SEASONAL VARIATION OF NUTRITIONAL HORMONES IN CAPTIVE FEMALE MOOSE

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ABSTRACT: The health status of animals may be inferred from the patterns of hormonal concentrations and other chemical characteristics in blood samples. Baseline endocrine data representing the nutritional and reproductive condition of moose are currently unknown. In this study, we examined the seasonal patterns of 3 nutritional hormones (leptin, ghrelin, insulin-like growth factor-1) in 3 captive, non-pregnant female moose (*Alces alces)* fed a maintenance diet from November to August. Plasma concentrations for leptin, ghrelin, and IGF-1 averaged 1.36 ± 0.81 ng/mL, 0.229 ± 0.110 ng/mL, and 114.0 ± 30.5 ng/mL, respectively; only ghrelin displayed a seasonal change. Plasma ghrelin concentration was significantly elevated ($P < 0.001$) during winter months suggesting it may be sensitive to seasonal changes and indicative of nutritional status.

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Northern latitudes are characterized by extreme seasonal differences in temperature, photoperiod, forage availability, and forage quality (Chapin et al. 1980, Risenhoover 1989). As a consequence, large herbivores such as moose (*Alces alces*) may experience seasonal nutritional constraints that can impact their health and fecundity (Cook et al. 2001, Tollefson et al. 2010). In south-central Alaska, pregnancy and calving rates of moose are positively associated with autumn body condition (Testa and Adams 1998). In northern Alaska, heavier caribou (*Rangifer tarandus*) in autumn were more likely to have a successful pregnancy (Cameron et al. 1993), suggesting that a threshold body condition must be attained to trigger reproduction (Thomas 1982). However, this evidence is largely circumstantial and fails to provide a mechanistic connection between body condition and reproduction. Our study focused on 3 hormones - leptin, ghrelin, and insulin-like growth factor-1 (IGF-1) - that relay satiety information to the central nervous system (CNS) and influence reproduction.

Leptin, an adipose-derived hormone (Zhang et al. 1994) and product of the *Ob* gene, is secreted by white adipose tissue in direct proportion to the adiposity of an animal (Delavaud et al. 2002, Geary et al. 2003) and influences appetite, energy expenditure, and reproductive function (Zieba et al. 2008, Friedman 2010). Correlations between plasma leptin concentration and adiposity are established in various animal species including cattle (*Bos taurus*; Block et al. 2001) and sheep (*Ovis aries*; Blache et al. 2000). Receptors for leptin exist at each tier of the

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hypothalamus-pituitary-gonadal (HPG) axis, suggesting a mechanism of action on these target tissues by leptin (Brann et al. 2002).

Ghrelin is a gut-derived hormone synthesized and secreted by the abomasal and ruminal tissues in ungulates (Hayashida et al. 2001, Geary et al. 2003). It is the natural ligand of the growth hormone secretagogue (a substance eliciting release of another substance) receptor, and elicits the release of growth hormone (GH) from the pituitary (Shintani et al. 2001). Ghrelin is a potent neuroendocrine integrator, influencing a myriad of endocrine and non-endocrine functions (Barreiro and Tena-Sempere 2004, Fernandez-Fernandez et al. 2006). Circulating ghrelin levels are elevated above baseline concentrations prior to feeding, and return rapidly to baseline after re-alimentation in sheep (Sugino et al. 2002) and rats (*Rattus norvegicus*; Toshinai et al. 2001). High ghrelin levels stimulate appetite, decrease energy expenditure, and hinder reproduction in several species (Tena-Sempere 2005, Budak et al. 2006).

IGF-1, similar to ghrelin and leptin, is a peptide hormone that is influenced by nutrition (Daftary and Gore 2005). In mammals, IGF-1 is primarily produced by the liver (Gluckman et al. 1991) and is synthesized and released in response to the presence of GH (Ketelslegers et al. 1995). In steers on a low plane of nutrition, IGF-1 concentrations are reduced and thought to help conserve energy during times of negative energy balance (Breier et al. 1986). It exerts positive effects on bone growth, protein synthesis, and somatic growth, and likely plays a role in regulating reproduction given that gonadotropin releasing hormone (GnRH) neurons in the brain express both IGF-1 and IGF-1 receptors (Suttie and Webster 1995, Daftary and Gore 2005).

Receptors for leptin, ghrelin, and IGF-1 are widely distributed throughout the body, including the hypothalamus, pituitary, and ovaries, indicating possible direct and indirect actions at all levels of the HPG axis (Gluckman et al. 1991, Hodgkinson et al. 1991, Brann et al. 2002, Tena-Sempere 2005, Zhang et al. 2008). Studies on sheep, rats, and cattle suggest that leptin and ghrelin are necessary for normal secretion patterns of GnRH (Hileman et al. 2000, Zieba et al. 2003) and pulsatile rhythms of luteinizing hormone (LH; Hileman et al. 2000, Nagatani et al. 2000). Moreover, evidence exists that leptin, ghrelin, and IGF-1 help regulate embryo implantation (Kawamura et al. 2003) and timing of puberty (Ahima et al. 1997, Chehab at al. 1997). Collectively, leptin, ghrelin, and IGF-1 appear to communicate nutritional status to the CNS, which in turn, influences reproductive function (Tena-Sempere 2005, Budak et al. 2006).

Understanding the role of nutritional hormones in regulating appetite, energy expenditure, and reproduction could provide an important tool for assessing nutritional and reproductive status of wild herbivores. Assessment of this potential role requires accurate assays and knowledge of baseline seasonal concentrations of these hormones. To date, leptin and ghrelin have not been characterized in moose. Our objectives were to: 1) adapt and validate assays for leptin, ghrelin, and IGF-1 in moose, 2) provide baseline measures for leptin, ghrelin and IGF-1, and 3) examine the influence of season on leptin, ghrelin, and IGF-1 in moose on a maintenance diet.

METHODS

This study was conducted at the University of Alaska Fairbanks (UAF) Matanuska Experiment Farm (MEF) near Palmer, Alaska (61º 33′ 57 N, 149º 15′ 05 W). The captive moose were maintained throughout the study in fenced 4-ha paddocks with access to a lake for water.

Design and species

Our study was designed to provide baseline seasonal profiles of the 3 nutritional

hormones in captive, tractable female moose (5 to 9 years of age) over a 10-month period beginning in November 2009. All animals were hand-raised orphans collected from the Matanuska-Susitna Borough and Municipality of Anchorage in south-central Alaska, and were conditioned to the experimental protocol such that moving the animals and drawing blood required neither sedation nor restraint. They were fed a nutritionally balanced pelleted ration (Alaska Mill & Feed Supply, Anchorage, Alaska) at a maintenance level of 1.25% of body weight. They were provided *ad libitum* access to ensiled brome (*Bromus inermis*) hay and pasture, and had access to native forages in the paddock including paper birch (*Betula papyrifera*), balsam poplar (*Populus balsamifera*), quaking aspen (*Populus tremuloides*), Scouler willow (*Salix scouleriana*), rose (*Rosa acicularis*), and low bush cranberry (*Vaccinium oxycoccos*). Availability of native forage was limited due to previous overbrowsing by captive moose.

Blood sampling and processing

The moose were moved once monthly from their paddocks to individual 4 x 20 m stalls where blood samples were collected immediately prior to the morning feeding, after which they were returned to the paddock. Blood (6 cc) was drawn in a few seconds from the jugular vein through a 21 gauge needle attached to a 10 cc syringe; this procedure was not stressful to the animals. Blood samples were evacuated into ethylenediaminetetraacetic acid (EDTA) treated blood collection tubes (7 mL, 12 mg EDTA). The blood was mixed by inversion and centrifuged at 4000-x g for 10 min to separate the plasma and cellular components. The plasma fraction was transferred with a long-stemmed Pasteur pipette into 5 mL cryovials (Fischer Scientific, USA) that were immediately transferred to a cooler containing ice packs for same day transport to the laboratory. Each sample was then aliquoted into 5, 1.5 mL snap cap vials (Fischer Scientific, USA) and stored at -80 ºC until assayed. All methods were approved by the University of Alaska Anchorage (Protocol #181596-2) and University of Alaska Fairbanks (Protocol #182744-2) Institutional Animal Care and Use Committees.

Hormone assays and validation

Hormones were assayed using either enzyme-linked immunosorbent (EIA) or radioactive immunosorbent assays (RIA). All plasma samples were assayed in duplicate, and each assay contained a pooled plasma sample for tests of inter- and intra-assay variation.

Ghrelin levels were measured with a commercially available, double-antibody, RIA kit (GHRT-89HK, Millipore Total ghrelin, St. Charles, Michigan, USA) following the manufacturer's recommended protocol with a slight modification to account for low sample volumes. The radioactive pellet was counted on a Laboratory Technologies Genesys gamma counter (Genii Model LTl1010, Maple Park, Illinois, USA). Kits were validated using standard tests of parallelism and accuracy on pooled plasma samples. Serial dilutions of pooled samples and standards exhibited parallelism for each respective hormone; specifically, ghrelin had a parallelism R^2 value of 0.983. Tests of accuracy for ghrelin had an $R^2 = 0.980$ with a minimum detectable concentration of 0.093 ng/mL. Intra- and inter-assay coefficients of variation (CV) for ghrelin were 12.45 and 9.71%, respectively.

Leptin and IGF-1 concentrations were measured in triplicate with a competitive, liquid-liquid phase, double-antibody leptin/IGF-1 RIA at the University of Missouri. The leptin RIA followed the procedure established for cattle (Delavaud et al. 2000) and modified for use with rabbit anti-ovine leptin primary anti-serum #7105. Minimum

detectable concentration was 0.1 ng/tube, and inter- and intra-assay CVs were 5%. The IGF-1 RIA followed the procedure established previously for cattle (Lalman et al. 2000). Minimum detectable concentration was 1.5 ng/tube, and inter- and intra-assay CVs were < 6%.

Descriptive statistics were computed (SPSS 17.0, Armonk, New York, USA) for each animal and hormone. To test for seasonal differences, winter was defined as November through April, and summer as May through August; these intervals were chosen to fit with astronomical seasons and temporal change in forage quality. Seasonal concentrations of hormones were analyzed in R 3.1.3 using a one-way ANOVA; all differences were considered significant at $\alpha \leq 0.05$.

RESULTS

Plasma leptin concentration averaged 1.36 ± 0.81 ng/mL (Table 1) and mean individual concentrations ranged from 0.73–1.99 ng/mL. Although no significant seasonal differences were found ($F_{1,28}$ = 0.0710, P = 0.792), individual leptin concentrations varied widely across time (Table 1, Fig. 1). Plasma ghrelin concentrations averaged 0.229 ± 0.110 ng/mL (Table 1) with individual concentrations ranging from 0.081–0.337 ng/mL. Mean ghrelin plasma concentrations were significantly higher in winter than summer ($F_{1,28}$ = 42.5, P < 0.001; Table 1, Fig. 2); concentrations declined in all moose in May, and except for 1 animal (AR) in June, remained < 0.200 ng/mL in June-August (Fig. 2). Plasma concentrations of IGF-1 averaged 114.0 ± 30.5 ng/mL (Table 1) with mean concentrations of individuals ranging from 84.7–149.0 ng/mL; no seasonal difference was found ($F_{1,28}$ = 3.32, P = 0.079; Fig. 3).

DISCUSSION

Leptin

Leptin concentrations in this study were lower than those reported in domestic cattle and sheep (Chilliard et al. 1998), but similar to the lower range (1.20 – 2.63 ng/mL) measured in male Iberian red deer (*Cervus elaphus hispanicus;* Gaspar-López et al. 2009) and reindeer (*Rangifer tarandus*; Soppela et al. 2008). In addition, plasma leptin concentrations in non-pregnant red deer (*Cervus elaphus*) hinds (Scott 2011) were higher than those of our moose, indicating that captive moose are in the lower range reported for other

Table 1. Mean monthly hormone concentrations with standard errors for leptin, ghrelin, and IGF-1 measured in 3 captive female moose at the Matanuska Experimental Farm in Palmer, Alaska, November to August 2010.

		Leptin (ng/mL)		Ghrelin (ng/mL)		IGF-1 (ng/mL)	
Month	n	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE
Nov	3	1.43	0.11	0.257	0.067	100.0	7.0
Dec	3	1.12	0.23	0.337	0.025	119.0	10.2
Jan	3	1.09	0.28	0.303	0.032	103.0	14.9
Feb	3	1.90	0.57	0.271	0.014	84.7	17.1
Mar	3	1.58	0.75	0.301	0.031	111.0	23.8
Apr	3	1.26	0.42	0.321	0.033	116.0	25.6
May	3	1.42	0.54	0.157	0.065	134.0	21.7
Jun	3	1.43	0.81	0.170	0.057	149.0	8.6
Jul	3	1.31	0.57	0.100	0.038	129.0	5.5
Aug	3	1.10	0.60	0.081	0.029	91.4	14.9

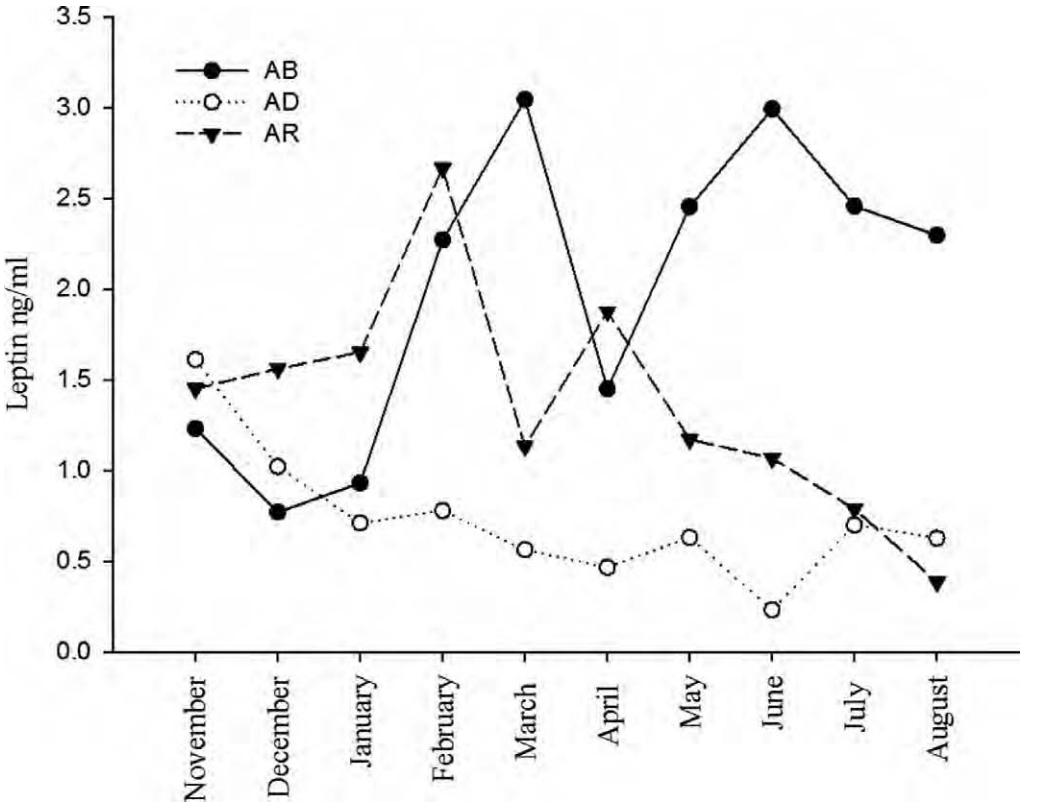

Fig. 1. Seasonal concentrations of leptin in 3 captive moose fed a maintenance diet at the Matanuska Experimental Farm in Palmer, Alaska, November to August 2010. Each letter combination represents an individual moose.

cervids. Although moose typically gain body fat during summer and fall and deplete these reserves over winter (Schwartz et al. 1987, Franzmann and Schwartz 1998), we did not expect the captive moose to exhibit these typical fluctuations in body condition given their high quality diet.

The highly variable leptin concentrations observed throughout the year suggest that leptin may not be regulated solely by adiposity level, and as a result, is not an adequate singular measure of fat mass in moose. While not investigated here, other studies indicate that leptin may respond to the overall nutritional status of an animal and its environment, rather than adiposity (Daniel et al. 2002), a possible explanation for the absence of seasonal change. In addition to nutritional status, photoperiod might be a seasonal cue capable of modifying leptin concentrations in ruminants and non-ruminants (Bocquier et al. 1998, Chilliard et al. 2005, Soppela et al. 2008). For example, leptin concentration in well-nourished reindeer declined in early winter as animals maintained body weight and feed intake, suggesting that photoperiod, rather than feed intake and quality, plays an important role in controlling leptin concentration (Soppela et al. 2008). Further, leptin declined in ovariectomized ewes exposed to short days regardless of nutritional status and changes in adipose mass (Bocquier et al. 1998). It is reasonable to expect that photoperiod could influence leptin concentrations in our moose given the extreme changes in daylength at northern latitudes.

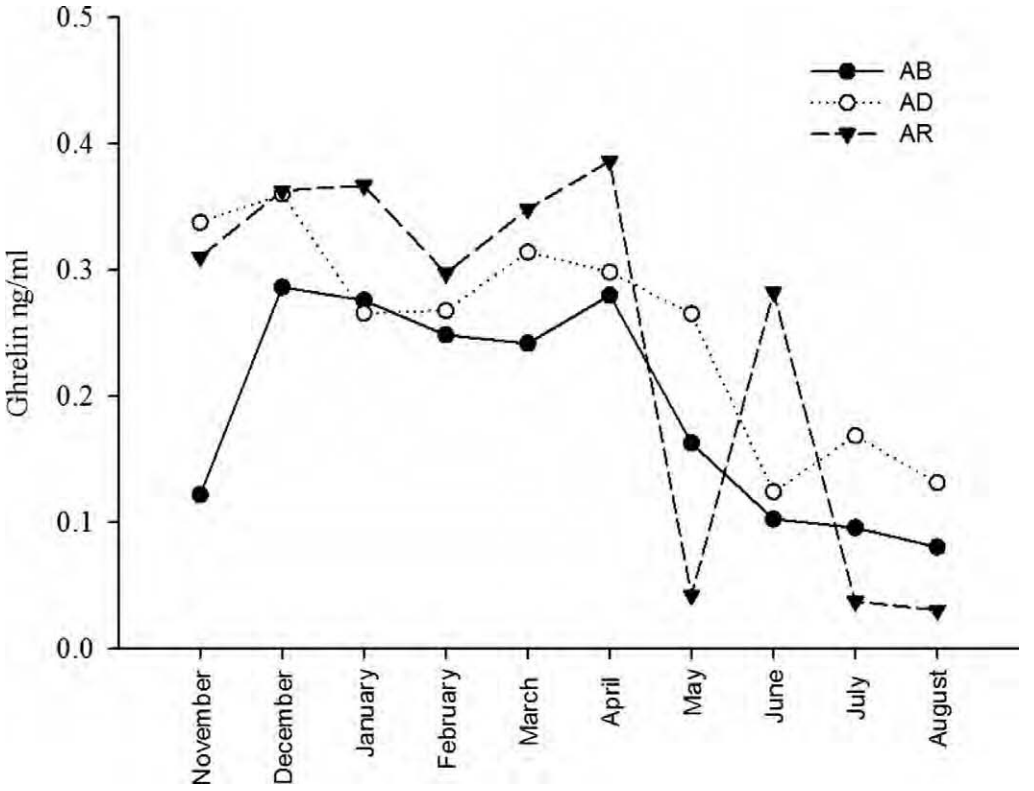

Fig. 2. Seasonal concentrations of ghrelin in 3 captive moose fed a maintenance diet at the Matanuska Experimental Farm in Palmer, Alaska, November to August 2010. Each letter combination represents an individual moose.

Ghrelin

Ghrelin is a gut-derived hormone that is one of many influences on appetite, fattening, and reproduction via input to the HPG axis (Gentry et al. 2003, Tena-Sempere 2008). Ghrelin fluctuates in response to change in the short-term nutritional state of animals, with plasma concentrations significantly elevated in food restricted animals (Gualillo et al. 2002, Bradford and Allen 2008). Mean ghrelin concentrations were lower in our moose compared to *ad libitum* fed steers (0.229 vs 0.123 ng/mL) and Holstein heifers (Wertz-Lutz et al. 2006, Field et al. 2013), and slightly lower than mean ghrelin concentrations in fasted Holstein heifers (Field et al. 2013).

We expected ghrelin concentration to remain stable because of the consumption of a high quality maintenance diet. Conversely, given the relationship between ghrelin and feeding and gut fill, we expect that concentrations would fluctuate seasonally in wild animals. Ghrelin concentration in our captive moose varied seasonally, with higher pre-feeding concentrations in winter than summer months for all animals, suggesting that in moose, ghrelin may not solely respond to changes in gut fill or appetite, but may be influenced by seasonal changes in energy expenditure and/or other physiological processes. This contrasts with research on rats (Toshinai et al. 2001) and Angus steers (Wertz-Lutz et al. 2008) that indicated ghrelin levels in food-deprived or food-restricted animals are significantly higher than in fed animals, presumably due to energy restriction. Additionally, the preprandial ghrelin surge

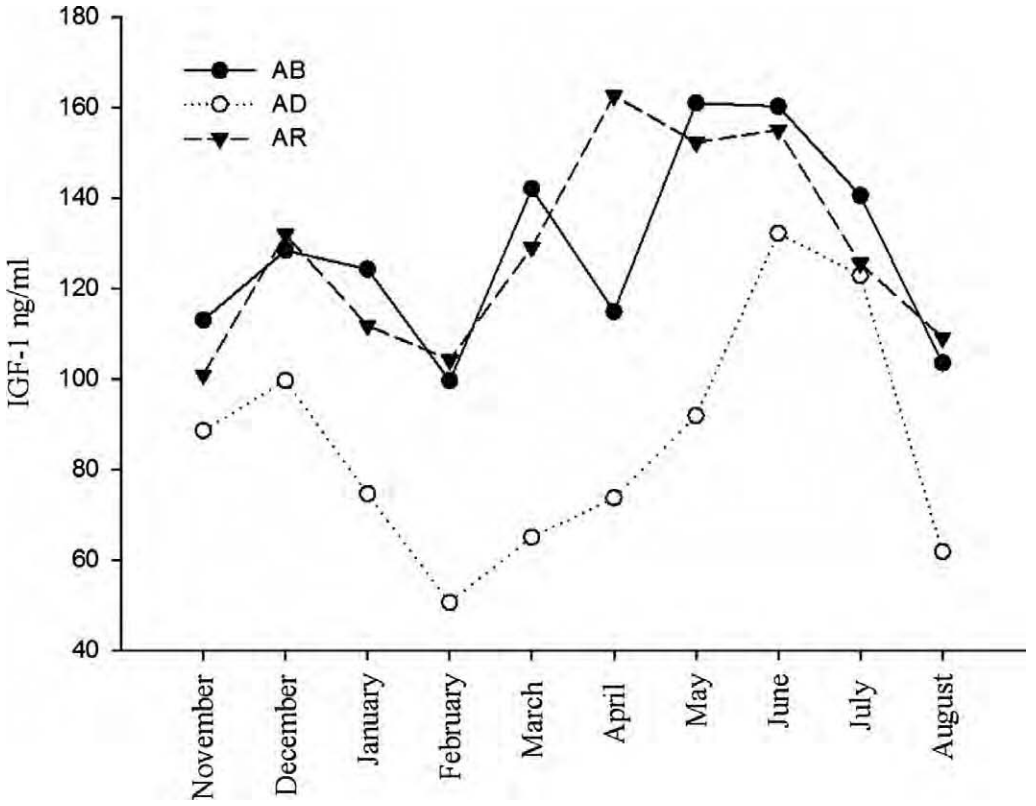

Fig. 3. Seasonal concentrations of IGF-1 in 3 captive moose fed a maintenance diet at the Matanuska Experimental Farm in Palmer, Alaska, November to August 2010. Each letter combination represents an individual moose.

in Suffolk rams can be modified by feeding restriction (Sugino et al. 2002). A seasonal response would correspond with seasonal changes in forage quality and consumption level, and facilitate elevated ghrelin concentrations during those seasons with poor forage quality and reduced consumption and energy expenditure. We believe ghrelin concentration is reflective of a longer temporal window, not just the immediate period prior to feeding. The ghrelin spike in moose "AR" during June remains inexplicable.

Insulin-like Growth Factor-1

IGF-1 is essential in stimulating bone growth and protein synthesis (Suttie and Webster 1995). Additionally, IGF-1 receptors are located throughout the body including sites on GnRH neurons, suggesting that it can influence reproduction via neuroendocrine pathways (Daftary and Gore 2005). Concentrations in the current study did not change between winter and summer months, and individual peak concentrations were similar to those in muskoxen (*Ovibos moschatus*; Adamczewski et al. 1997) and reindeer (Suttie and Webster 1995). The overall mean concentration was similar to those reported in wild moose sampled from late fall through late winter in south-central and southeast Alaska (90.7–135.0 ng/mL; Parillo 2010). In contrast, although captive muskoxen had similar mean concentrations as our moose, wild muskoxen had lower concentrations (Adamczewski et al. 1997). Reindeer had higher peak levels overall (Bubenik et al. 1998), with their bottom range similar to our values.

The absence of any seasonal change in IGF-1, and the minor/lack of change in body condition supports the idea that IGF-1 in moose is influenced, in part, by active and energetically expensive physiological processes as with other species (e.g., the active rebuilding of body stores and growth). Other ungulate species exhibit increasing IGF-1 concentration in conjunction with growth in young animals, restoration of lean body mass in adults, increase in photoperiod, and the timing of forage quality and quantity (Kerr et al. 1991, Suttie et al. 1991, Adamczewski et al. 1992, Ditchkoff et al. 2001). In contrast, Parillo (2010) reported higher IGF-1 concentrations in winter than fall in wild Alaskan moose, although the winter values were based on a single, rather than multiple collections across the entire season.

Conclusion

Our results indicate that leptin, ghrelin, and IGF-1 can be measured with accuracy and precision in moose using standard protocols with certain modifications. Plasma leptin concentrations were highly variable between and within moose and without a seasonal pattern over the 10-month period, suggesting that leptin is not regulated singularly by adiposity, but by other hormonal/physiological mechanisms. In contrast, plasma ghrelin concentrations exhibited a seasonal pattern over the same period, with higher concentrations measured during winter months; however, it is difficult to determine if this was an artifact of sample size or physiological response. Lastly, IGF-1 concentrations reflected neither monthly nor seasonal changes; given our small animal sample, it could not be determined if this was due to individual variation or a seasonal response. Using any of these hormones as definitive indicators of nutritional status or reproductive condition of moose is not appropriate because levels varied considerably over time and among individuals. Further investigations are warranted given the complexity of hormonal regulation in the physiological and reproductive processes in moose.

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