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Bovine dopamine type-2 receptor SNP has no effect on growth, semen characteristics and prolactin concentrations in beef bulls treated with a dopamine agonist

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ABSTRACT

A dopamine type-2 receptor (DRD2) SNP, previously found to be correlated with serum prolactin (PRL) concentrations in cattle, was evaluated for impact on growth traits, serum prolactin concentration, and semen quality. Over a four-year period, yearling beef bulls were allowed diets containing or lacking ergot alkaloids (EA). Every 21 or 28 d semen was collected for semen motility and morphology assessment and blood samples were collected to measure serum PRL concentrations. In addition, body condition score and scrotal circumference were evaluated. Serum PRL concentrations were assessed using a radioimmunoassay. In the first year, all bulls were sacrificed at the end of a 126-day study. Testicles and epididymis were collected at the end of the study or 60 days after removal from treatment. Immunohistochemistry was performed on testis, epididymis, and sperm cells, incubated with or without a primary antibody for DRD2 and counterstained with DAPI. Isolation of DNA was performed on sperm pellets using DNAzol (Thermo Fisher Scientific, Waltham, MA, USA) methods. Polymerase chain reaction was performed to amplify the region of the DRD2 gene containing the SNP of interest. The products were subjected to restriction fragment length polymorphism analysis. Further, all samples were subjected to genotyping using a custom Taqman genotyping assay (Applied Biosystems, Foster city, CA, USA). The presence of DRD2 was detected in the testis, epididymis, and sperm cells. The DRD2 genotype was not associated with semen quality, serum PRL, or growth traits. Consumption of EA resulted in lesser PRL serum concentrations but had no effect on values for other variable examined.

1. Introduction

Prolactin (PRL) is an ~ 22 kDa peptide produced primarily by pituitary lactotrophs and is regulated by dopamine through tonic inhibition (Ben-Jonathan and Hnasko, 2001). Prolactin modulates more than 300 biological functions across species (Bole-Feysot et al., 1998; Freeman et al., 2000). Hyperprolactinemia is associated with erectile dysfunction as well as infertility in men which improves through treatment with a dopamine agonist (Segal et al., 1979). Dopamine is the main regulator of PRL *via* tonic inhibition (Ben-Jonathan and Hnasko, 2001) and the bovine dopamine type-2 receptor (DRD2) gene possesses several single nucleotide

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polymorphisms (SNP), with one SNP reported to be correlated with serum PRL concentrations (Campbell et al., 2014). This SNP is located within Intron 3 and, therefore, does not likely affect protein structure or function and currently there is little information available on the mechanism in which this SNP located in a non-coding portion of the gene (Campbell et al., 2014) acts. Currently all information is associative and little direct evaluation of the DRD2 SNP on PRL gene expression is available as well as there has been little to no evaluation of this SNP's effect on bull growth and reproduction.

The effect of dopamine type-2 receptor genotype on PRL serum concentrations and hair coat scores was evaluated in steers consuming or not consuming forage containing ergot alkaloids (EA) (Campbell et al., 2014). The DRD2 SNP located within Intron 3 at position 404,365 on chromosome 15, includes a base substitution between adenine and guanine at position 534 (Campbell et al., 2014). The dopamine type-2 receptor genotype was correlated with serum PRL concentrations, in which homozygous AA steers were associated with greater serum PRL concentrations compared to GG steers in April and May but not June months. While the DRD2 SNP (Campbell et al., 2014) is located within a non-coding region and is not likely involved in protein folding or structure, the SNP may be involved in altering gene expression within the PRL pathway. Interestingly, the SNP does not appear to represent an alternate acceptor site to generate alternative splice variants of the DRD2 mRNA, nor has splice variants been reported for the bovine DRD2. Alternate splicing of DRD2 does occur to form long and short forms of the receptor to facilitate coupling of G-proteins and has been reported in the rat and human pituitary and brain (Dal Toso et al., 1989). Both, long and short forms of DRD2 are able to suppress PRL transcription (McChesney et al., 1991). This alternative splicing of DRD2, however, has not yet been reported in cattle. Evidence for effects on the PRL pathway due to dopaminergic SNPs exist with some continued differences in severity of effects in male physiology. Homozygosity of the polymorphism located on Exon 7 of DRD2, was found to be associated with hyperprolactinemia in humans (Hansen et al., 2005). Furthermore, an insertion-deletion located within the DRD2 promoter region was associated with sexual dysfunction and lesser serum prolactin concentrations in human male patients (Zhang et al., 2011). The aforementioned polymorphisms, however, were not located within a non-coding region such as the SNP of interest in this study.

Cattle grazing a forage producing EA, a naturally occurring dopamine agonist, have relatively lesser serum PRL concentrations (Schillo et al., 1988; Strickland et al., 2011). Ergot alkaloids can bind many neurotransmitter receptors (Larson et al., 1995; Mizinga et al., 1993; Schoning et al., 2001; Wang et al., 2009) to elicit physiological responses in cattle that consume them. Ergot alkaloids function agonistically with DRD2, inhibiting the synthesis and secretion of prolactin (Caron et al., 1978; Sibley and Creese, 1983; Thompson and Stuedemann, 1993; Paterson et al., 1995). Ergot alkaloid consumption may negatively affect semen motility and morphology (Looper et al., 2009; Pratt et al., 2015a), as well as affect bull fertility (Schuenemann et al., 2005). There, however, are inconsistencies in results among studies. There are some reports where there was no change in semen quality (Schuenemann et al., 2005; Stowe et al., 2013) or *in vivo* fertility in PRL knockout mice (Horseman et al., 1997). It is possible that EA affects the male physiology by binding to DRD2 receptors located in male reproductive tissues. Furthermore, DRD2 is localized in the acrosome and tail regions of spermatazoa in cattle, rats, and mice, and is also present in spermatocytes and spermtids within the testis of rats. Furthermore, dopamine type-2 receptors are also present in human spermatazoa, with the exception of the acrosome (Otth et al., 2007). This finding is consistent across species with DRD2 receptors present in male reproductive tissues (Otth et al., 2007; Ramírez et al., 2009; Gonzalez et al., 2015).

Results to date indicate there are negative effects on growth traits in cattle treated with or consuming a dopamine agonist. In some studies, there was a consistent and marked decrease in average daily gain (ADG) in cattle grazing EA (Hoveland, 1993; Crawford et al., 1989) while in other studies there was no difference in BW or BCS between treatments of bulls treated and those not treated with a dopamine agonist (Stowe et al., 2013). The decreased growth in animals grazing EA may be attributed to lesser concentrations of growth hormone (GH). The GH concentrations, however, have been reported to increase with treatment of EA (Thompsonm et al., 1987) or remain unchanged (Paterson et al., 1995).

With much variation in reports on growth and semen characteristics in cattle, clarification of the role of DRD2 genotypic effects is warranted. The objective of this study is to assess the presence of DRD2 in the testis, epididymis, and sperm of bulls and to assess if the DRD2 SNP (Campbell et al., 2014) has any effect on growth, semen quality, or serum PRL concentrations in bulls treated with a naturally occurring dopaminergic agonist, EA.

2. Materials and methods

2.1. Experimental design

All animal research was approved by the Clemson University Institutional Animal Care and Use Committee (AUP #2010-068 and AUP # 2014-60).

2.2. Treatment

Over 4 years, 2011 (n = 14), 2012 (n = 21), 2014 (n = 25) and 2015 (n = 29), yearling beef bulls were fed a ration that included or excluded ergot alkaloids. In 2011, bulls were fed a concentrate ration which contained or lacked tall fescue seeds containing or lacking EA at 0.8 μg/g dry matter (DM) (Stowe et al., 2013). In all other years, bulls were subjected to grazing forage that produced or lacked EA. Grazing treatments were performed according to Burnett et al. (2017). Bulls were subjected to electroejaculation every 21 d for all animals in year 2011 with a total grazing period of 126 d. For all other years, electroejaculation was performed every 28 d with a total of 155 d for 2012 and 168 d for years 2014 and 2015. Breeding soundness exams (BSE) were performed on all bulls to assess semen motility and semen morphology, scrotal circumference (SC), body condition (BCS) and structural soundness. Bulls

passing the BSE were allotted to treatment and blocked according to body weight (BW) and BCS. Semen samples were centrifuged, separated into cell and fluid portions, and each individually stored at - 80 °C until used for DNA isolation. Caudal venipuncture was performed to collect blood which was processed for serum collection. Blood was allowed to clot overnight at 4 °C and serum was collected by whole blood centrifugation at 2000 x *g* for 15 min at 4 °C and serum was stored at – 20 °C. All bulls were sacrificed at the end of a 126-day study and testicles and epididymis collected either immediately at the end of the study (Group A with five and three bulls on E- and E + diet, respectively) or 60 days after removal from treatment (Group B with three bulls from each treatment) for year 2011 only. Data for growth, SC and semen quality from all animals in all years were assessed across Periods 2 (0 d) through 5 (84 d).

2.3. Radioimmunoassay

Serum PRL concentrations were determined using a previously validated radioimmunoassay (RIA) performed in the F. Neal Schrick laboratory (Bernard et al., 1993). The intra- assay coefficient was 9.7% and the inter-assay coefficient of variation was 6.0% (Burnett et al., 2017).

2.4. Immunohistochemistry and western blotting

Formalin-fixed samples were processed and IHC performed as described by Calcatera et al. (2011). Slides were washed in phosphate buffered saline (PBS) three times, and incubated in blocking solution of 10% goat serum/PBS for 15 min. Blocking serum was removed and sections were incubated with primary antibody for 1 h at room temperature. A primary mouse monoclonal Ab for DRD2 (Sc-5303; Santa Cruz Biotechnology Inc., Dallas, Texas, USA) was diluted in blocking solution to a concentration of 4 μg/mL. Negative controls were treated with blocking solution in place of primary antibody. Following incubation in primary Ab, slides were washed in blocking solution followed by a final 1 -h incubation using secondary antibody, Alexafluor 594-conjugated goat anti-mouse IgG (A11032; Invitrogen, Carlsbad, CA, USA), diluted in blocking solution (10 μg/mL). After incubation, secondary antibody was removed and slides were washed in PBS. Nuclei staining was conducted using DAPI (0.33 μg/mL; Sigma, St Louis, MO, USA) and was followed by a PBS wash as previously described in this manuscript. Slides were mounted with PBS, cover slips were applied, and immediately examined.

Protein extracts were produced from cattle testis, epididymis and pituitary tissues by homogenization in a 1X RIPA buffer (Alfa Aesar, Ward Hill, MA) with addition of proteinase and phosphatase inhibitors at 10 μl/mL (HALT; Thermo Scientific, Waltham, MA, USA). Concentrations of extracts were obtained using the DC microplate assay (Bio-Rad; Hercules, CA, USA). Samples were processed by boiling 5 min in a 2X laemeli buffer with 1/8th volume beta mercaptoethanol. Denatured samples were centrifuged briefly and then loaded at 45 μg total mass per well on a 12% mini protean TGX precast gel (Bio-Rad; Hercules, CA, USA). Proteins were blotted onto a 0.2 μm nitrocellulose membrane (Bio-Rad; Hercules, CA, USA). The membrane was blocked in 2.5% non-fat dry milk Tris buffered saline with added 0.1% Tween 20 solution (TBST). Immunodetection was conducted using a primary antibody against DRD2 (B-10) at 1:20 dilution (5303; Santa Cruz Biotechnology, Dallas, TX, USA) overnight at 4 °C followed by three washes in TBST and an incubation with a goat anti-mouse IgG HRP conjugated secondary antibody (Sc-2031; Santa Cruz Biotechnology, Dallas, TX, USA) for 1 h at room temperature. The membrane was washed again three times in TBST followed by incubation with a clarity ECL western substrate (Bio-Rad; Hercules, CA, USA) for 5 min at room temperature. Chemiluminescent detection was performed in a Fluor Chem FC2 imager (Alpha Innotech; San Leandro, CA, USA) for 10 min.

2.5. RNA isolation and RT-PCR

Total RNA was isolated from testis and epididymis using the mirVana mRNA isolation kit (Ambion, Austin, Texas,USA). Purity of RNA was assessed by the nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) to obtain A260:A280 ratio. Quality was assessed using the Agilent 2100 bioanlayzer (Agilent Technologies Inc, Santa Clara, California, USA) ribonucleic acid integrity number (RIN), utilizing samples with an 8.0 or greater for analysis. Complimentary DNA was generated by reverse transcription reaction using Superscript II Frist-Strand kit (Invitrogen, Carlsbad, Ca, USA). Primers for DRD_2 were generated through IDT PrimerQuest Tool (San Jose, CA, USA) for use in polymerase chain reaction (PCR), specific for bovine DRD2 (NM_174043.2) corresponding to nucleotide positions 510–531 forward primer and 576–597 reverse primer of the mRNA DRD2 sequence. End-point PCR was performed using GoTaq green (Promega Madison, WI, USA) and the resulting amplified cDNA was subjected to slab gel electrophoresis on 1.5% agarose gels. In addition to size analysis PCR products were ligated into pDrive cloning vectors and used to transform competent E. Coli (Qiagen, Valencia, CA). Transformations were plated and subsequent colonies selected, propagated, and plasmid DNA isolated and subjected to dideoxysequencing.

2.6. DNA isolation

Sperm pellets were suspended in PBS to approximately 1×10^6 cells, as assessed by sperm quality analyzer (SQA) (Advanced Agricultural Technologies) or semen samples were aliquoted to 100 μl for use in DNA isolation. Samples were centrifuged at 10,000 x *g* for 1 min, supernatant removed, and the sperm pellet washed in 100 μl of 1X PBS. For cell lysis, 1 mL DNAzol (Thermo Fisher Scientific, Waltham, MA, USA) was added to each 100 μl semen sample with 1/8th volume of betamercaptoethanol (BME). Cells were lysed by gentle pipetting. DNA was precipitated by addition of 500 μl of 100% ethanol to each lysed sample. Samples were inverted

until spooling of DNA was visible with a white precipitate. The DNA was sedimented by centrifugation at 4000 x *g* for 1 min. The supernatant was discarded and the pellet washed in 80% ethanol (500 µl), centrifuged at 4000 x *g* for 1 min for a total of two washes. After the last wash, the DNA pellet was allowed to dry on the benchtop for 1 min, then re-suspended in 100 µl 8 mM NaOH. Samples were stored up to 1 week at 4 °C until complete solubilization of the DNA was achieved. Samples were subjected to Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) to determine A260:A280 ratio to assess purity.

2.7. Genotyping

Genotypes for each bull were obtained through restriction fragment length polymorphism (RFLP) analysis (Campbell et al., 2014) and Taqman SNP genotyping assay. In brief, polymerase chain reactions were generated for a previously identified dopamine SNP region (Campbell et al., 2014). The subsequent products were purified using the wizard SV gel and PCR clean-up system (PROMEGA, Durham, NC, USA) and were subjected to slab gel electrophoresis for amplification of the dopamine SNP region (Campbell et al., 2014) to ensure amplification was successful. Purified products (200 ng) were digested using a Pfel (Tfil) restriction enzyme (New England Biolabs, Ipswich, MA, USA), allowed to digest at 37 °C for 30 min. Slab gel electrophoresis was performed with each sample, digested and undigested. Digested samples yielded three genotypes with different size products; AA (532 and 261bp), AG (793, 532 and 261 bp), and GG (793 bp). A custom Taqman SNP genotyping assay (Applied Biosystems, Foster city, CA, USA) specific to the DRD2 SNP region (Campbell et al., 2014) was performed using 10 ng of the purified PCR products. Samples were run in 96-well format and run on the CFX Real-Time System (BIO-RAD Hercules, CA, USA) for a single step at 95 °C for 10 min followed by 40 cycles of denaturation at 92 °C for 15 s and annealing and extension at 60 °C for 1 min. Allelic discrimination analysis was performed using CFX Maestro software (BIO-RAD Hercules, CA, USA) to determine genotype. Sample of known genotype for each of the three genotypes were run on each plate as positive controls as well as a no template control (NTC). These results were compared to the RFLP analysis to ensure efficiency in genotype determination.

2.8. Statistical analysis

For serum PRL concentration, BW, BCS and semen characteristics, Analysis of Variance, followed by pairwise student's t-tests among LSMeans was used to assess the effect of genotype and EA treatment on semen quality and growth traits. The model for the Analysis of Variance included fixed effects for treatment, genotype, time on treatment and the interactions; and year as a random effect. The time on treatment effect was included in the model since animals in each year were assessed across periods 2 (0 d) through 5 (84 d). Equality of variances were examined by Levene's test ($P > 0.05$). The JMP software (SAS Institute Inc.) was used for all statistical calculations.

3. Results

3.1. DRD2 gene expression in bull reproductive tissues

The DRD2 receptor was identified to be present in the bovine testis, epididymis, and sperm using IHC (Fig. 1); however, attempts using the same antibody in western blotting did not yield definitive results (data not shown). To further confirm the presences of DRD2 gene products, end point (RT-PCR) and dideoxysequencing of the amplified product (Fig. 1) were conducted and the expected product size and sequence were produced. The receptor was present in all samples examined regardless of treatment or bull genotype.

3.2. Genotype frequencies

Genotypic frequency was determined to be 18% AA, 61% AG, and 21% GG using RFLP and 21% AA, 59% AG, 20% GG using the custom Taqman genotyping methods across all years. Comparison of values with use of the RFLP and Taqman DRD2 genotyping methods are depicted in Fig. 3. Genotypes were identical for all bulls with the exception of three animals across all years in the study. Values for a representative sample are depicted in Fig. 4 with use of RFLP and Taqman.

3.3. Growth, semen characteristics, and PRL serum concentrations

Prolactin concentrations were similar for animals with the DRD2 genotype but there was a treatment effect, with greater concentrations of serum prolactin in bulls not consuming EA compared to bulls consuming EA (144.02 ng/mL ± 49.3; 53.10 ng/mL ± 49.3, respectively ($P < 0.05$; Fig. 2). There was a year by treatment by genotype random effect for PRL concentrations with 37.6% of the total variance attributed to this interaction ($P = 0.02$). As expected, period had an effect ($P < 0.05$) on BCS and BW with animals having a greater BCS and BW earlier in the treatment period. Period also affected scrotal circumference, with bulls having a lesser scrotal circumference (35.4 ± 0.41; $P < 0.05$) at the start of the study, increasing up to 84 d (36.3 ± 0.44; $P < 0.05$; Table 1). A random interaction including year, treatment, genotype, and period accounted for 9.7% of the total variance for BCS ($P = 0.03$) and 0% for BW and SC ($P < 0.0001$). There were no significant differences in semen concentration due to treatment, period, genotype or their interactions. There were no differences in sperm motility, sperm progressive motility, number of motile sperm cells or number of progressively motile sperm cells due to treatment, period, genotype or the interactions. Period, however, affected velocity with bulls having been treated for longer periods having greater sperm velocity ($P < 0.05$). Sperm cell morphology

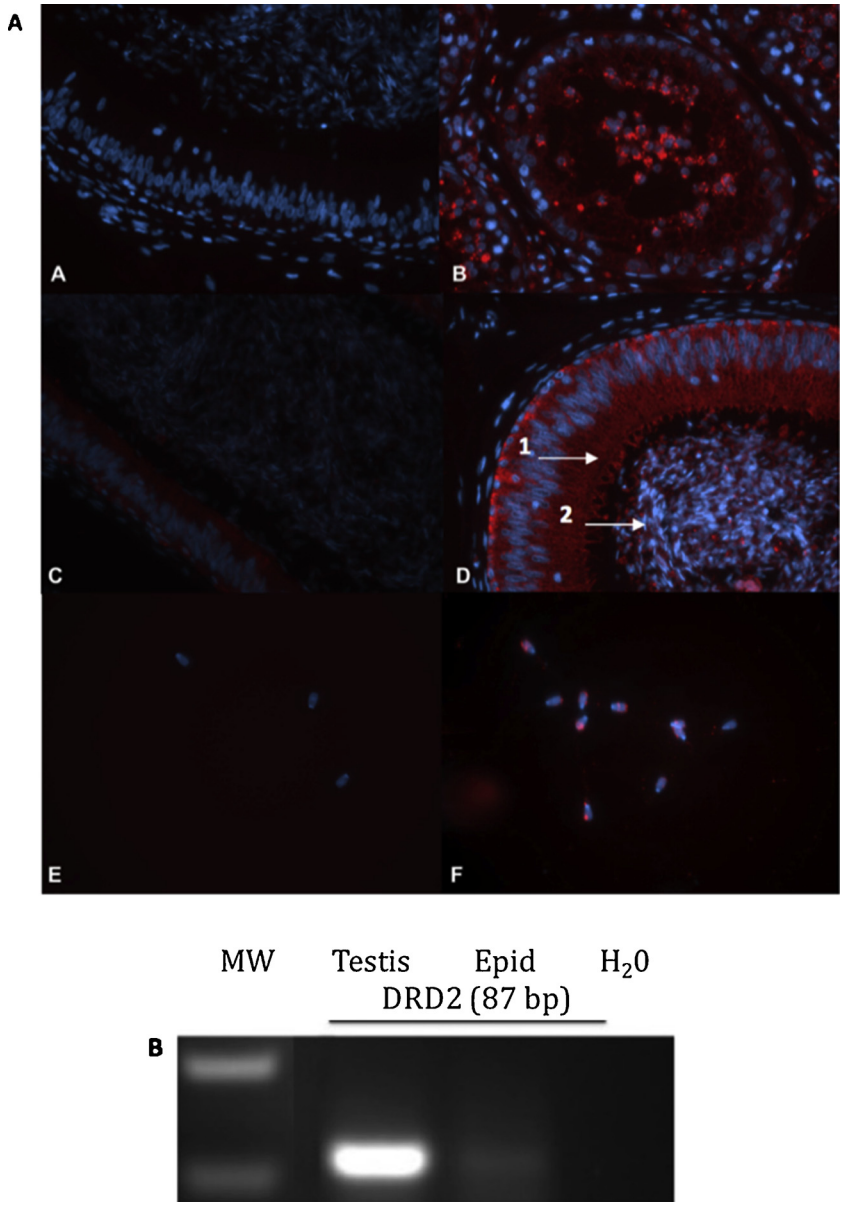

Fig. 1. Immunohistochemical detection and end-point PCR of DRD2 in testis and epididymis. Panels are labeled at the top left in bold. Panel (A) Immunohistochemistry of DRD2: Panels (A), (C) and (E) are negative controls for the primary Ab and panels (B), (D) and (F) were incubated with anti-DRD2 Ab. Dopamine receptor type-2 staining is shown in red. Slides were counterstained with DAPI (blue) for visualization of the nuclei. Both stains are indicated by white arrows in panel (D) with staining for DRD2 and DAPI indicated by arrows labeled 1 and 2, respectively. Tissue types are as follows: panels (A) and (B) are testis, panels (C) and (D) are epididymis, panels (E) and (F) are sperm cells. All images were taken at 40x magnification. Ab, antibody; DRD2, Dopamine type-2 receptor. Panel (B) End-point PCR: An 87 bp amplicon for DRD2 mRNA abundance is shown in testis and epididymis. MW, molecular weight ladder; DRD2, Dopamine type-2 receptor.

as well as the total number of sperm cells per ejaculate were not different as a result of treatment. A random effect for cell morphology including treatment, year, genotype, and period accounted for none of the percent total variance ($P = 0.009$). Furthermore, there was no effect on total motile sperm or total progressive motile sperm cells per ejaculate in relation to genotype, treatment, period or the interactions (Table 2).

4. Discussion

There are inconsistences in results among studies of dopamine agonist effects on male reproduction and physiology. The reason for the variability may be attributed to genetic makeup of animals utilized in the studies. In the present study, there was evaluation of

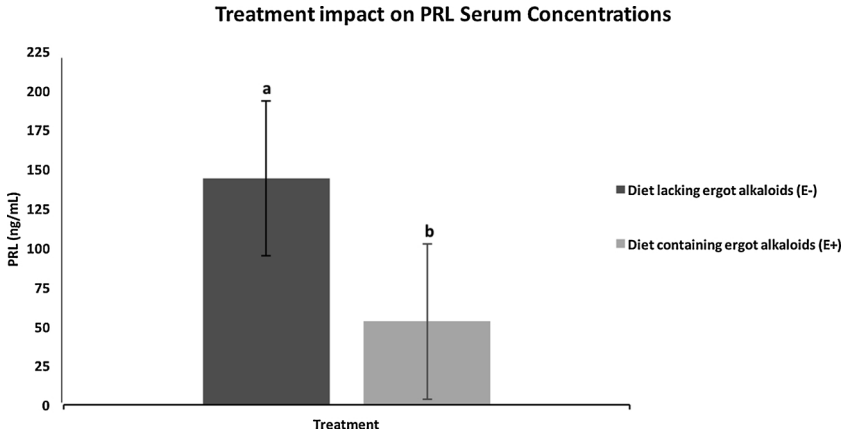

Fig. 2. Treatment effect on prolactin serum concentrations of bulls grazing E + or E-. Treated bulls had lesser overall serum concentrations of PRL than E- bulls. Different letters indicate significance ($P = 0.02$) due to treatment.

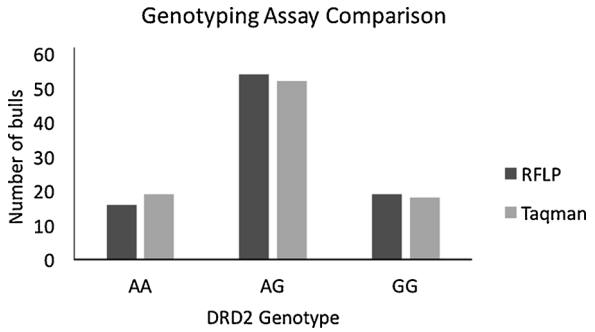

Fig. 3. Comparison in genotype calls for RFLP and the custom Taqman genotyping assays.

the presence of DRD2 gene expression in male reproductive tissues of bulls and assessment of genotypic differences in animals for the DRD2 gene in an attempt to determine any possible differences in bull physiology due to a particular DRD2 SNP (Campbell et al., 2014) on serum PRL concentration, growth traits, and semen quality in bulls grazing a dopamine agonist. Using IHC techniques, the DRD2 receptor was found to be present in bull testis, epididymis and sperm. This is similar to observations in other species where the DRD2 receptor was present throughout the male reproductive system, including sperm cells (Otth et al., 2007; Ramírez et al., 2009; Gonzalez et al., 2015). To further verify the expression of DRD2 receptor gene in male reproductive tissues, end-point RT-PCR identified products of the correct size and sequence. The primers utilized in RT-PCR flank the intronic region the SNP of interest is located. A single product was identified indicating no splice variants present due to genotype; however, this does not negate the possibility of alternative spliced forms of DRD2 present in cattle. Alternate splicing of DRD2 has been reported in rats and humans, resulting in long and short forms of the receptor; however, both forms are functionally coupled to G-proteins and can suppress PRL gene expression (Dal Toso et al., 1989; McChesney et al., 1991).

Results from the present study indicate EA consumption could affect male reproductive physiology as a result of EA binding to the DRD2 receptor in the testis and accessory glands. This binding could alter spermatogenesis, sperm maturation or the concentrations or composition of constituents of seminal fluid affecting sperm physiology and subsequent fertility. Further, if the toxin is present in seminal fluid, sperm physiology could be altered through binding to the DRD2 receptor or other receptor types which may be present and that are capable of binding EA. Dopamine receptors have been well established to have functions in regulation of male physiology in both rats and humans. Treatments with dopamine type-2 receptor agonists and antagonists have consistently been associated with DRD2 effects in initiation of ejaculation (Ferrari and Giuliani, 1994; Clement et al., 2006; Stafford and Coote, 2006; Peeters and Giuliano, 2008). The results of the present retrospective study indicate there is little, if any, effect on bull growth and semen quality at time of collection due to EA consumption. In contrast, results of two previous studies indicate there are effects of EA consumption on the capacity of bull sperm to survive normal extension and freezing procedures (Pratt et al., 2015b; Burnett et al., 2017), supporting the observation of Gallagher and Senger (1989), where incubation of sperm with dopamine agonists decreased sperm viability when there was cryopreservation and thawing imposed.

Genotyping was originally attempted by nested PCR. In brief, end-point PCR was performed for the F1-R793 DRD2 region (Campbell et al., 2014). The resulting products were subjected to nested PCR with primers flanking the SNP site (Campbell et al., 2014) for genotype (AA, AG, GG) on either the forward or reverse primer. The specific forward and reverse primer processes were conducted using primers flanking either the end of the sequence at 793 bp or the beginning of the sequence, 1 bp, respectively. The

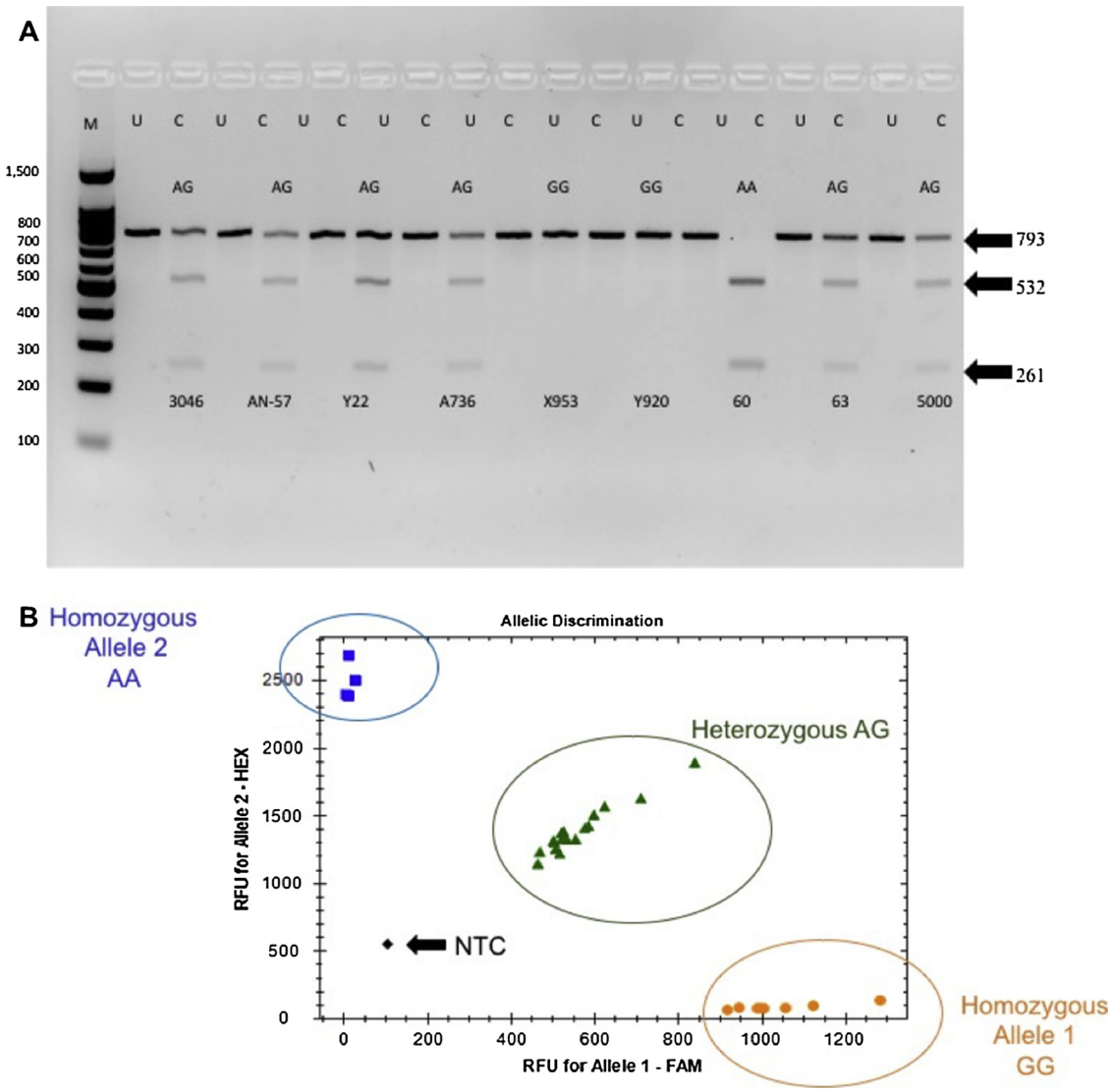

Fig. 4. A representative sample of bulls analyzed by RFLP and Taqman analysis for DRD2 genotypes. Panels are denoted on the top left in bold. Panel (A) RFLP analysis: molecular weight marker is on the left and product sizes are on the right. Bull IDs are below each lane. All products were incubated in the absence (U) or presence (C) of TfiI restriction enzyme. A single band present at 793 bp indicates a lack of the TfiI restriction site and is genotyped as GG. The presence of two bands at 532 and 261 indicate homozygous for the TFiI site and genotyped as AA. The presence of all three bands are genotyped as AG. Panel (B) Taqman analysis: The allelic discrimination plot from the custom Taqman genotyping assay for the DRD2 SNP region contains a shape, each representative of a single animal with each cluster represented by a different color, representing a genotype. Plots are represented as relative fluorescence units (RFU) for each allele. Allele 1 representing RFU for 'G' on the horizontal axis and Allele 2 representing 'A' on the vertical axis. Genotypes are represented as the following: Orange circles, GG (lower right corner); Green triangles, AG (middle); Blue squares, AA (upper left corner); Black dot, non-template control (NTC).

Table 1
Mean body weight, body condition and scrotal circumference of bulls grazing ergot alkaloids (E+) or a diet lacking ergot alkaloids (E-) up to 84 days.

Parameter	d 0	d 21-28	d 42-56	d 84	P-Value
Body weight (BW; in kg)	508.56 ± 11.8^{c}	520.90 ± 11.8^{bc}	534.6 ± 11.8^{b}	551.8 ± 12.3^{a}	< 0.001
Scrotal Circumference (SC; in cm)	35.4 ± 0.41^{c}	35.8 ± 0.41^{bc}	36.5 ± 0.41^{a}	36.3 ± 0.44^{ab}	0.0053
Body Condition (BCS)	5.9 ± 0.21^{a}	5.5 ± 0.21^{b}	5.5 ± 0.21^{b}	5.6 ± 0.22^{b}	0.0009

$^{a-c}$Means within the same row possessing a different superscript lowercase letter differ due to day.

Table 2
Mean semen characteristics of bulls grazing ergot alkaloids (E+) or a diet lacking ergot alkaloids (E-) up to 84 days.

Parameter	d 0	d 21-28	d 42-56	d 84	P-Value
Concentration (million/mL)	383.2 ± 54.9[a]	377 ± 54.6[a]	435.3 ± 56.5[a]	448.5 ± 59.7[a]	0.42
PROG Motility (%)	66.5 ± 2.96[a]	71.5 ± 2.95[a]	71.6 ± 3.13[a]	72.9 ± 3.49[a]	0.35
Motility (%)	76.5 ± 2.49[a]	82.1 ± 2.46[a]	82.3 ± 2.74[a]	84.2 ± 3.13[a]	0.27
Normal Morphology (%)	85.6 ± 4.91[a]	84.9 ± 4.73[a]	84.2 ± 5.00[a]	92.2 ± 5.42[a]	0.55
Motile sperm cells (x 10^{-6})	304.1 ± 34.9[a]	298.0 ± 34.7[a]	351.2 ± 36.2[a]	345.7 ± 38.8[a]	0.31
Velocity (∞/sec)	51.5 ± 3.77[b]	56.2 ± 3.74[ab]	62.8 ± 3.96[a]	63.6 ± 4.39[a]	0.04
Total number sperm cells (10^{-6})	2.08 ± 0.639[a]	2.65 ± 0.637[a]	3.11 ± 0.654[a]	2.47 ± 0.682[a]	0.24
Total Motile Sperm (million/mL)	1.68 ± 0.423[a]	2.04 ± 0.421[a]	2.42 ± 0.432[a]	1.90 ± .452[a]	0.17
Total Progressive sperm cells (million/mL)	1.49 ± 0.348[a]	1.80 ± 0.346[a]	2.15 ± 0.356[a]	1.64 ± 0.376[a]	0.16

[a−b]Means within the same row possessing a different superscript lowercase letter differ due to day.

resulting products should have amplified only for the specific genotype, but when procedures were conducted with known genotyped animals, results were inconclusive. The RFLP method was then conducted and the resulting products were subjected to dideoxysequencing along with subcloned samples of known DRD2 genotype to assess issues with specific amplification of the prior methods. Results were again inconclusive, with clones of known genotype and resulting products of RFLP unable to read 'G' in all samples sequenced, suggesting the polymerase may be unable to read through the 'G' 'C' rich region of this particular site. The custom Taqman geontyping assay was utilized to compare to RFLP methods. With use of both procedures, there were nearly identical results in known genotyped animals, with results for three animals differing between methods, accounting for a 3.4% error rate among calls. These three samples may have differed due to possible contamination issues, with genotypes all resulting in AA in the taqman genotyping assay. These samples were limiting, thus, subsequent genotyping was not possible. An attempt to isolate DNA from urine samples from these same animals was made but was unsuccessful. The DNA isolated from urine never resulted in quality template due to contaminants and sediment in the urine. The error in genotypic calls, however, is extremely low and does not directly affect the overall distribution within the study. This comparison of results with use of Taqman methods to RFLP led to the conclusion that both methods' could be reliable for use in further studies for genotyping.

Body weight and BCS in relation to period are consistent with results from previous studies in the same laboratory as that of the present study in which BW and BCS were unaffected by treatment alone, with BCS effected by day (Stowe et al., 2013). When examining a subset of bulls in 2011, it was found that SC was less at the end of a 126 d study when bulls were consuming a greater concentration of EA; however, considering data across all 4 years SC was not effected by treatment. A lack of effect on SC may be due to the duration of the periods analyzed, as this current study only occurred for an 84 day period whereas Stowe et al. (2013) extended the analysis to 126 days. A change in SC was observed in all bulls due to period, with prolonged time on study resulting in a greater SC. The methods used in these two studies differ in treatment delivery, with the approach in the current study being the use of grazing for consumption of EA, with the exception of year 2011, and in the previous study (Stowe et al., 2013) a concentrate diet was fed to deliver EA throughout the study. Furthermore, the current observations on SC are consistent with conclusions by Looper et al. (2009) and Schuenemann et al. (2005) where there was also no change in SC due to EA treatments.

The current observations are consistent with the consensus based on results from previous studies in which animals grazing a dopamine agonist have a decrease in circulating serum PRL concentrations (Schillo et al., 1988; Porter and Thompson, 1992; Pratt et al., 2015a). The conclusions on serum PRL concentration, however, are inconsistent with the findings of Campbell et al. (2014) who observed differences in serum PRL concentrations that were related to DRD2 genotype. In the current study, PRL concentrations remained consistent across DRD2 genotypes. Campbell et al. (2014) observed a correlation with serum PRL concentrations with GG genotypes where there was lesser PRL and in steers with the AA genotype there were greater PRL concentrations when grazing pasture containing EA in May. In this previous study, however, there was not the same consistent effect across all months that animals were on treatment. Results during the month of June were attributed to the effects of ambient heat masking any genotypic advantage, with the results during this month of the study being more consistent with the findings in the current study in which genotype had no effect on PRL concentrations. Furthermore, in the present study there was assessment of the effect of period on cattle grazing containing or lacking EA continuing from April to August or February to August. Period was found to have no significant effect on PRL serum concentrations in the present study.

Results from previous studies are inconsistent when the effects of EA on semen quality of bulls were assessed. Ergot alkaloids affect semen quality, with results of previous studies indicating there is a lesser sperm concentration in semen as a result of EA treatment (Pratt et al., 2015a). Furthermore, there have been reports of EA effects on sperm motility and morphology (Looper et al., 2009; Pratt et al., 2015a) while there are other reports where there were no effects on sperm or semen characteristics (Schuenemann et al., 2005; Stowe et al., 2013). findings in the present study nsupport these later observations, with no effect of EA treatment or period on sperm motility, concentration or morphology. Furthermore, from results of the present study it can be concluded that DRD2 genotype has no effect on semen quality. Interestingly, sperm velocity was affected by period, with bulls longer on study exhibiting an increase in semen velocity. This has been observed in previous studies where bulls were grazing a pasture where dopamine agonist was present in which sperm velocity was affected by day (Burnett et al., 2017) and most likely these results were attributed to increasing bull age and/or seasonal change from winter to spring temperatures. These results are inconsistent with those in hyperprolactinemia human patients, in which sperm velocity is increased when there are relatively greater circulating serum PRL

concetrations (Panidis et al., 1997).

5. Conclusion

Results from the present research indicate that genotype of DRD2 (Campbell et al., 2014) does not have a role in male physiology pertaining to semen quality, growth, or circulating concentrations of serum PRL. These data are, however, consistent with a treatment effect on serum PRL concentrations in bulls grazing EA. Although these results are inconsistent with previous findings regarding this SNP (Campbell et al., 2014). This SNP is located within a non-coding region, so the current observations are plausible because with the DRD2 genotype there are no direct effects on male growth and semen characteristics or indirectly through alterations of serum concentrations of PRL.

Conflict of interest

None.

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References

Ben-Jonathan, N., Hnasko, R., 2001. Dopamine as a prolactin (PRL) inhibitor. Endocr. Rev. 22, 724–763. https://doi.org/10.1210/edrv.22.6.0451.

Bernard, J., Chestnut, A., Erickson, B., Kelly, F., 1993. Effects of prepartum consumption of endophyte-infested tall fescue on serum prolactin and subsequent milk-production of Holstein cows. J. Dairy Sci. 76, 1928–1933.

Bole-Feysot, C., Goffin, V., Edery, M., Binart, N., Kelly, P., 1998. Prolactin (PRL) and its receptor: actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. Endocr. Rev. 19, 225–268. https://doi.org/10.1210/edrv.19.3.0334.

Burnett, C.R., Bridges, W.C., Pratt, S.L., 2017. Effects of grazing tall fescue containing ergot alkaloids on bull sperm cryopreservation. Anim. Reprod. Sci. 181, 24–29. https://doi.org/10.1016/j.anireprosci.2017.03.011.

Calcatera, S.M., Korn, N., Ellis, S., Pratt, S.L., 2011. Immunohistochemical detection of Argonaute 2 in bovine and porcine tissues. J. Histotechnol. 34, 74–78. https://doi.org/10.1179/204602311X13007117736023.

Campbell, B.T., Kojima, C.J., Cooper, T.A., Bastin, B.C., Wojakiewicz, L., Kallenbach, R.L., Schrick, F.N., Waller, J.C., 2014. A single nucleotide polymorphism in the dopamine receptor D2 gene may be informative for resistance to fescue toxicosis in angus-based cattle. Anim. Biotechnol. 25, 1–12. https://doi.org/10.1080/10495398.2013.796960.

Caron, M., Beaulieu, M., Raymond, V., Gagn, B., Drouin, J., Lefkowitz, R., Labrie, F., 1978. Dopaminergic receptors in the anterior pituitary gland. J. Biol. Chem. 253, 2244–2253.

Clement, P., Bernabe, J., Kia, H.K., Alexandre, L., Giuliano, F., 2006. D2-like receptors mediate the expulsion phase of ejaculation elicited by 8-hydroxy-2-(di-N-propylamino) tetralin in rats. J. Pharmacol. Exp. Ther. 316, 830–834. https://doi.org/10.1124/jpet.105.092411.

Crawford, R.J., Forwood, J.R., Belyea, R.L., Garner, G.B., 1989. Relationship between level of endophyte infection and cattle gains on tall fescue. J. Prod. Agric. 2, 147–151.

Dal Toso, R., Sommer, B., Ewert, M., Herb, A., Pritchett, D., Bach, A., Shivers, B., Seeburg, P., 1989. The dopamine D2 receptor: two molecular forms generated by alternative splicing. EMBO J. 8, 4025–4034.

Ferrari, F., Giuliani, D., 1994. The selective D2 dopamine receptor antagonist eticlopride counteracts the ejaculation praecox induced by the selective D2 dopamine agonist SND 919 in the rat. Life Sci. 55, 1155–1162.

Freeman, M.E., Kanyicska, L.A., Lerant, A., Nagy, G., 2000. Prolactin: structure, function, and regulation of secretion. Physiol. Rev. 80, 1523–1631.

Gallagher, G.R., Senger, P.L., 1989. Effect of phenylephrine, ergonovine, oxytocin and norepinephrine as an extender ingredient on viability of bovine spermatozoa. J. Anim. Sci. 67, 1573–1576. https://doi.org/10.2527/jas1989.6761573x.

Gonzalez, C., Gonzalez, B., Matzkin, M., Muniz, J., Cadet, J., Garcia-Rill, E., Urbano, F., Vitullo, A., Bisagno, V., 2015. Psychostimulant-induced testicular toxicity in mice: evidence of cocaine and caffeine effects on the local dopaminergic system. PLoS One 10, e0142713. https://doi.org/10.1371/journal.pone.0142713.

Hansen, K., Zhang, Y., Colver, R., Tho, S., Plouffe, L., McDonough, P., 2005. The dopamine receptor D2 genotype is associated with hyperprolactinemia. Fertil. Steril. 84, 711–718. https://doi.org/10.1016/j.fertnstert.2005.03.040.

Horseman, N.D., Zhao, W., Montecino-Rodriguez, E., Tanaka, M., Nakashima, K., Engle, S.J., Smith, F., Markoff, E., Dorshkind, K., 1997. Defective mammopoiesis, but normal hematopoiesis, in mice with a targeted disruption of the prolactin gene. EMBO J. 16, 6926–6935. https://doi.org/10.1093/emboj/16.23.6926.

Hoveland, C.S., 1993. Importance and economic significance of the Acremonium endophytes to performance of animals and grass plant. Agric. Ecosyst. Environ. 44, 3–12. https://doi.org/10.1016/0167-8809(93)90036-O.

Larson, B.T., Samford, M.D., Camden, J.M., Piper, E.L., Kerley, M.S., Paterson, J.A., Turner, J.T., 1995. Ergovaline binding and activation of D2 dopamine receptors in GH4ZR7 cells. J. Anim. Sci. 73, 1396–1400.

Looper, M.L., Rorie, R.W., Person, C.N., Lester, T.D., Hallford, D.M., Aiken, G.E., Roberts, C.A., Rottinghaus, G.E., Rosenkrans Jr, C.F., 2009. Influence of toxic endophyte-infected fescue on sperm characteristics and endocrine factors of yearling brahman-influenced bulls. J. Anim. Sci. 87, 1184–1191. https://doi.org/10.2527/jas.2008-1258.

McChesney, R., Sealfon, S., Tsutsumi, M., Dong, K., Roberts, J., Bancroft, C., 1991. Rapid paper either isoform of the dopamine D2 receptor can mediate dopaminergic repression of the rat prolactin promoter. Mol. Cell. Endocrinol. 79, R1–R7.

Mizinga, K., Thompson, F., Steudemann, J., Edwards, G., 1993. Neural dopamine D2 receptors in rats fed endophyte-infected fescue seed. Toxicol. Environ. Chem. Rev. 16, 307–319.

Otth, C., Torres, M., Ramírez, A., Fernandez, J., Castro, M., Rauch, M., Brito, M., Yañez, A., Rodríguez-Gil, J., Slebe, J., Concha, I., 2007. Novel identification of peripheral dopaminergic D2 receptor in male germ cells. J. Cell. Biochem. 100, 141–150. https://doi.org/10.1002/jcb.21037.

Panidis, D., Rousso, D., Skiadopoulos, S., Panidou, E., Mamopoulos, M., 1997. Evaluation of semen parameters in man with hyperprolactinemia induced by

metoclopramide. Arch. Androl. 39, 237–242. https://doi.org/10.3109/01485019708987922.
Paterson, J., Forcherio, C., Larson, B., Samford, M., Kerley, M., 1995. The effects of fescue toxicosis on beef-cattle productivity. J. Anim. Sci. 73, 889–898.
Peeters, M., Giuliano, F., 2008. Central neurophysiology and dopaminergic control of ejaculation. Neurosci. Biobehav. Rev. 32, 438–453. https://doi.org/10.1016/j.neubiorev.2007.07.013.
Porter, J.K., Thompson, F., 1992. Effects of fescue toxicosis on reproduction in livestock. J. Anim. Sci. 70, 1594–1603.
Pratt, S.L., Calcatera, S.M., Stowe, H.M., Dimmick, M.A., Schrick, F.N., Duckett, S.K., Andrae, J.G., 2015a. Identification of bovine prolactin in seminal fluid, and expression and localization of the prolactin receptor and prolactin-inducible protein in the testis and epididymis of bulls exposed to ergot alkaloids. Theriogenology 83, 662–669. https://doi.org/10.1016/j.theriogenology.2014.10.031.
Pratt, S.L., Stowe, H.M., Whitlock, B.K., Strickland, L., Miller, M., Calcatera, S.M., Dimmick, M.D., Aiken, G.E., Schrick, F.N., Long, N.M., Duckett, S.K., 2015b. Bulls grazing kentucky 31 tall fescue exhibit impaired growth, semen quality, and decreased semen freezing potential. Theriogenology 83, 408–414. https://doi.org/10.1016/j.theriogenology.2014.10.001.
Ramírez, A., Castro, M., Angulo, C., Ramió, L., Rivera, M., Torres, M., Rigau, T., Rodríguez-Gil, J., Concha, I., 2009. The presence and function of dopamine type 2 receptors in boar sperm: a possible role for dopamine in viability, capacitation, and modulation of sperm motility. Biol. Reprod. 80, 753–761. https://doi.org/10.1095/biolreprod.108.070961.
Schillo, K., Leshin, L., Boling, J., Gay, N., 1988. Effects of endophyte-infected fescue on concentrations of prolactin in blood sera and the anterior pituitary and concentrations of dopamine and dopamine metabolites in brains of steers. J. Anim. Sci. 3, 713–718.
Schoning, C., Flieger, M., Pertz, H.H., 2001. Complex interaction of ergovaline with 5-ht2A, 5-ht1b/1d, and alpha1 receptors in isolated arteries of rat and guinea pig. J. Anim. Sci. 79, 2202–2209.
Schuenemann, G.M., Edwards, J.L., Hopkins, F.M., Rohrbach, N.R., Adair, H.S., Scenna, F.N., Waller, J.C., Oliver, J.W., Saxton, A.M., Schrick, F.N., 2005. Fertility aspects in yearling beef bulls grazing endophyte-infected tall fescue pastures. Reprod. Fertil. Dev. 17, 479–486. https://doi.org/10.1071/RD05005.
Segal, S., Yaffe, H., Laufer, N., Ben-David, M., 1979. Male hyperprolactinemia: effects on fertility. Fertil. Steril. 32, 556–561. https://doi.org/10.1016/S0015-0282(16)44359-1.
Sibley, D., Creese, I., 1983. Interactions of ergot alkaloids with anterior pituitary D-2 dopamine receptors. Mol. Pharmacol. 23, 585–593.
Stafford, S.A., Coote, J.H., 2006. Activation of D2-like receptors induces sympathetic climactic-like responses in male and female anaesthetised rats. Br. J. Pharmacol. 148, 510–516. https://doi.org/10.1038/sj.bjp.0706763.
Stowe, H.M., Miller, M., Burns, M.G., Calcatera, S.M., Andrae, J.G., Aiken, G.E., Schrick, F.N., Cushing, T., Bridges, W.C., Pratt, S.L., 2013. Effects of fescue toxicosis on bull growth, semen characteristics, and breeding soundness evaluation. J. Anim. Sci. 91, 3686–3692. https://doi.org/10.2527/jas.2012-6078.
Strickland, J.R., Looper, M.L., Matthews, J.C., Rosenkrans Jr, C.F., Flythe, M.D., Brown, K.R., 2011. Board-invited review: St. Anthony's fire in livestock: causes, mechanisms, and potential solutions. J. Anim. Sci. 89, 1603–1626. https://doi.org/10.2527/jas.2010-3478.
Thompson, F.N., Stuedemann, J.A., 1993. Pathophysiology of fescue toxicosis. Agric. Ecosyst. Environ. 44, 263–281. https://doi.org/10.1016/0167-8809(93)90050-Y.
Thompsonm, F., Stuedemann, J., Sartin, J., Belesky, D., Devine, O., 1987. Selected hormonal changes with summer fescue toxicosis. J. Anim. Sci. 65, 727–733. https://doi.org/10.2527/jas1987.653727x.
Wang, H., Looper, M.L., Johnson, Z.B., Rorie, R.W., Rosenkrans, C.F., 2009. Involvement of signaling pathways in bovine sperm motility, and effect of ergot alkaloids. Vitr. Cell. Dev. Biol. 45, 483–489.
Zhang, X., Zhang, Z., Zhu, R., Yuan, T., Jenkins, T., Reynolds, G., 2011. Sexual dysfunction in male schizophrenia: influence of antipsychotic drugs, prolactin and polymorphisms of the dopamine D2 receptor genes. Pharmacogenomics 12, 1127–1136.