls were significantly higher ( $P < 0.01$ ) than that of 3-, 6- and 8-months bulls.

The groups did not differ significantly in either basal or stimulated LH levels ( $P > 0.10$ ). However, the treated groups were more responsive to GnRH.

Prolactin levels were not significantly different among treatment groups ( $P > 0.10$ ).

Neither basal nor stimulated T were significantly different ( $P > 0.10$ ) among the three groups (Table XIV). The high group, however, had the highest basal and stimulated concentrations of T. Unlike the T,  $\Delta 4A$  levels in the control group were the highest among treatments, yet the difference was not significant ( $P > 0.10$ ). Intergroup differences in testicular weights were not significant, ( $P > 0.10$ ), but it is of interest that testis weights in the treated groups were less than those of the control group.

Table XIII. Basal and GnRH-induced Secretion of Gonadotropins and PRL  
in Bulls of 12 Months of Age <sup>‡</sup>

| Levels of Infestation | Number of Bulls <sup>†</sup> | FSH (ng/ml) <sup>‡</sup> |            | LH (ng/ml) <sup>‡</sup> |            | PRL (ng/ml) <sup>‡</sup> |
|-----------------------|------------------------------|--------------------------|------------|-------------------------|------------|--------------------------|
|                       |                              | Basal                    | Stimulated | Basal                   | Stimulated |                          |
| Control (0%)          | 3                            | 3.1 ± 0.4                | 3.8 ± 0.3  | 0.8 ± 0.1               | 2.7 ± 1.0  | 121.3 ± 24               |
| Low (50-70%)          | 11                           | 3.1 ± 0.2                | 4.1 ± 0.3  | 0.9 ± 0.1               | 6.7 ± 1.5  | 154.9 ± 29               |
| High (80-84%)         | 8                            | 3.0 ± 0.3                | 3.5 ± 0.3  | 0.8 ± 0.3               | 4.7 ± 1.2  | 94.0 ± 16                |

<sup>‡</sup>Mean ± Standard Error

<sup>†</sup>1987 Bulls

<sup>‡</sup>No significant difference between treatment groups (P>0.10)

Table XIV. Testicular Weight and Basal and GnRH-induced Androgens Secretion in Bulls of 12 Months of Age\*

| Levels of Infestation | Number of Bulls <sup>†</sup> | T (ng/ml) <sup>‡</sup> |            | $\Delta$ 4A (ng/ml) <sup>‡</sup> |             | Testicular Weight (g) <sup>‡</sup> |
|-----------------------|------------------------------|------------------------|------------|----------------------------------|-------------|------------------------------------|
|                       |                              | Basal                  | Stimulated | Basal                            | Stimulated  |                                    |
| Control (0%)          | 3                            | 2.4 ± 0.7              | 13.8 ± 4.8 | 0.14 ± 0.04                      | 0.39 ± 0.10 | 381 ± 36                           |
| Low (50-70%)          | 11                           | 2.2 ± 0.4              | 13.1 ± 1.6 | 0.12 ± 0.01                      | 0.36 ± 0.04 | 335 ± 30                           |
| High (80-84%)         | 8                            | 2.8 ± 0.4              | 14.1 ± 2.8 | 0.11 ± 0.01                      | 0.35 ± 0.06 | 362 ± 12                           |

\*Mean ± Standard Error

<sup>†</sup>1987 Bulls

<sup>‡</sup>No significant difference between treatment groups (P>0.10)

## CHAPTER V

## DISCUSSION

Effect of Fungal-infestation on PRL Release.

PRL levels were significantly reduced ( $P < 0.01$ ) in bulls grazing fungus infested fescue regardless of whether they received zeranol or not at three months of age (Tables I and V).

At 5 months of age, PRL release was reduced by fungal infestation in the Zeranol-treated bulls but the difference was not significant (Table II). However, PRL levels were significantly reduced ( $P < 0.07$ ) in the nonzeranol treated bulls at 6 months of age (Table VIII). This might be related to differences in year of study since percentage of infected plants in some pastures increased during the 3 years of study. This discrepancy in PRL release also could be attributed to the supplementation of late summer pastures with low fungal-infestation hay. This would dilute the dose of ergot alkaloids.

Prolactin secretions were significantly ( $P < 0.05$ ) reduced by fungal infestation in both the zeranol and the nonzeranol treated bulls at 7 and 8 months of age respectively (Tables III and IV). However, in the nonzeranol treated bulls the magnitude of PRL suppression was higher than that of the zeranol treated bulls. This might be attributed to environmental factors. The sampling time was in September for the zeranol-treated bulls and in November for the nonzeranol treated bulls. It has been reported that photoperiod affects

PRL levels in the bovine (Schams, 1972). Hence, the reduced light of November interacted with fungus to greatly suppress PRL in the nonzeranol treated bulls.

Hurely et al. (1981) and Evans et al. (1988) reported a reduction in PRL release in calves fed infected fescue. Ergot alkaloids that were associated with infected fescue have been shown to stimulate dopamine receptors, reduce dopamine turnover and increase dopamine release (Fuxe et al., 1978; Fluckiger, 1980; Porter et al., 1985; Henson et al., 1987). Thus, the decrease in PRL could be attributed to the activity of fungus-associated ergot alkaloids. In the zeranol-treated bulls, PRL was more severely suppressed by the fungus treatment than in the nonzeranol treated bulls at 3 months of age. Zeranol is an estrogen agonist (Katzenellenbogen et al., 1979). Active immunization against estradiol in the ram resulted in a reduction in dopamine release (Jenkins et al., 1986). This indicates that estradiol has stimulatory effects on dopamine release. Thus, both ergot alkaloids and the zeranol may have acted together on the pituitary to suppress PRL release. The significant interaction between age x treatment that affected PRL secretions in the zeranol-treated bulls might be due to pasture supplementation with hay during the summer. This might have diluted the ergot alkaloids and consequently affected PRL levels at 5 months of age.

Body weights were reduced in both the zeranol and nonzeranol implanted bulls as a result of grazing endophyte infested fescue pasture.

However, the zeranol implanted bulls were more severely affected by the fungus than nonzeranol treated bulls. This might be related to the additive effects of zeranol and the fungus. Endogenous PRL is a growth stimulant (Nicoll et al., 1980). Thus, reduction in PRL secretions in the treated population might have contributed to the body weight reduction.

#### Effects of Fungal-infestation on FSH Secretion.

Fungal infestation did not significantly affect either basal or GnRH stimulated FSH secretion in either the presence or absence of zeranol in bulls of 3 months of age (Tables I and V).

However, in contrast to what was seen at 3 months, fungal ingestion slightly diminished basal FSH and significantly reduced stimulated FSH levels ( $P < 0.06$ ) in zeranol treated bulls of 5 months of age (Table II). Basal FSH secretion in the nonzeranol treated bulls was not affected by the fungus at 6 months of age (Table VIII).

Basal FSH secretion was also reduced in a dose-dependent manner in zeranol-treated bulls of 7 months of age (Table III) but the differences were not significant ( $P > 0.10$ ). A similar condition existed in the nonzeranol treated bull of 8 months of age (Table IX), and added to this was a nonsignificantly reduced GnRH stimulated secretion of FSH in the high-fungus group (Table IX). Taken together, these data suggest that fungus-associated alkaloids may diminish FSH secretion in the prepuberal bull. Fungal infestation did not

permanently affect FSH release since, FSH levels were not diminished in the 12 month old bull (Table XIII).

This decrease in stimulated FSH secretion in bulls treated with fungus and zeranol could be due to the effects of ergot alkaloids on GnRH secretion. Wuttke et al. (1971) reported that ergot alkaloid might act on the hypothalamus to suppress GnRH release. The reason that zeranol-implanted bulls were affected more severely by the fungus could be due to the estrogenic effects of zeranol on FSH release. Estrogen is a potent FSH regulator in the bull (Schanbacher, 1987a). Thus, ergot alkaloids might have added to the negative effects of  $E_2$  on FSH suppression.

The reason that there was no fungus effects at 3 months of age could be due to the short time exposure of the bulls to the fungus or age-related sensitivity to the fungus.

GnRH-stimulated FSH secretion in the zeranol treated bulls was higher than that of the nonzeranol treated bulls. Deschamps et al. (1987) reported that bulls implanted with zeranol at birth showed a significant increase in GnRH-stimulated FSH release at 4 months of age. Estrogen is a potent suppressor of FSH release in the bull (Schanbacher et al., 1987a). Therefore, this suppression in FSH release would increase the pituitary content of FSH resulting in an increase in GnRH-stimulated FSH.

Effects of Fungal Infestation on LH Secretion.

At 3 months of age, there was a significant reduction in both basal ( $P < 0.01$ ) and stimulated ( $P < 0.06$ ) LH secretion in the zeranol plus fungus treated bulls (Table I). On the other hand, neither basal nor stimulated LH in the nonzeranol treated bulls were affected by the ingestion of fungus (Table V). As was the case at 3 months, basal LH levels were significantly reduced ( $P < 0.08$ ) at 7 months in the zeranol plus fungus treated bulls (Table III). Thus, again, fungus plus zeranol resulted in reduced gonadotropins release.

LH secretion was not affected by the fungus at 6 months of age in the nonzeranol treated bulls (Table VIII) but GnRH stimulated LH release may have been reduced in the nonzeranol bulls of 8 months of age (Table IX). Thus fungus alone may also have a measurable effect on LH synthesis and secretion.

Ergot alkaloids can suppress LH release through GnRH suppression in the rat (Wuttke et al., 1971). Feeding infected fescue has been shown to increase dopamine levels (Henson et al., 1987), and dopamine infusion inhibited the increase in LH release that follows a  $\text{PGF}_2\alpha$  injection in the ewe (Deaver and Daily, 1982). Thus, the decrease in LH secretion could be attributed to dopaminergic factors produced by the fungus. Also, active immunization against estradiol resulted in a decrease in dopamine levels in the ram (Jenkins et al., 1986). This indicates a stimulatory effect of estrogen on dopamine secretion and this could be contributed to the suppression of LH in the zeranol-treated bulls. In the zeranol-implanted bulls, the hypothalamus and pituitary

were under suppressive effects of zeranol. Thus, zeranol and ergot alkaloids acted together to suppress both basal and stimulated LH.

Effects of Fungal Infestation on Androgen Secretions in Nonzeranol Treated Bulls.

Basal T production was nonsignificantly reduced by the fungus in bulls of 3 months of age but GnRH-stimulated T release was greatly reduced ( $P < 0.05$ ) (Table VI). Neither basal nor stimulated androstenedione secretion was, however, affected. Reduced T secretion in the treated groups could be a result of a fungus induced delay of Leydig cell differentiation but the absence of an effect on  $\Delta 4A$  is not readily explained. Testosterone secretion was not affected in 8-month-old bulls (Table X), which supports delay in Leydig cell differentiation as an explanation for the effect seen at 3 months. Both basal and stimulated  $\Delta 4A$  secretion were nonsignificantly higher in the treated 8-month-old bulls than they were in the control bulls (Table X); but, again, what this may suggest as to cellular effects is not clear.

In addition to the delay in Leydig cells differentiation, prolactin could also be a factor involved in the response seen at 3-months of age since PRL was reported to increase LH receptors in the rodent's testis (Aragona et al., 1977) and to potentiate the effect of LH on T production in mice (Bartke, 1971b). Long-day-induced increases in PRL levels in calves have been shown to increase T levels (Stanisiewski et al., 1987). They attributed this increase in T to an

increase in PRL. However, treatment of mature bulls with bromocriptine significantly reduced PRL levels without affecting T production (Stolla et al., 1979). This indicates that PRL may have age-related stimulatory effects on T production in the bull and this could explain the lack of a PRL effect on T secretion at 6 and 8 months.

#### Effects of Fungal Infestation on Testicular Development.

Sertoli-cell number was suppressed by 31% in the zeranol-implanted bulls ( $P < 0.01$ ) and by 23% in the nonzeranol treated bulls ( $P < 0.05$ ) at 7 and 8 months respectively (Tables IV and XI) when bulls grazed infested fescue. The greater decrease in SC in the zeranol-treated bulls could be attributed to the added effects of zeranol suppression of gonadotropins secretion.

In the nonzeranol treated bulls, the total population of SC were nonsignificantly ( $P > 0.10$ ) reduced by 5% in the low group and by 38% in the high infestation group at 3 months of age (Table VII). Sertoli cell multiplication is affected by gonadotropins and nutrition (Hochereau-de Reviers et al., 1987). In some instances gonadotropins were reduced by the fungus in this study. This might have contributed to decrements in SC counts. Also, the reduction in PRL by the fungal infestation might have contributed to an inhibition in Sertoli cell mitosis. Birth weight of calves born to dams grazing fungus-infected fescue was lower than that of non-infected fescue (Bolt and

Bond, 1986) and most of SC division occur during fetal life (Orth, 1982).

Thus, prenatal effects of infected-fescue on SC cannot be ignored.

Number of A-spermatogonia was not affected by fungal infestation at 3 months of age (Table VII). Also spermatogonia number was not influenced by the treatment in either the zeranol or nonzeranol implanted bulls (Tables IV, and XI) at 7 or 8 months of age, respectively. However, in the zeranol treated bulls, there was a tendency for the number of A-spermatogonia to decrease as infestation increased.

When number of cross sections without germ cells, with spermatogonia only and with spermatocytes as the most advanced germ cell were pooled, the treated groups were significantly ( $P < 0.04$ ) higher than the control group (Table XII). At the same time, cross sections with more advanced germ cells (round and elongated spermatids) were significantly more numerous ( $P < 0.06$ ) in the control than that in the treated population (Table XII). These data suggest that spermatogenesis and testicular development are delayed by the fungal infected fescue. Zavos et al. (1990) reported a decrease in daily sperm production in mice fed infected fescue. This may suggest a possible direct effects of ergot alkaloids on spermatogenesis. Barenton and Pelleter (1980) reported that bromocriptine treatment to rams resulted in a delay in the beginning of testicular growth. This might be attributed to ergot alkaloids effects on testicular growth or as a result of PRL reduction.

At 7 months, TW were significantly ( $P < 0.07$ ) reduced (20%) by the fungal infestation in zeranol-treated bulls (Table IV.). Testis weights at 8 months were also reduced (22%) in the nonzeranol treated bulls (Table XI) but differences were not significant ( $P > 0.10$ ). This might be related to the high variability within animals in the nonzeranol treated bulls (Table IX) and the added gonadotropins suppression in the zeranol-treated bulls.

Testicular weights in the high infestation group were reduced to 60% of the control value yet the difference was not significant because of high variability within groups at 3 months of age in the nonzeranol bulls (Table VII). These findings are also supported by Zavos et al. (1986 and 1990) who observed a reduction in testicular weight in rats and mice fed infected-fescue seed. They attributed this reduction to the toxic factors present in infected seed. However, Evans et al. (1988) have reported that feeding fungus infected hay to Holstein bulls from 2 to 13 months of age did not affect testicular weights. In the present study, bulls were born to dams grazing on infected fescue. Therefore, the animals were exposed to the fungus during the fetal period of development. Also, in the study of Evans, et al (1988), bulls were supplemented with grains and this might have reduced the toxic effects associated with tall fescue.

Testicular weights were nonsignificantly reduced by 12% in the low group and by 5% in the high infestation group at 12 months of age. This

might be related to decrements in Sertoli cells population as seen in the 8 month old bulls (Table XI).

It is concluded that feeding infested fescue significantly reduced PRL levels in the bull. Fungal infestation significantly affected gonadotropin secretion in the zeranol treated bull and They also reduced in the nonzeranol treated bull, but the difference was not significant. Testosterone secretion was reduced in the treated bulls in the early prepubertal period. Testicular weights and Sertoli cell number were decreased by fungal ingestion both with and without zeranol. Also, testicular development was delayed by fungus as evidenced by the histological evaluation. Decrements in number of Sertoli cells could lead to a permanent impairment of testicular function.

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## APPENDICES

APPENDIX 1  
LAWYER AND  
COUNCIL

## APPENDIX 1

## APPENDIX 1

## RADIOIODINATION PROCEDURES

LH: Chloramine-T Technique (Brown et al., 1983)

Protein: LER 1072-2

Materials needed:

Plastic 1.5 ml Microcentrifuge vial  
0.5 M Phosphate buffer (pH 7.5) (PB)  
0.05 M Phosphate buffer (pH 7.5) (PB)  
Chloramine-T ( $1\mu\text{g}/\mu\text{l}$  in 0.05 M PB)  
Sodium Metabisulfite ( $2\mu\text{g}/\mu\text{l}$  in 0.05 M PB)  
Bovine Serum Albumin (BSA)  
G 25-M Sephadex column

Proceed in a step-wise addition:

1.  $2.5\ \mu\text{g}$  LER 1072-2 in  $25\ \mu\text{l}$  of 0.05 M PB (pH 7.5)
2. Add  $500\ \mu\text{Ci}$   $^{125}\text{I}$  (Amersham Co.)
3. Add  $15\ \mu\text{g}$  of Chloramine-T
4. Mix gently, for 1 minute
5. Add  $60\ \mu\text{l}$  of Sodium Metabisulfite
6. Add  $100\ \mu\text{l}$  of eluent buffer (0.05 M PB + 0.1% BSA)
7. Load onto a G 25-M Sephadex column for separation
8. Elute with 0.05 M PB + 0.1% BSA

FSH: Iodogen Technique (Bolt and Rollins, 1983)

Protein: USDA bFSH-BP3

**Materials needed:**

Wheaton 1.5 ml flat bottom serum vial, acid washed \*

0.5 M PB (pH 7.4)

0.25 M PB (pH 7.4)

0.50 M PB (pH 7.4)

0.05 M Sodium Diphosphate solution

Sodium Metabisulfite (25 $\mu$ g/ml in 0.25 M PB)

Potassium Iodide (1 mg/ml in 0.5 M Sodium Diphosphate)

BSA

G 25-M Sephadex column

\*Plate the reaction vessel with a solution of iodogen in chloroform at a concentration of 2  $\mu$ g/50  $\mu$ l.

1. 5 mg in 5 ml; iodogen/chloroform

2. 40  $\mu$ l of #1 on 940  $\mu$ l of chloroform

Proceed in a step-wise addition:

1. 2.5  $\mu$  bFSH-BP3 in 35  $\mu$ l of 0.5 M PB

2. Add 400 uCi 125-I (Amersham Co.)

3. Vortex for 10 minutes (thorough, but gentle)

4. Add 200  $\mu$ l Sodium Metabisulfite

5. Add 200  $\mu$ l Potassium Iodide

6. Load on G 25-M Sephadex column for separation

7. Elute with 0.05 M PB + 0.1% BSA

Prolactin: Iodogen Technique (Bolt and Rollins, 1983, modified)

Protein: USDA-bPRL-I-1

**Materials needed:**

Wheaton 1.5 ml flat bottom serum vial, acid washed\*

0.5 M PB (pH 7.4)

0.25 M PB (pH 7.4)

0.05 M PB (pH 7.4)

0.5 M Sodium Diphosphate solution

Sodium Metabisulfite (25  $\mu$ g/ml in 0.25 M PB)

Potassium Iodide (1 mg/ml in 0.5 M Sodium

Diphosphate)

BSA

G 25-M Sephadex Column

\* Plate the reaction vessel with a solution of iodogen in chloroform at a concentration of 4  $\mu$ g/50  $\mu$ l.

1. 50 mg in 5 ml; iodogen/chloroform
2. 80  $\mu$ l of #1 on 900  $\mu$ l of chloroform

Proceed in a step-wise addition:

1. 5.0  $\mu$ g bPRL-I-1 in 35  $\mu$ l of 0.5 M PB (pH 7.4)
2. Add 400  $\mu$ Ci 125-I (Amersham Co.)
3. Vortex 5 minutes (thorough, but gentle)
4. Add 200  $\mu$ l Sodium Metabisulfite
5. Add 200  $\mu$ l Potassium Iodide
6. Load on G 25-M Sephadex column for separation
7. Elute with 0.05 M PB + 0.1% BSA

**Column Preparation:**

Gel filtration was used to separate LH, FSH and PRL radiolabelled compounds. Sephadex G 25-M gel (Pharmacia

Co.) suspended in 0.05 M sodium phosphate buffer was placed into a 1 x 20 cm glass column. Approximately 10 ml of the elution buffer (0.05 M sodium phosphate buffer + 0.1% BSA) was used to rinse the column just prior to use. Buffer pH for LH was 7.5 and for FSH and PRL was 7.4

#### Isolation Technique:

Upon loading iodinated material into the column, isolation of the labelled protein is accomplished through fractionating the eluted material in 1 ml fractions. Radioactivity is eluted from the column in two peaks; the first peak is the protein fraction and the second peak is the free iodide. The labelled protein is eluted after approximately 11-13 fractions with the free iodide coming off the column at approximately 20-25 fractions.

#### TRACER VERIFICATION

##### Trichloroacetic Acid Test:

##### Materials:

Diluted radiolabelled protein (= 32,000 cpm)

Four 1.5 ml plastic microcentrifuge tubes

18% Trichloroacetic acid in 0.09 M PB

1% BSA in 0.5 M PB (pH 7.5)

##### Procedure:

1. Add 25  $\mu$ l of the diluted tracer to the microcentrifuge tubes
2. Set aside two tubes as Controls
3. To the remaining tubes, add 100  $\mu$ l of cold (4°C)

1% BSA, then add 200  $\mu$ l TCA

4. Vortex rapidly for 10 seconds

5. Centrifuge treated tubes at 3,000 rpm for 5 minutes

6. Aspirate supernatant from the treated tubes and dispose of properly

7. Count the Control and Treated tubes on a gamma counter

Calculation of Labelled Hormone:

To calculate % labelled hormone:

avg cpm of TREATED TUBES  $\div$  avg cpm of CONTROL TUBES

## APPENDIX 2

## APPENDIX 2

## RADIOIMMUNOASSAY TECHNIQUES

Protocol for LH: Niswender as modified by D. J. Bolt (1981)

| Sequence:      |               | 1                 | 2             | 3               | 4             | 5             |
|----------------|---------------|-------------------|---------------|-----------------|---------------|---------------|
| <u>TUBE ID</u> | <u>TUBE #</u> | <u>RIA BUFFER</u> | <u>SAMPLE</u> | <u>STOCK Ab</u> | <u>1ST Ab</u> | <u>TRACER</u> |
| TC             | 1,2,3         | ---               | ---           | ---             | ---           | 100 ul        |
| NSB            | 4,5,6         | 700 ul            | ---           | ---             | ---           | 100 ul        |
| BO             | 7,8,9         | 500 ul            | ---           | ---             | 200 ul        | 100 ul        |
| stand          | 10-33         | 300 ul            | 200 ul        | ---             | 200 ul        | 100 ul        |
| QC             | 34-37         | 100 ul            | 400 ul        | ---             | 200 ul        | 100 ul        |
| BLANK          | 38            | 500 ul            | ---           | ---             | 200 ul        | 100 ul        |
| SAMPLE         | 39 +          | 100ul*            | 400 ul        | ---             | 200 ul        | 100 ul        |

\*Sample volume (sample + buffer) must equal 500 ul;

therefore, if 400 ul of sample is used, then add 100 ul of buffer or if 300 ul of sample is used add 200 ul of buffer.

TC = Total count or the total amount (counts per minute) of tracer added to each tube.

NSB = Nonspecific binding, amount of interference from impurities in the assay tubes, buffer, etc.

BO = Total binding (%) capacity of the working dilution of tracer and antibody to be used as the basis for determining hormone concentrations.

Standards = Known amounts of hormone used to construct the standard reference curve.

QC = A standard unknown plasma sample used in assay to

control intra-and interassay variation.

Sample = Unknown amount of hormone that is to be measured.

To mix Standard: (LH Standard ampule contains 32 ng/800 $\mu$ l).

Number the Standard tubes: 31, 62, 125, 250, 500, 1,000, 2,000, 4,000.

Place 800  $\mu$ l of RIA Buffer in each tube then add the contents of the ampule to the tube numbered 4,000. Mix well and perform serial dilutions (i.e., 4,000, 2,000, 1,000, etc.) by placing 800  $\mu$ l from the previous tube into the next and so on.

Proceed in a step-wise addition:

1. Add sample or standard to numbered assay tubes
2. Add 200  $\mu$ l of anti-LH antibody (1:40,000) to all tubes except TC and NSB tubes.
3. Add 100  $\mu$ l of LH-I<sub>125</sub>
4. Vortex
5. Incubate for 48 hours at room temperature
6. Add 400  $\mu$ l Normal Rabbit Serum (NRS) (Miles Scientific, Inc.) to all tubes except TC
7. Add 200  $\mu$ l 2nd Ab (Miles Scientific, Inc.) to all tubes except TC
8. Vortex
9. Add 1 ml PEG (Sigma Chemical Co.) to all tubes except TC
10. Incubate for 3-16 hours at 4°C

11. Centrifuge at 3,000 rpm for 20 minutes
12. Decant supernatant and discard properly
13. Count the "pill" on the gamma counter

See Appendix 3 for RIA buffer, Ab buffer, NRS and PEG buffer recipes.

First Ab recipe:

$$\frac{x}{300} = \frac{\text{total volume}}{\text{Ab concentration needed}} = \text{volume of stock Ab}$$

Total volume = [# of tubes + 25 (for error)] x 200 ul

Ab concentration = 1:10,000

Then take:

Total volume - volume of stock needed = amt of buffer needed

Tracer recipe:

[# of tubes + 30] x 18,000 cpm/tube = total cpm needed

Total cpm - stock cpm/ul (usually 32,000 cpm) = volume of stock tracer needed

Total volume = [# of tubes + 30] x 100 ul

Total volume - stock volume = amount of RIA buffer needed

Second Ab recipe:

Total volume = [# of tubes + 25] x 200 ul

Total volume / 5 = volume of 2nd Ab stock needed

Total volume - 2nd Ab volume = amount of RIA buffer required

### Protocol for FSH: Bolt and Rollins, 1983

The procedure is the same as that for LH except for the standard curve parameters, Ab dilution and tracer cpm.

To mix standards: (FSH Standard ampule contains 1024 ng/800 ul) (provided by D. J. Bolt, USDA) Number the standard tubes: 1, 2, 4, 8, 16, 32, 64, 128 ng. Place 800 ul of RIA Buffer in each tube, then add the contents of the ampule to the tube numvered "128". Mix well and perform serial dilution (i.e. 128 64 32 etc.). Discard the remaining 800 ul from the 1 ng tube.

Working Ab dilution for FSH is 1:10,000 (provided by D. J. Bolt, USDA)

Tracer: 20,000 cpm

### Testosterone and Androstenedione

Proceed in a step-wise addition:

1. Add 100 ul of sample to extraction tube for testosterone or 150 ul for androstenedione determination
2. Add 2 ml of Benzene
3. Vortex for 1 minute
4. Freeze the aqueous layer with liquid nitrogen
5. Decant organic layer into evaporation tube
6. Evaporate organic layer with nitrogen gas and a warm water bath
7. Add 200 ul assay buffer
8. Vortex

9. Add 100 ul of each standard (10-1000 pg)
10. Add 300 ul of assay buffer to TC, 300 ul to NSB,  
200 to Bo and 100 ul to each standard tube
11. Add 100 ul of Ab to all tubes except TC and NSB
12. Add 100 ul of the tracer.
13. Vortex
14. Incubate overnight at 4°C
15. Add 200 ul of Dextran coated charcoal to all tubes  
except TC
16. Vortex
17. Incubate for 15-20 minutes at 4°C
18. Centerfuge at 300 rpm for 10 minutes
19. Decant supernatant into scintillation vials
20. Add 6 ml of Scintillation fluid
21. Shake the vials
22. Count in liquid scintillation counter for 1  
minute.

### APPENDIX 3

## APPENDIX 3

## BUFFERS AND REAGENTS

## Sodium Phosphate Buffers

1. Dibasic F. W. = 268.07 (hydrous or anhydrous)  
To make 500 ml of 0.5 M Sodium Phosphate Buffer, dibasic: Add 67 gm Sodium Phosphate (dibasic) to a 500 ml volumetric flask then fill halfway with double distilled water. Stir until dissolved, then fill to volume with double distilled water and stir for an additional 10-15 minutes. Filter the solution into the 0.45 um filter storage container (a vacuum is required). Store at room temperature. Buffer will keep 4-6 weeks.
2. Monobasic F.W. = 137.99 (hydrous or anhydrous)  
To make 500 ml of 0.5 M Sodium Phosphate Buffer, monobasic: Add 34.49 gm Sodium Phosphate (monobasic) to a 500 ml volumetric flask and fill halfway with double distilled water. Stir until dissolved, then fill to volume with double distilled water and stir for an additional 10-15 minutes. Filter the solution into the 0.45 um filter storage container (a vacuum is required). Store at room temperature. Buffer will keep 4-6 weeks.

## Reagents for LH and FSH RIA

1. Phosphate buffer (PB): (see phosphate buffer chart for mixture.) Use 0.5 M Sodium Phosphates and dilute with double distilled water to obtain desired molarity of buffer. NOTE: to adjust pH use 1 M, 3 M, or 10 M NaOH (base) or 5% sulfosalicylic acid (acid).
2. Basic RIA Buffer: Use a volumetric flask. Mix solution using 0.01M PBS with a pH of 7.5; readjust pH after mixing. Add material to flask before adding liquid; only fill halfway with PBS and dissolve materials before completing the mixture.

| <u>MATERIALS</u>           | <u>LITER</u> | <u>HALF LITER</u> |
|----------------------------|--------------|-------------------|
| 0.1% BSA                   | 1 gm         | 0.5 gm            |
| 0.135 M NaCl               | 7.89 gm      | 3.95 gm           |
| 0.005 M NaHCO <sub>3</sub> | 0.42 gm      | 0.21 gm           |
| 0.01% Thimerosal           | 100 gm       | 50 mg             |

3. 2% Normal Rabbit Serum (NRS) v/v: Mix using a 0.01 M PBS with a pH of 7.5; readjust pH after mixing solution.

| <u>MATERIALS</u>    | <u>LITER</u> | <u>HALF LITER</u> |
|---------------------|--------------|-------------------|
| Normal Rabbit Serum | 20 ml        | 10 ml             |
| 0.01% Thimerosal    | 100 mg       | 50 mg             |

4. Polyethylene glycol (PEG) v/v: Mix a 5% solution in 0.01 M PBS with a pH of 7.5; readjust after mixing.

| <u>MATERIALS</u> | <u>LITER</u> | <u>HALF LITER</u> |
|------------------|--------------|-------------------|
| PEG              | 50 gm        | 25 gm             |
| 0.01% Thimerosal | 100 mg       | 50 mg             |

5. First Ab Buffer: Use a volumetric flask. Mix using 0.01M PBS with a pH of 7.5; readjust pH after mixing.

| <u>MATERIALS</u>           | <u>LITER</u> | <u>HALF LITER</u> |
|----------------------------|--------------|-------------------|
| 0.135 M NaCL               | 7.89 gm      | 3.95 gm           |
| 0.005 M NaHCO <sub>3</sub> | 0.42 gm      | 0.21 gm           |
| 0.05 M (Di) NaEDTA         | 18.6 gm      | 9.3 gm            |
| 1300 NRS                   | 3.3 ml       | 1.65 ml           |
| 0.01 Thimerosal            | 100 mg       | 50 mg             |

#### Reagents for Steroids RIA

1. Dextran coated charcoal:

Mix: 0.625 gm Norit A charcoal (Sigma Chemical Co.)  
 0.0625 gm Dextran t-70 (Pharmacia Co.)  
 100 ml Assay Buffer

2. Steroid buffer: (for 2 liters)

Mix: 10.8 gm Sodium Phosphate (monobasic) F.W. = 137.99  
 32.7 gm Sodium Phosphate (dibasic) F. W. = 268.07  
 2 gm Sodium azide  
 18 gm Sodium chloride  
 2 gm gelatin

## VITA

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In June 1985 he joined the department of Animal Production at King Faisal University where he worked as a demonstrator. In September 1986 he came to the United States and joined the English Language Institute, The University of Tennessee. He joined the department of Animal Science in September 1987 and he received his Master of Science degree in May 1990.