AN ABSTRACT OF THE THESIS OF

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Title: Impacts of Stocking Density on Development and Puberty Attainment of Replacement Beef Heifers.

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Reinaldo Fernandes Cooke

The following experiment presented herein compared growth, physical activity, stress-related responses, and puberty attainment in heifers reared on high- (HIDENS) or low-stocking densities (LOWDENS). Sixty Angus x Hereford heifers were ranked by age and BW (210 ± 2 d and 220 ± 2 kg, respectively) on d 0, and assigned to: a) 1 of 3 drylot pens (10 × 14 m pens; 10 heifers/pen) resulting in a stocking density of 14 m^2/heifer (HIDENS; n = 3), or b) 1 of 3 pastures (25-ha pastures; 10 heifers/pasture), resulting in a stocking density of 25,000 m^2/heifer (LOWDENS; n = 3). Pastures utilized herein were harvested for hay prior to the beginning of this experiment, and negligible forage was available for grazing to LOWDENS heifers throughout the experimental period (d 0 to 182). Heifers received the same limit-fed diet during the experiment, which averaged (DM basis) 4.0 kg/heifer daily of alfalfa-grass hay and 3.0 kg/heifer daily of a corn-based concentrate. Heifer shrunk BW was recorded after 16 h of feed and water withdrawal on d -3 and d 183 for ADG calculation. On d 0, heifers were fitted with a pedometer fixed behind their right shoulder. Each week during the experiment, pedometer results were

recorded and blood samples were collected for puberty evaluation via plasma progesterone. Plasma samples collected on d 0, 28, 56, 84, 112, 140, 161, and 182 were also analyzed for concentrations of cortisol and IGF-I. On d 28, 102, and 175, blood samples were also collected for RNA isolation and analysis of heat shock protein (HSP) 70 and HSP72 mRNA expression. On d 0, 49, 98, 147, and 182, hair samples were collected from the tail switch for analysis of hair cortisol concentrations. No treatment effects were detected ($P = 0.66$) for heifer BW or ADG. Heifers from LOWDENS had more ($P < 0.01$) steps/week compared with HIDENS. Heifers from LOWDENS had greater ($P = 0.05$) mRNA expression of HSP72, and tended ($P = 0.10$) to have greater mRNA expression of HSP70 compared with HIDENS. Plasma concentrations of cortisol and IGF-I were often greater ($P \leq 0.05$) in LOWDENS vs. HIDENS heifers (treatment × day interaction; $P < 0.01$). Hair cortisol concentrations were greater ($P < 0.01$) for HIDENS vs. LOWDENS heifers beginning on d 98 (treatment × day interaction; $P < 0.01$). Heifers from HIDENS experienced delayed puberty attainment and had a lower ($P < 0.01$) proportion of pubertal heifers on d 182 compared with LOWDENS (treatment × day interaction; $P < 0.01$). In summary, HIDENS negatively impacted heifer stress-related and physiological responses, and delayed puberty attainment compared with LOWDENS.

IMPACTS OF STOCKING DENSITY ON DEVELOPMENT AND PUBERTY ATTAINMENT OF REPLACEMENT BEEF HEIFERS

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I understand that my thesis will become part of the collection of Oregon State University.

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Kelsey M. Schubach, Author

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CHAPTER I
INTRODUCTION

Public scrutiny of beef production systems is growing rapidly, and cattle welfare is one of the main targets for attention (Grandin, 2014). Cattle producers are currently challenged with improving production efficiency while fostering animal well-being (Thornton, 2010). Hence, management practices that increase beef cattle productivity and promote animal welfare are warranted to enhance profitability in beef cattle systems, address the current and projected increases in beef demand, and satisfy industry and public requirements for proper animal care.

Stocking density is one example of a management practice that can impact welfare and productive efficiency in cattle operations (Fraser et al., 2013). In U.S. spring-calving cowherds, replacement heifers are weaned in the fall and exposed to their first breeding season the following spring. Hence, these heifers are commonly reared in drylot systems to facilitate feeding and management during the fall and winter (Olson et al., 1992). However, rearing cattle in areas with elevated stocking density is known to stimulate stress reactions (Grandin, 2014), while acute and chronic stress directly impairs reproductive function in beef cattle (Dobson and Smith, 2000). Accordingly, Petersen et al. (2014) reported that heifers developed in drylots (11 m^2/heifer) gained more BW but had increased heart rate and rested less compared with contemporary heifers reared on native range (7,400 m^2/heifer). Mulliniks et al. (2013) also indicated that heifers reared in drylots had greater ADG, but reduced pregnancy rates compared with cohorts reared on range pastures.

CHAPTER II
LITERATURE REVIEW

1. Importance of replacement heifer development

Cow-calf systems rely on the ability of females to initiate and maintain estrous cyclicity and produce a calf each year (Velazquez et al., 2008). Replacement beef heifers represent the opportunity to incorporate genetics into the herd that will benefit efficiency and profitability of the cow-calf system. In order for heifers to be efficacious, they need to be efficient reproductively without incurring excessive development costs (Gasser, 2013). It is difficult to quantify economic losses associated with reproductive delay, however it is estimated that reproductive diseases and conditions cost the United States beef industry over $5 million annually (Bellows et al., 2002). A substantial amount is invested in replacement heifers, and they should be managed to reach puberty and become pregnant early during the breeding season in order for producers to get a return on this investment (Engelken, 2008).

1.1 Timing of puberty attainment

It is recommended that heifers reach puberty by 12 months of age (Patterson et al., 1992), and by 13 to 15 months of age have maximized conception rates to first breeding (Gasser, 2013). Breeding heifers at pubertal estrus results in lower pregnancy rates compared to those bred at third or subsequent estrus (Byerley et al., 1987). Thus, heifers that reach puberty by 12 months of age should be able to conceive by 15 months of age,

at second or third estrus, and calve at 24 months. If a heifer fails to reach puberty and conceive during the breeding season, the producer faces economic losses such as decreased weaning weight and milk production, and increased overall costs (Bellows et al., 2002). Lesmeister et al. (1973) found heifers that calved as 2-year-olds calved earlier and produced more calves throughout their lifetime than heifers that calved as 3-year-olds. Additionally, due to older age as well as faster pre-weaning rate of gain of calves, those heifers that calved earlier in the breeding season produced heavier calves at weaning (Lesmeister et al., 1973). Therefore, heifers that calve earlier tend to be more efficient both biologically and economically (Marshall et al., 1989). Most factors related to reproductive efficiency can be attributed to management rather than genetic components of fertility, since these components are not highly heritable (Patterson et al., 1992), and should be manipulated to increase efficiency and profitability of the system.

2. Puberty

Puberty is defined practically as being complete when a heifer ovulates and exhibits her first estrus, which is followed by a normal luteal phase (Moran et al., 1990). Reproduction in heifers is largely controlled by the gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH), which are released from the pituitary gland into the blood stream (Moran et al., 1990). Release of LH and FSH is dependent on synthesis and release of gonadotropin releasing hormone (GnRH) from the hypothalamus (Vizcarra et al., 1997).

Follicular development in cattle occurs in waves, however, in prepubertal heifers prior to the inaugural ovulation all follicular waves are anovulatory (Adams et al., 1994). During the late prepubertal period, waves of follicular growth are associated with peaks in FSH concentrations. Therefore there are no dramatic changes in growth of follicles as ovulation approaches; hence endocrine mechanisms controlling this growth are in place prior to puberty (Evans et al., 1994). Adams et al. (1994) concluded wave emergence and selection of the dominant follicle respectively, are temporally associated with the surge in plasma FSH and are similar between prepubertal and sexually mature heifers. However, follicle diameter is smaller in prepubertal heifers, suggesting reproductive maturation occurs past the first ovulation (Adams et al., 1994; Evans et al., 1994).

Since emergence of follicular waves are similar pre- and post- puberty and no change in FSH secretion is evident prior to ovulation, it can be concluded that FSH has a permissive role in sexual maturation (Evans et al., 1994; Dodson et al., 1988). Prior to puberty, LH secretion is more sensitive to estradiol negative feedback when compared to pubertal heifers. This negative feedback of estradiol on secretion of LH decreases during pubertal development (Day et al., 1984). Therefore, the onset of puberty in the heifer is a result of decreased sensitivity to estradiol negative feedback and an increase in LH pulse frequency (Day et al., 1984; Evans et al., 1994). Eventually these pulses culminate in a surge of LH and that induces ovulation. It is common for first ovulation to be followed by a short lived corpus luteum and subsequent short interestrous interval, whereas the subsequent ovulation is usually associated with the first observed estrus and followed by the first normal estrous cycle (Gasser, 2013) indicating the heifer is pubertal.

Another hormone involved in sexual maturation in beef females is progesterone. Berardinelli et al. (1979) described two increases in progesterone prior to puberty, the source of both being luteal tissue within the ovary. Anderson et al. (1996) suggest exposure to progestins prior to puberty suppresses estradiol receptors within the hypothalamus. Therefore, upon withdrawal of exogenous progestins, secretion of LH is hastened and puberty occurs shortly after (Anderson et al., 1996). However, it should be noted that this effect was only observed in heifers in which estradiol negative feedback has already begun to decline (Anderson et al., 1996). Gonzalez-Padilla et al. (1975) report administration of estradiol induced LH release in prepubertal heifers, however ovulation did not occur in those not pretreated with progesterone. Therefore, the increase in progesterone prior to puberty is believed to sensitize the ovary to the effects of estradiol positive feedback, resulting in the LH surge (Gonzalez-Padilla et al., 1975).

2.1 Relationship between nutrition and puberty

Nutrition plays a pivotal role in heifer development and puberty attainment. Endocrine activity involved in puberty appears to be suppressed until the heifer is of a sufficient size and body composition to be reproductively successful (Rawlings et al., 2003). Prepubertal heifers fed to accelerate body weight gain exhibit greater frequency of LH pulses and an advancement of puberty onset (Cardoso et al., 2014). Typically, it is recommended that heifers reach 60-65% of their mature body weight by puberty (Patterson et al., 1992). However, more recent research found that reproduction and calf production traits were not negatively affected when heifers were developed to 53% of

their mature body weight when compared to 58% (Funston and Deutscher, 2004). Additionally, reproductive performance was not adversely affected when heifers achieved 55% of mature body weight as opposed to 62% (Lardner et al., 2014). Regardless, sufficient body weight must be attained for the heifer to be reproductively successful (Rawlings et al., 2003). Feeding programs should be developed for cattle of different biological types in order for heifers to reach optimal body weight and size (Yelich et al., 1996).

2.1.1 Energy

Favorable metabolic status during the juvenile period results in greater LH pulsatility, and hence advances puberty onset (Cardoso et al., 2014). Hill et al. (1970) concluded heifers fed 85% of maintenance requirements for both energy and protein had impaired follicle growth compared to those receiving 100% of maintenance requirements. Similarly, Staigmiller et al. (1979) reported decreased ovulation rates in underfed cows, possibly due to lack of hormonal stimulation necessary for ovulation or incompetent follicles. Heifers subjected to restricted energy intake exhibit prolonged suppression of LH secretion by estradiol negative feedback (Kurz et al., 1990). Additionally, Kurz et al. (1990) found LH secretion was altered in ovariectomized heifers as well, and normal LH patterns were observed with increased energy intake. It follows that metabolic status plays a substantial role in LH secretory mechanisms in prepubertal heifers (Kurz et al., 1990), and therefore puberty onset. However, body weight gain is not the only mechanism by which energy intake influences puberty attainment, as metabolic status is

known to modulate hormones that mediate the puberty process such as insulin-like growth factor-1 (IGF-1, Schillo et al., 1992).

2.1.2 Insulin-Like Growth Factor-1

Insulin-like growth factor-1 is a 70 amino acid peptide that is part of the IGF family of polypeptides that are structurally and evolutionarily related to proinsulin (Gluckman et al., 1987). This hormone is produced in a variety of tissues; however the liver is the main organ responsible for production of IGF-1 that is measured in the blood (Pfaffl et al., 1998), and production is governed by presence of growth hormone (Simpson et al., 1991). During periods of under nutrition, such as fasting, GH receptors are reduced in the liver leading to decreased plasma IGF-1 concentration (Gluckman et al., 1987). Unique to peptide hormones, IGF-1 bioavailability is dependent upon IGF-1 binding proteins (IGFBPs), and these circulating complexes represent the storage form of IGF-1 (Gluckman et al., 1987).

Metabolic status is communicated to the hypothalamic-pituitary-ovarian axis via a variety of hormones including IGF-1 (Flores et al., 2008). IGF-1 acts as a chemical mediator, influencing reproductive events according to the physiological state of the animal, allowing these events to occur when nutritional conditions are suitable for successful reproduction (Simpson et al., 1991; Velazquez et al. 2008). Peripheral IGF-1 concentrations increase as heifers approach puberty (Garcia et al., 2002), indicating IGF-1 is involved in puberty onset.

Receptors for IGF-1 can be expressed by GnRH neurons, thereby providing a mechanism by which IGF-1 exerts control over GnRH neurons (Daftary and Gore, 2004), and hence stimulates LH and FSH release necessary for reproductive maturation to occur (Zulu et al., 2002). In addition to the liver, the ovary also synthesizes and secretes IGF-1 (Spicer and Echternkamp, 1995), indicating the IGF-1 functions in not only an endocrine manner, but has paracrine/autocrine components as well. Follicle diameter is correlated with serum concentrations of IGF-1 (Flores et al., 2008), and low serum IGF-1 is correlated with decreased follicle numbers and arrested follicular development without changes in FSH, LH, and estradiol concentrations (Chase et al., 1998). The IGF-1 stimulates proliferation and differentiation of granulosa cells (Monget and Monniaux, 1994) as well as steroidogenesis of follicular cells via stimulation of the aromatase system (Zulu et al., 2002). IGF binding proteins are also secreted in the ovary, and affect ovarian function (Zulu et al., 2002). The proportion of IGFBPs differs between small and large preovulatory follicles, with higher concentrations of IGFBP activity found in smaller, atretic follicles (Schams et al., 1999), reflecting the increased potency and bioavailability of IGFs in large preovulatory follicles (Zulu et al., 2002).

3. Stress

Stress can be defined as the displacement of homeostasis by external body forces (Stott, 1981), and can affect a wide range of aspects such as fertility, growth rate, and disease resistance (Dobson and Smith, 2000). Stress can be acute or chronic, with chronic stimulation being more frequent in animal husbandry systems where animals are exposed

to the same stressors or repeated stressors for extended periods (Dantzer and Mormède, 1983). A variety of environmental and management conditions experienced by farm animals have the potential to be perceived as stressful, activating the hypothalamo-pituitary-adrenocortical axis as well as the sympatho-adrenal medullary axis (Minton, 1994), through which the stress response is controlled (Salak-Johnson and McGlone, 2007). This activation elicits physiologic responses designed to reestablish homeostasis (Carroll and Forsberg, 2007).

3.1 The hypothalamic-pituitary-adrenocortical axis

Activation of the hypothalamic-pituitary-adrenocortical (HPA) axis leads to secretion of corticotropin-releasing hormone (CRH) from the paraventricular nucleus of the hypothalamus (Swanson and Sawchenko 1980), as well as vasopressin (VP, Carroll and Forsberg, 2007). Corticotropin-releasing hormone and VP can independently mediate glucocorticoid secretion from the adrenal glands; however, working together they control the magnitude and duration of the glucocorticoid response (Carroll and Forsberg, 2007). The ability of these neurohormones to potentiate the glucocorticoid response is species specific, with CRH being the primary stimulator of ACTH secretion in cattle (Carroll et al., 2007).

Corticotropin-releasing hormone, upon binding to its distinct membrane-bound receptors, induces cells of the anterior pituitary to begin synthesizing and secreting adrenocorticotrophic hormone (ACTH, Salak-Johnson and McGlone, 2007). Primarily, ACTH acts on the adrenal glands to stimulate synthesis and release of steroids, as well as

promoting the uptake of cholesterol and converting it to pregnenolone (Carroll and Forsberg, 2007). The adrenal glands consist of the adrenal cortex and the adrenal medulla. The adrenal cortex can be divided into three zones; the zona glomerulosa, the zona fasciculata, and the zona reticularis. Glucocorticoids, mineralocorticoids, as well as androgens and progesterone are synthesized within the adrenal cortex (Brown 1994 book). Mineralocorticoids and glucocorticoids are necessary for survival, whereas adrenal androgens are thought to play a role in reproductive performance. Mineralocorticoids, such as aldosterone, maintain sodium and extracellular fluid balance within the body, which are critical to maintain homeostasis (Carroll and Forsberg, 2007).

The adrenal medulla synthesizes catecholamines, epinephrine and norepinephrine, in response to sympathetic nervous system stimulation. Epinephrine, secreted directly into the circulatory system, results in increased heart rate and blood flow as well as conversion of glycogen to glucose within the liver and via adipolysis. Norepinephrine on the other hand results in vasoconstrictive effects, which increase blood pressure (Carroll and Forsberg, 2007). Catecholamines also influence the HPA axis through regulation of ACTH release as well as stimulation of cortisol release from the adrenal cortex (Axelrod and Reisine, 1984).

3.1.1. Cortisol

ACTH acts on cells of the zona fasciculata of the adrenal cortex to induce secretion of glucocorticoids (Fulford and Harbuz, 2005). An appropriate concentration of glucocorticoids within the body, mediated via actions of ACTH, is essential for

maintenance of homeostasis. However, CRH and VP also play roles in the regulation of glucocorticoid production via paracrine actions on the adrenal glands (Carroll and Forsberg, 2007). In cattle, cortisol is the primary glucocorticoid released from the adrenal cortex in response to HPA activation (Minton, 1994).

Glucocorticoids (i.e. cortisol), elicit a variety of effects on the body in response to stress. During stressful conditions, cortisol increases blood glucose concentrations through stimulation of the liver to convert fat and protein to metabolites, which are ultimately converted to glucose, providing energy (Carroll and Forsberg, 2007). In addition to increased levels of blood glucose, cortisol also stimulates secretion of catecholamines from the adrenal medulla, which control processes such as heart rate and increased glucose production by the liver (Carroll and Forsberg, 2007). Cortisol suppresses the inflammatory and immune systems as well, which prevents chronic and excessive stimulation, which could result in death (Munck et al., 1984). Chronic stress, or chronic exposure to increased concentrations of cortisol can be severely detrimental, causing excessive protein catabolism, hyperglycemia, immunosuppression (Carroll and Forsberg, 2007), and suboptimal reproduction (Dobson et al., 2001).

3.1.2 Hair Cortisol

As mentioned previously, chronic stress or chronic exposure to increased blood concentrations of cortisol can be severely detrimental (Dobson and Smith, 2000). Therefore, it is necessary to obtain a reliable method for assessing chronic stress. However, cortisol, being used as a diagnostic marker for the stress response is most

frequently analyzed from blood, saliva, or feces. Routine management and handling procedures can induce the stress response, thereby increasing circulating cortisol concentrations and only providing an indication of stress experienced at that point in time. Recently, cortisol concentrations in hair have been used as an alternative to traditional sampling methods. This method has proved successful in assessing long-term changes in cortisol levels, or chronic stress, for a variety of species including humans (Sauvé et al., 2007) and dogs (Bennett and Hayssen, 2010). Recently, Burnett et al., 2014 showed that cortisol could be quantified in hair clipped from the tail switch of cattle. Cortisol is accumulated gradually in growing tail hair via passive diffusion from the blood into growing hair follicle cells (Combs, 1987) as well as from apocrine and sebaceous glands after the hair shaft is formed (Cone, 1996). Therefore, hair cortisol concentrations may be used to assess long term HPA activity and hence chronic stress experienced by the animal.

3.2 Heat shock proteins

Cells have developed a variety of strategies to cope with stress. However, cells from all organisms exhibit a similar response to sudden increases in growth temperature; characterized by extremely rapid synthesis of a select group of proteins termed heat shock proteins (HSPs). Heat shock proteins are a group of highly conserved proteins, defined based on molecular mass and classified into 2 major families; large HSPs (including HSP60, HSP90, and HSP70) and small HSPs (including HSP27). However, this response is not limited to heat shock, since increased expression of HSPs occurs

when cells are exposed to a variety of stressors, thereby indicating a stress response at the cellular level (Welch, 1992). Heat shock proteins are molecular chaperones, doing so by interacting with other proteins, thereby minimizing the possibility that these proteins will interact inappropriately together (Feder and Hofmann, 1999). Heat shock proteins serve to enhance stress resistance and cell recovery rate when an organism is exposed to various stressors, as well as acting as protective agents for cell activities during stressful conditions (Deng et al., 2013).

In the unstressed cell, HSPs are expressed constitutively, however a select few of these proteins are expressed in stressful situations and can be used as a diagnostic marker of stress (Welch, 1992). In fact, Deng et al. (2013) found the expression of HSP70 to be positively correlated with plasma cortisol and ACTH levels. This is not surprising given that HSP70 expression is enhanced in response to stress-related accumulation of unfolded proteins (Park et al., 2007) and is the most highly induced HSP (Welch, 1992). HSP90, one of the most abundant proteins in cells of mammals, is also increased after stress (Welch, 1992). In fact, it is suggested that HSP90 interacts with portions of steroid hormone receptor binding domains that are critical for necessary dimerization, thereby preventing inappropriate interaction with DNA in the absence of the steroid hormone (DeMarzo et al., 1991).

3.3 Effect of stress on puberty

As indicated previously, stress can affect a wide range of biological processes within the body, including reproductive function. Dobson and Smith (2000) suggest that

stressors interfere with mechanisms involved with regulation of the precise timing of events within the follicular phase, thereby decreasing fertility. For example, Dobson et al. (1999) found activation of the HPA axis via transport during the follicular phase interrupted the surge of LH necessary for ovulation to occur. This indicates stress interferes with GnRH pulsatile secretion mediated through effects on the hypothalamus or at the level of the pituitary gland. Additionally, Phogat et al. (1997) showed increased ACTH concentrations resulted in reduction of amount of LH released by challenges with small doses of GnRH. These results suggest ACTH interferes with GnRH self-priming and estradiol sensitization, thereby causing a disruption of estradiol induced LH synthesis and release (Phogat et al., 1997). Activation of the hypothalamic-pituitary-adrenal axis by stressors reduces pulsatility of both GnRH and LH through actions at the hypothalamus as well as the pituitary gland, depriving the growing ovarian follicle of necessary LH support and hence resulting in decreased estradiol production by follicles (Dobson and Smith, 2000). Decreased ovarian estradiol production interferes with the positive estradiol feedback to the hypothalamus and pituitary gland, delaying the LH surge and further reducing LH amplitude (Smith and Dobson, 2002).

As mentioned above, in addition to glucocorticoids and mineralocorticoids, adrenal androgens are also produced within the adrenal cortex (Brown, 1994). Adrenal steroidogenesis begins with cholesterol, most of which is provided from the circulation as low-density-lipoproteins via specific receptors present on adrenal tissue. Cholesterol is transported from the outer to the inner mitochondrial membrane by steroidogenic acute regulatory protein (StAR), which is considered the rate-limiting step. Once within the mitochondrion, cholesterol is cleaved by cholesterol desmolase resulting in

pregnenolone. Pregnenolone is converted to 17-hydroxypregnenolone by the enzyme 17α-hydroxylase or to progesterone by the enzyme β-hydroxysteroid dehydrogenase. Both products can be converted into 17-hydroxyprogesterone and directed towards synthesis of cortisol or androstenedione. However, only progesterone can be directed towards synthesis of aldosterone (Stewart, 2003). Therefore, under stimulation by ACTH, the adrenal cortex may synthesize and secrete sexual steroids in addition to glucocorticoids and mineralocorticoids, which could in turn negatively affect reproductive function.

4. Stocking density, a stressor that impacts puberty attainment in beef heifers

Stocking density, defined as the number of animals per unit area of land at any point in time (Heitschmidt and Taylor Jr, 1991), is one example of management that may affect productivity and welfare of cattle. Changes in behavior are observed when stocking density is increased; including decreased lying time and increased aggression at the feed bunk (Krawczel et al., 2012). Additionally, productivity of animals is affected by stocking density, as evidenced by decreased milk production reported for dairy cows housed in higher stocking densities (Nordlund et al., 2006). This decrease may be attributed to insufficient bunk or resting space, which could result in cows that are unable to maintain adequate nutrient intake to meet energy and essential nutrient requirements (Hosseinkhani et al., 2008). Cows exhibit a greater corticoid response to an ACTH challenge when freestall availability was reduced (Friend et al., 1979), and when access to feed was decreased (González et al., 2003). These results indicate that altered behavior

and reduced feed activity which results from increased stocking density may be stressful for cows (Krawczel et al., 2012).

The impact of stocking density on reproductive development of beef heifers is unknown. However, research is warranted given the impacts stress and energy intake have on puberty attainment. Data from Montana showed heifers developed on drylot gained more weight, but had increased heart rate and rested less compared with cohorts reared on native range (Petersen et al., 2014). Similarly, data from New Mexico found heifers reared in drylots had greater average daily gain, but reduced pregnancy rates compared with contemporary heifers reared on range pastures (Mulliniks et al., 2013). These data indicate higher stocking density negatively impacts stress as well as productivity of heifers.

In Oregon, spring-calving operations wean replacement heifers in the fall and expose them to their first breeding season the following spring, Therefore, replacement heifers are often reared in drylot systems during the fall, winter, and early spring. This facilitates management as well as supply of feed and water to growing heifers. However, rearing cattle in confinement is known to stimulate stress reactions (Grandin, 2014), which could potentially affect puberty attainment in these heifers. Hence, the experiment described herein was done to compare growth, physical activity, stress related responses, and puberty attainment in heifers reared on high- or low-stocking densities from weaning until their first breeding season.

CHAPTER III

IMPACTS OF STOCKING DENSITY ON DEVELOPMENT AND PUBERTY ATTAINMENT OF REPLACEMENT BEEF HEIFERS

1. Abstract

Sixty Angus x Hereford heifers were ranked by age and BW (210 ± 2 d and 220 ± 2 kg, respectively) on d 0, and assigned to: a) 1 of 3 drylot pens (10 × 14 m pens; 10 heifers/pen) resulting in a stocking density of 14 m^2/heifer (**HIDENS**; n = 3), or b) 1 of 3 pastures (25-ha pastures; 10 heifers/pasture), resulting in a stocking density of 25,000 m^2/heifer (**LOWDENS**; n = 3). Pastures utilized herein were harvested for hay prior to the beginning of this experiment, and negligible forage was available for grazing to LOWDENS heifers throughout the experimental period (d 0 to 182). Heifers received the same limit-fed diet during the experiment, which averaged (DM basis) 4.0 kg/heifer daily of alfalfa-grass hay and 3.0 kg/heifer daily of a corn-based concentrate. Heifer shrunk BW was recorded after 16 h of feed and water withdrawal on d -3 and d 183 for ADG calculation. On d 0, heifers were fitted with a pedometer fixed behind their right shoulder. Each week during the experiment, pedometer results were recorded and blood samples were collected for puberty evaluation via plasma progesterone. Plasma samples collected on d 0, 28, 56, 84, 112, 140, 161, and 182 were also analyzed for concentrations of cortisol and IGF-I. On d 28, 102, and 175, blood samples were also collected for RNA isolation and analysis of heat shock protein (**HSP**) 70 and HSP72 mRNA expression. On d 0, 49, 98, 147, and 182, hair samples were collected from the tail switch for analysis of hair cortisol concentrations. No treatment effects

were detected (P = 0.66) for heifer BW and ADG. Heifers from LOWDENS had more (P < 0.01) steps/week compared with HIDENS. Heifers from LOWDENS had greater (P = 0.05) mRNA expression of HSP72, and tended (P = 0.10) to have greater mRNA expression of HSP70 compared with HIDENS. Plasma concentrations of cortisol and IGF-I were often greater ($P \leq 0.05$) in LOWDENS vs. HIDENS heifers (treatment × day interaction; P < 0.01). Hair cortisol concentrations were greater (P < 0.01) for HIDENS vs. LOWDENS heifers beginning on d 98 (treatment × day interaction; P < 0.01). Heifers from HIDENS experienced delayed puberty attainment and had less (P < 0.01) proportion of pubertal heifers on d 182 compared with LOWDENS (treatment × day interaction; P < 0.01). In summary, HIDENS negatively impacted heifer stress-related and physiological responses, and delayed puberty attainment compared with LOWDENS.

Key Words: Beef heifers, growth, puberty, stocking density, stress.

2. Materials and methods

This experiment was conducted at the Oregon State University - Eastern Agricultural Research Center (Burns, OR) from September 2015 until March 2016 (d 0 to 182). All animals were cared for in accordance with acceptable practices and experimental protocols reviewed and approved by the Oregon State University Institutional Animal Care and Use Committee (#4757).

Animals and treatments

On day 0 of the experiment, 60 Angus x Hereford heifers were ranked by age and BW (initial age = 210 ± 2 d; initial BW = 220 ± 2 kg) and allocated to: a) 1 of 3 drylot pens (10 x 14 m pens; 10 heifers/pen), resulting in a stocking density of 14 m^2/heifer (**HIDENS**; n = 3), or b) 1 of 3 meadow foxtail (*Alopecurus pratensis* L.) dominated pastures (25-ha pastures; 10 heifers/pasture), resulting in a stocking density of 25,000 m^2/heifer (**LOWDENS**; n = 3). Pasture and drylot pens were located approximately 800 m and 80 m, respectively, from the handling facility where cattle were processed during the experiment. Treatments were designed to represent stocking densities of drylot- or pasture-based heifer development programs utilized in our research station and representative of commercial cow-calf operations (Cooke et al., 2012; Cappellozza et al., 2014; Reis et al., 2015). In addition, HIDENS heifers were exposed to the stocking density recommended for growing cattle reared in drylot systems (Hurnik, 1991; Albin and Thompson, 1996).

All pastures utilized herein were harvested for hay prior to the beginning of this experiment, and negligible forage was available for grazing to LOWDENS heifers throughout the experimental period. Heifers were weaned 7 d prior to the beginning of the

experiment, and maintained as a single group within a 6-ha pasture with ad libitum access to alfalfa-grass hay until d 0. During the experimental period (d 0 to 182), all heifers received the same limit-fed diet described in Table 1, in addition to ad libitum access to water and a commercial mineral and vitamin mix (Cattleman's Choice; Performix Nutrition Systems, Nampa, ID) containing 14 % Ca, 10 % P, 16 % NaCl, 1.5 % Mg, 6000 ppm Zn, 3200 ppm Cu, 65 ppm I, 900 ppm Mn, 140 ppm Se, 136 IU/g of vitamin A, 13 IU/g of vitamin D3, and 0.05 IU/g of vitamin E. Diets were offered daily at 0800 h in feed bunks with similar linear space across treatments (0.7 m/heifer). Hay was offered separated from concentrate, and the entire diet was completely consumed within 24 h after being offered.

Sampling

Hay and concentrate samples were collected at the beginning of the experiment, and analyzed for nutrient content by a commercial laboratory (Dairy One Forage Laboratory, Ithaca, NY, USA). Samples were analyzed in triplicate by wet chemistry procedures for CP (method 984.13; AOAC 2006), ADF (method 973.18 modified for use in an Ankom 200 fiber analyzer, Ankom Technology Corp., Fairport, NY; AOAC 2006), and NDF (Van Soest et al., 1991; method for use in an Ankom 200 fiber analyzer, Ankom Technology Corp.). Calculations for TDN used the equations proposed by Weiss et al. (1992), whereas NE_m and NE_l were calculated with the equations proposed by the NRC (2000). Nutritional profile of diets is described in Table 1.

Heifer shrunk BW was recorded after 16 h of feed and water withdrawal on d -3 and d 183 for ADG calculation. Heifer temperament was assessed via chute score, exit velocity, and overall temperament score as described by Cooke et al. (2014) on d 0 and

182. On d 0, heifers were also fitted with a pedometer (HJ-321; Omron Healthcare, Inc., Bannockburn, IL) placed inside a polyester patch (Heat Watch II; Cow Chips, LLC, Manalapan, NJ) fixed behind their right shoulder to assess physical activity (Haley et al., 2005; Knight et al., 2015). Pedometers had the capability to store daily data for 7 consecutive days, and remained on heifers throughout the experimental period.

Each week during the experiment (day 0 to 182), heifer full BW and pedometer results were recorded, and blood samples were collected via jugular venipuncture into commercial blood collection tubes (Vacutainer, 10 mL; Becton Dickinson, Franklin Lakes, NJ) with 158 USP units of freeze-dried sodium heparin for plasma collection. Steps on days when heifers were processed for sampling were not recorded. If a pedometer malfunctioned or was lost, a new pedometer was inserted during the weekly handling and data from the previous week was considered missing. All plasma samples were analyzed for progesterone concentrations to estimate onset of puberty. Heifers were considered pubertal once plasma progesterone concentrations were $\geq$ 1.0 ng/mL, followed by a cyclic pattern of plasma progesterone $<$ and $\geq$ 1.0 ng/mL suggestive of normal estrous cycles (Day et al., 1984). Puberty attainment was declared at the first sampling that resulted in plasma progesterone $\geq$ 1.0 ng/mL. Heifer age and BW at puberty was calculated based on weekly full BW measurements and heifer age at the week of puberty attainment. Plasma samples collected on d 0, 28, 56, 84, 112, 140, 161, and 182 were also analyzed for concentrations of cortisol and IGF-I.

On d 28, 102, and 175, blood samples were also collected via jugular venipuncture into PAXgene tubes (BD Diagnostics, Sparks, MD) for subsequent RNA isolation and

analysis of heat shock protein (**HSP**) 70, HSP72, *ribosomal protein 9*, and *β2-microglobulin* mRNA expression in blood cells via real-time quantitative reverse transcription (**RT**)-PCR. On d 0, 49, 98, 147, and 182, hair samples were collected from the tail switch (Burnett et al., 2014) for analysis of hair cortisol concentrations. Within each sampling, hair was collected from an area that has not been previously sampled. Hair was collected using scissors as close to the skin as possible, and the hair material closest to the skin (2.5 cm of length, 300 mg of weight) was stored at -80°C until processed for cortisol extraction.

Laboratorial analyses

For plasma collection, blood samples were placed immediately on ice after sampling, subsequently centrifuged (2,500 × *g* for 30 min; 4°C), and plasma stored at -80°C on the same day of collection. Plasma concentrations of progesterone, cortisol, and IGF-I were analyzed using chemiluminescent enzyme immunoassays (Immulite 1000; Siemens Medical Solutions Diagnostics, Los Angeles, CA). The intra- and interassay CV were, respectively, 5.1 and 5.8% for progesterone, 4.8 and 7.0% for cortisol, and 7.0 and 4.2% for IGF-I.

Upon collection, PAXgene tubes were stored at room temperature overnight and then at -80°C until RNA isolation. Total RNA was extracted from blood samples using the PAXgene Blood RNA Kit (Qiagen, Valencia, CA). Quantity and quality of isolated RNA were assessed via UV absorbance (NanoDrop Lite; Thermo Fisher Scientific, Wilmington, DE) at 260 nm and 260/280 nm ratio, respectively (Fleige and Pfaffl, 2006). Extracted blood RNA (120 ng) was reverse transcribed using the High Capacity cDNA

Reverse Transcription Kit with random hexamers (Applied Biosystems, Foster City, CA). Real-time RT-PCR was completed using the Fast SYBR Green Master Mix (Applied Biosystems) and gene-specific primers (20 pM each; Table 2) with the StepOne Real-time PCR system (Applied Biosystems), according to procedures described by Rodrigues et al. (2015). At the end of each RT-PCR, amplified products were subjected to a dissociation gradient (95°C for 15 s, 60°C for 30 s, and 95°C for 15 s) to verify the amplification of a single product by denaturation at the anticipated temperature. Responses were quantified based on the threshold cycle ($\mathbf{C_T}$), the number of PCR cycles required for target amplification to reach a predetermined threshold. The C_T responses from *HSP70 and HSP72* were normalized to the geometrical mean of C_T values from *ribosomal protein 9* and *β2-microglobulin* (Vandesompele et al., 2002). The CV for the geometrical mean of *ribosomal protein 9* and *β2-microglobulin* C_T values across all samples was 2.5%. Results are expressed as relative fold change ($2^{-\Delta\Delta CT}$), as described by Ocón-Grove et al. (2008).

Cortisol was extracted from hair samples based on the procedures described by Moya et al. (2013). Briefly, hair samples were cleaned with warm water (37°C) for 30 min, and dried at room temperature for 24 h. Hair samples were then washed twice with isopropanol, dried at room temperature for 120 h, and ground in a 10-mL stainless steel milling cup with a 12-mm stainless steel ball (Retsch Mixer Mill MM400 ball mill; Retsch, Hannover, Germany) for 5 min at a frequency of 30 repetitions/s. Twenty mg of ground hair and 1 mL of methanol were combined into a 7-mL glass scintillation vial, sonicated for 30 min, and incubated for 18 h at 50°C and 100 rpm for steroid extraction. Upon incubation, 0.8 mL of methanol was transferred to a 2-mL microcentrifuge tube

and evaporated at 45°C. Samples were reconstituted in 100 μL of the PBS supplied with a salivary cortisol ELISA kit (Salimetrics Expanded Range, High Sensitivity 1-E3002, State College, PA), and stored at -80°C. Samples were analyzed for cortisol concentrations using the aforementioned ELISA kit, whereas intra- and inter-assay CV were, respectively, 5.8 and 7.3%.

Statistical analysis

All data were analyzed using pen or pasture (3 replications/treatment) as experimental unit, with the MIXED or GLIMMIX procedure of SAS (SAS Inst., Inc., Cary, NC, USA) for quantitative and binary data, respectively, and Satterthwaite approximation to determine the denominator df for the tests of fixed effects. All data were analyzed using replication(treatment) and heifer(replication) as random effects. The model statement used for ADG, initial and final BW, as well as heifer BW and age at puberty contained the effects of treatment. The model statement for puberty attainment, physical activity, temperament and physiological variables contained the effects of treatment, day, and the treatment × day interaction. The specified term used in the repeated statement was day, the subject was heifer(replication), and the covariance structure utilized was autoregressive, which provided the best fit for these analyses according to the Akaike information criterion. Results are reported as least square means. Significance was set at $P \leq 0.05$ and tendencies were determined if $P > 0.05$ and ≤ 0.10. Results are reported according to effect of treatment if no interactions are significant, or according to the highest order interaction detected.

3. Results and Discussion

A treatment effect was detected ($P < 0.01$) for physical activity, given that LOWDENS had more steps/week compared with HIDENS heifers throughout the experiment (Table 3). This outcome can be attributed to the greater area that LOWDENS heifer had available for movement. Others have also reported greater physical activity in heifers reared on pasture compared with drylot cohorts (Petersen et al., 2014; Perry et al., 2015). However, elevated physical activity may increase maintenance requirements and reduce growth rates in cattle (Cooke et al., 2009), whereas no treatment differences were detected for heifer BW and ADG during the experimental period (Table 3). According to the NRC model (2000) and the stocking density assigned to LOWDENS, their NE_m requirements could be up to 15% greater compared with NE_m requirements of HIDENS heifers. Petersen et al. (2014) and Perry et al. (2015) also reported greater ADG in heifers reared in drylot compared with pastures, although nutritional management differed among heifer groups. In this experiment, pasture availability and grazing activity of LOWDENS heifers was deemed negligible due to previous hay harvest and wintery conditions; hence, it is unlikely that LOWDENS heifers consumed pasture in amounts that fulfilled potential increases in their NE_m requirements. Given that HIDENS and LOWDENS heifers were offered and completely consumed the same limit-fed diet, BW and ADG results indicate that the stocking densities evaluated herein did not impact growth rates in beef heifers receiving the same dietary regimen.

Treatment × day interactions were detected ($P < 0.01$) for plasma concentrations of cortisol and IGF-I (Figure 1). Plasma cortisol concentrations were greater in LOWDENS vs. HIDENS heifers on d 84, 140, 161, and 182 of the experiment. These outcomes were

unexpected according to previous research and our hypothesis that reared cattle in elevated stocking density experience increased adrenocortical stress response (Huzzey et al., 2006; Huzzey et al., 2012; Grandin, 2014). Plasma IGF-I concentrations were greater in LOWDENS vs. HIDENS heifers on d 84 and 140 but greater in HIDENS vs. LOWDENS heifers on d 161, which also does not corroborate with similar dietary management and ADG among treatments (Ellenberger et al., 1989; Yelich et al., 1995; Cappellozza et al., 2014). Nevertheless, plasma concentrations of IGF-I and cortisol are promptly increased in response to physical activity (Schwarz et al., 1996; Raastad et al., 2000; Hill et al., 2008). Hence, treatment effects for plasma cortisol and IGF-I concentrations can be attributed, at least partially, to the additional activity of gathering and bringing the LOWDENS heifers from pasture to the handling facility, whereas HIDENS heifers were grouped in drylot pens adjacent to the handling facility. Yet, treatment differences for plasma IGF-I and cortisol concentrations were inconsistently detected during the experimental period, which cannot be fully explained by the previous rationale or any other variables evaluated herein.

A treatment × day interaction was detected ($P < 0.01$) for hair cortisol concentrations, which were greater for HIDENS vs. LOWDENS heifers on d 98, 147, and 182 (Figure 2). Cortisol concentration in hair from the tail switch has been recently validated as biomarker of chronic stress in cattle (Burnett et al., 2014; Marti et al., 2015; Moya et al., 2015), given that cortisol is gradually accumulated in the emerging tail hair and its concentration represents long-term adrenocortical activity (Moya et al., 2013). Further, measuring cortisol in hair from the tail switch eliminates the confounding effects that gathering and handling cattle exert on plasma cortisol concentrations as observed herein (Figure 1; Moya et al., 2013, Moya et al., 2015). Hence, treatment differences

detected for this variable support our hypothesis that chronic stress and adrenocortical activity were indeed greater in HIDENS compared with LOWDENS heifers. Such outcomes were only noted beginning on d 98 of the experiment, which might be associated with the time required for elevated stocking density to be perceived as a stressor by HIDENS heifers, as well as the time required for hair with elevated cortisol concentration to cross the skin line and become available for collection (Burnett et al., 2014). Treatment effects on hair cortisol concentrations may also help explain the similar ADG among HIDENS and LOWDENS heifers. The greater chronic stress experienced by HIDENS heifers during the experiment may have increased their basal metabolism and maintenance requirements to the same level that physical activity increased these parameters in LOWDENS heifers (NRC, 2000; Petersen et al., 2014).

Heifers from the LOWDENS group had greater (P = 0.05) mRNA expression of HSP72, and tended (P = 0.10) to have greater mRNA expression of HSP70 compared with HIDENS heifers during the experiment (Table 3). Expression of HSP in blood cells can also be used as diagnostic marker of stress, given that HSP are rapidly synthesized when cells are exposed to a variety of stressors (Welch, 1992). Hence, treatment effects of blood mRNA expression of HSP70 and HSP72 do not corroborate with our hypothesis and treatment effects detected for hair cortisol concentrations. Nevertheless, HSP mRNA expression may not be directly regulated by elevated circulating cortisol, given that exogenous cortisol administration to fish did not increase mRNA expression of hepatic HSP70 (Deane et al., 1999; Basu et al., 2001). Conversely, exercise has been shown to stimulate mRNA expression and circulating concentrations of these HSP in rodents and humans (Naito et al., 2001; Febbraio et al., 2002; Milne and Noble, 2002). Exercise

activates the heat shock response via several mechanisms including increased muscle temperature, exercise-related production of reactive oxygen species, and muscle ATP depletion (Noble et al., 2008). Hence, treatment effects detected for mRNA expression of HSP70 and HSP72 should be attributed to the greater physical activity of LOWDENS vs. HIDENS heifers, either on a daily basis according to differences in stocking rate and steps/week (Table 3), or during gathering for weekly samplings corroborating with plasma cortisol and IGF-I outcomes (Figure 1).

Rearing cattle in intensive systems, such as drylot with elevated stocking density, results in increased human-animal interaction, which has been shown to impact cattle temperament and subsequent productivity (Fordyce et al., 1988; Cooke et al., 2014). However, LOWDENS and HIDENS heifers had similar ($P \geq 0.26$) chute score, exit velocity, and overall temperament score during the experiment (Table 3), suggesting that treatments evaluated herein were not sufficient to impact heifer temperament variables. In addition, cattle temperament has been directly associated with neuroendocrine reactions and subsequent circulating cortisol concentrations (Cooke et al., 2014). Hence, treatment differences detected for plasma and hair cortisol concentrations should not be attributed to heifer temperament, which in turn did not impact any of the heifer performance parameters evaluated herein.

A treatment x day interaction was detected ($P < 0.01$) for puberty attainment. In cattle, age at puberty is highly determined by BW and growth rate (Schillo et al., 1992); however, HIDENS heifers experienced delayed puberty attainment compared with LOWDENS heifers (Figure 3) despite their similar ADG (Table 2). At the end of the experimental period, a greater ($P < 0.01$; Figure 3) number of LOWDENS were pubertal

compared to HIDENS heifers (66.5 vs. 31.9 % pubertal heifers/total heifers; SEM = 5.8). Within heifers that reached puberty during the experiment, HIDENS were heavier (P = 0.05) and older ($P < 0.01$) at puberty attainment compared with LOWDENS heifers (328 vs. 363 d of age, SEM = 12; 319 vs. 372 kg of BW, SEM = 11; respectively). Collectively, these results indicate that rearing heifers in high stocking density delayed their onset of puberty despite adequate age and BW development, and reasons for this outcome likely include treatment differences among physical activity and chronic stress parameters. Regarding physical activity, exercise stimuli alter circulating concentrations of endogenous opioids that modulate gonadotropin secretion and consequent onset of puberty, cyclicity, and fertility in cattle (Harber and Sutton, 1984; Mahmoud et al., 1989). Accordingly, Lamb et al. (1979) reported that prepartum exercise regimens enhanced subsequent reproductive efficiency in dairy heifers without impacting BW change. Regarding stress and puberty attainment, chronic stress and the resultant increase in adrenocortical activity impairs gonadotrophin synthesis and release (Li and Wagner, 1983; Dobson et al., 2000) and reduces the sensitivity of the brain to estrogen (Hein and Allrich, 1992). Hence, the reduced physical activity and increased adrenocortical activity of HIDENS heifers, as evidenced by treatment differences on steps/week, blood mRNA expression of HSP, and hair cortisol concentrations, likely contributed to their delayed puberty attainment compared with LOWDENS cohorts.

In conclusion, rearing replacement beef heifers in drylots with high stocking density negatively impacted stress-related and physiological responses, and delayed puberty attainment compared with rearing heifers in pastures with low stocking density. Moreover, these outcomes were independent of heifer nutritional status and growth rate,

but were associated with reduced physical activity and increased chronic stress caused by high stocking density. Therefore, stocking density should be considered in heifer development programs to optimize reproductive and overall efficiency of cow-calf operations.

Table 1. Composition and nutrient profile (DM basis) of diets offered to heifers during the experiment.

	d 0 to 69	d 70 to 139	d 140 to 182
Ingredients, kg/hd/d			
Alfalfa-grass hay	4.1	4.1	4.0
Whole corn	2.5	3.0	3.5
Soybean meal	0.0	0.0	0.2
Nutrient profile[1]			
DM, %	91.8	86.0	91.7
TDN,[2] %	70.0	71.3	72.5
NDF, %	40.1	38.0	35.4
ADF, %	26.0	24.3	22.4
$NE_{m,}$[3] Mcal/kg	1.57	1.62	1.66
$NE_{l,}$[3] Mcal/kg	0.96	1.00	1.04
CP, %	10.9	10.9	11.9
Nutrient intake[2]			
DM, kg/d	6.60	7.10	7.80
TDN,[2] kg/d	4.62	5.06	5.66
NDF, kg/d	2.65	2.70	2.76
ADF, kg/d	1.72	1.73	1.75
$NE_{m,}$[3] Mcal/d	10.4	11.5	13.0
$NE_{l,}$[3] Mcal/d	6.34	7.10	8.11
CP, kg/d	0.72	0.77	0.93

[1] Based on feed analyses by a commercial laboratory wet chemistry analysis (Dairy One Forage Laboratory, Ithaca, NY, USA).
[2] Calculated according to the equations described by Weiss et al. (1992).
[3] Calculated with the following equations (NRC, 2000): Calculated with the equation (NRC, 2000): $NE_m = 1.37\ ME - 0.138\ ME^2 + 0.0105\ ME^3 - 1.12$; $NE_l = 1.42\ ME - 0.174(ME)^2 + 0.0122(ME)^3 - 0.165$. Given that ME = DE × 0.82, and 1 kg of TDN = 4.4 Mcal of DE.

Table 2. Primer sequences for all gene transcripts analyzed by quantitative real-time RT-PCR.

Target gene	Primer sequence 5' to 3'	Accession no.	Reference
Heat shock protein 70			
Forward	CGGCTTAGTCCGTGAGAACA	BTU09861	Liu et al. (2014)
Reverse	CCGCTCGGTATCGGTGAA		
Heat shock protein 72			
Forward	AACATGAAGAGCGCCGTGGAGG	U02892	Lacetera et al. (2006)
Reverse	GTTACACACCTGCTCCAGCTCC		
Ribosomal protein 9			
Forward	ACATCCCGTCCTTCATCGT	NM001101152	Liu et al. (2014)
Reverse	GCCCTTCTTGGCGTTCTT		
β2-microglobulin			
Forward	GGGCTGCTGTCGCTGTCT	NM_173893	Silva et al. (2008)
Reverse	TCTTCTGGTGGGTGTCTTGAGT		

Table 3. Growth parameters, activity and temperament variables, and blood mRNA expression of heat shock proteins (**HSP**) in heifers reared in low stocking density (25,000 m^2/heifer; **LOWDENS**, n = 3) or high stocking density (14 m^2/heifer; **HIDENS**, n = 3).[1]

Item	LOWDENS	HIDENS	SEM	*P* =
Growth parameters				
Initial BW (d -3), kg	211	212	3	0.78
Final BW (d 183), kg	355	358	5	0.70
ADG,[2] kg/d	0.77	0.78	0.02	0.66
Activity				
Steps/week[3]	19,839	3,147	628	< 0.01
Temperament variables[4]				
Chute score	1.91	1.84	0.09	0.58
Exit velocity, m/s	2.15	1.97	0.16	0.43
Temperament score	2.52	2.37	0.14	0.47
HSP mRNA expression[5]				
HSP70	3.80	2.40	0.48	0.10
HSP72	3.52	2.77	0.20	0.05

[1] From d 0 to 182, HIDENS heifers were reared in 1 of 3 drylot pens (10 x 14 m pens; 10 heifers/pen) and LOWDENS heifers were reared in 1 of 3 meadow foxtail (*Alopecurus pratensis* L.) dominated pastures (25-ha pastures; 10 heifers/pasture).
[2] Calculated using initial (d -3) and final (d 183) shrunk BW, which was recorded after 16 h of feed and water withdrawal.
[3] Based on pedometers (HJ-321; Omron Healthcare, Inc., Bannockburn, IL) assessed every 7 d during the experimental period.
[4] According to the techniques described by Cooke et al. (2014), and evaluated on d 0 and 182 of the experiment.
[5] Samples collected on d 28, 102, and 175 of the experiment, processed and evaluated for mRNA expression according to Rodrigues et al. (2015).

Figure 1. Plasma concentrations of cortisol (Panel A) and IGF-I (Panel B) from heifers reared in low stocking density (25,000 m^2/heifer; **LOWDENS**) or high stocking density (14 m^2/heifer; **HIDENS**) from d 0 to 182 the experiment. A treatment × day interaction was detected ($P < 0.01$). Within days, * $P \leq 0.05$, ** $P \leq 0.01$.

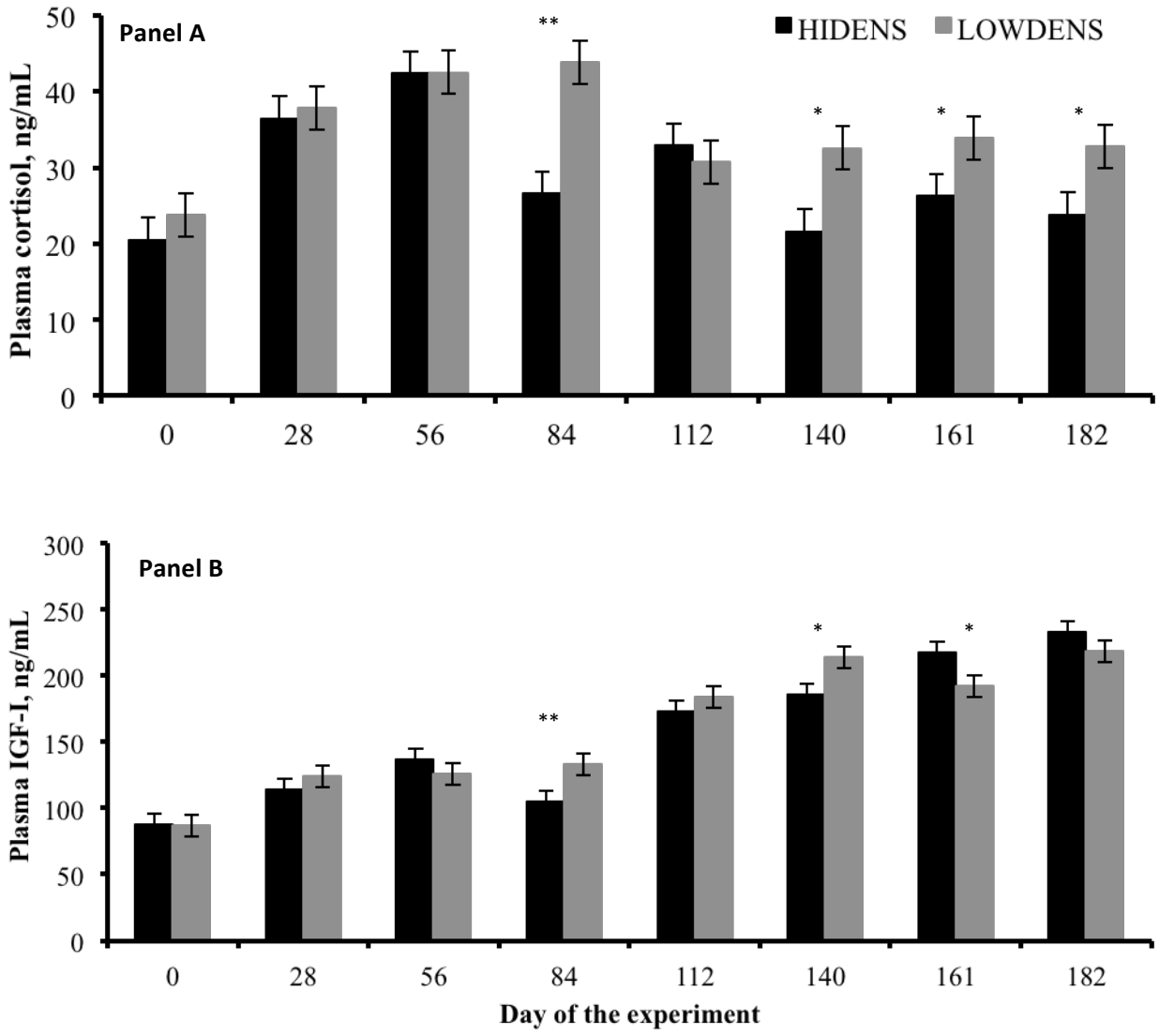


Figure 2. Cortisol concentrations in tail switch hair from heifers reared in low stocking density (25,000 m^2/heifer; **LOWDENS**) or high stocking density (14 m^2/heifer; **HIDENS**) from d 0 to 182 the experiment. A treatment × day interaction was detected ($P < 0.01$). Within days, ** $P \leq 0.01$.

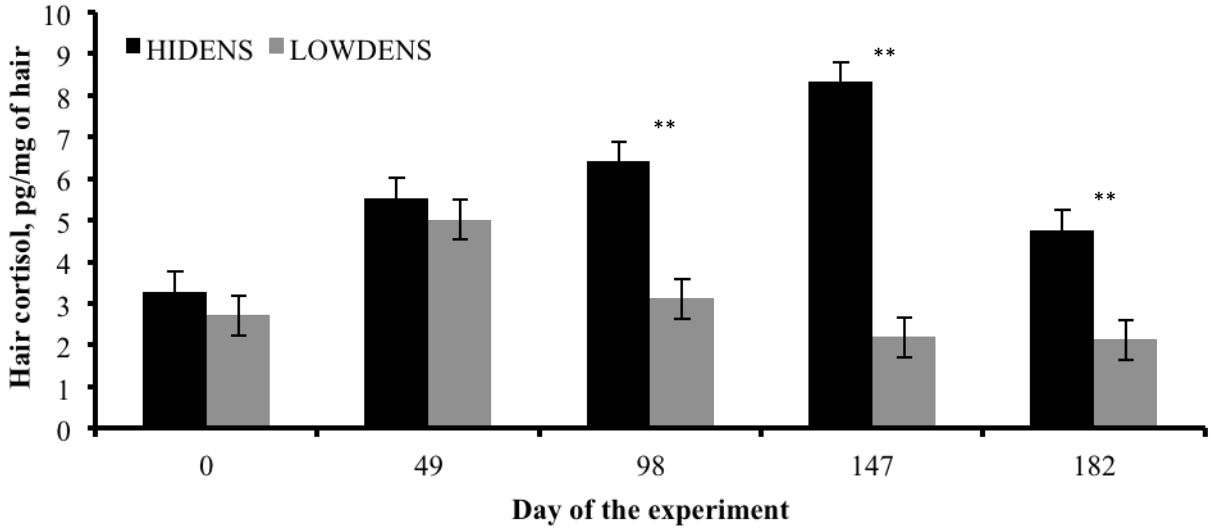
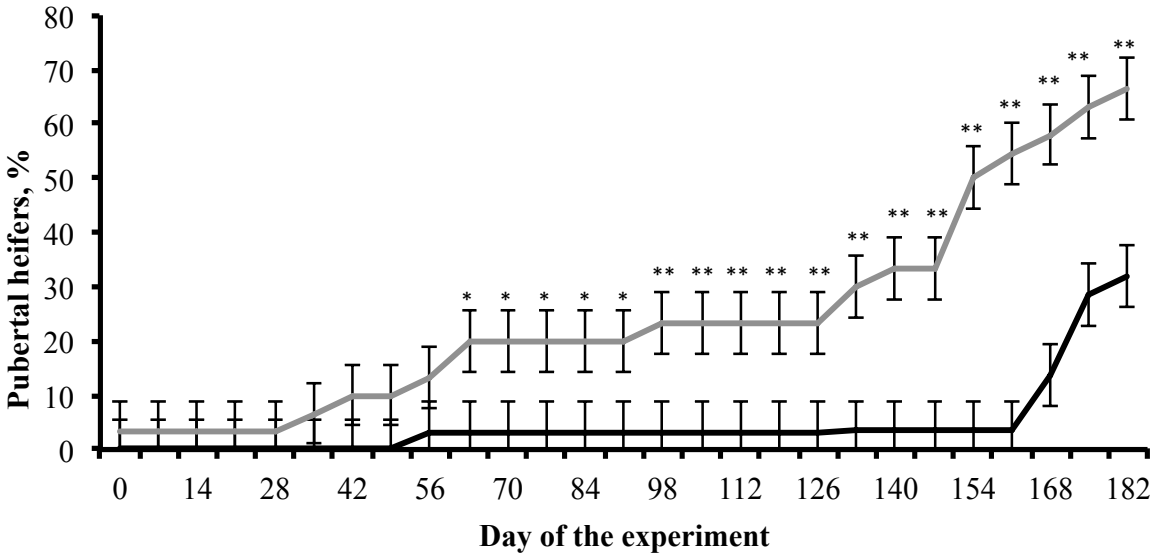


Figure 3. Puberty attainment in heifers reared in low stocking density (25,000 m^2/heifer; **LOWDENS**) or high stocking density (14 m^2/heifer; **HIDENS**) from d 0 to 182 the experiment.

Puberty was evaluated according to plasma progesterone concentrations in samples collected weekly during the experiment. Heifers were considered pubertal once plasma progesterone concentrations were ≥ 1.0 ng/mL, followed by a cyclic pattern of plasma progesterone < and ≥ 1.0 ng/mL suggestive of normal estrous cycles (Day et al., 1984). Puberty attainment was declared at the first sampling that resulted in plasma progesterone ≥ 1.0 ng/mL. A treatment × day interaction was detected ($P < 0.01$). Within days, ** $P \leq 0.01$.



CHAPTER IV

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