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ABSTRACT

Objectives of this project were to evaluate polymorphisms in upstream elements of the lactate dehydrogenase B (LDHB) gene in crossbred dairy heifers (n = 27) and their effects on immune function and heifer growth when grazing endophyte-infected tall fescue. Two cultivars of tall fescue were utilized: Kentucky 31 (KY31), a wild-type endophyte-infected tall fescue, and HiMag 4 (HiMag), a domesticated non-toxic endophyte-infected tall fescue. Crossbred dairy heifers (Holstein × Jersey) were stratified by weight and randomly allotted to forage. The LDHB gene codes for one subunit of lactate dehydrogenase (LDH), an enzyme that catalyzes pyruvate to lactate and back to pyruvate. Forward primer used for amplification was 5'-ACACACCAGCAGCATCTCAG-3' and reverse primer was 5'- GATAAGGGCTGCACGAAGAC-3'. The amplicon size for this LDHB primer set was 457 base pairs. Sequenced amplicons were aligned with Clustal2W for polymorphism detection and genotype assignment. Heifers that had a heterozygous genotype and grazed HiMag were heavier when compared with other heifer groups. Number of red blood cells and hemoglobin concentrations for heifers grazing KY31 were greater when compared to heifers grazing HiMag. Distribution of white blood cells was affected by LDHB genotype. Two dairy heifer management tools, stockpiled tall fescue and LDHB genotyping, were assessed in this study, both of which impacted heifer growth and immune function as assessed by blood cell differentials.

[*] Rachel Henry received her B.S. degree in May 2011 majoring in Animal Science. This paper is based on her undergraduate honors research project.
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MEET THE STUDENT-AUTHOR

Rachel Henry

I grew up in Fort Smith, Ark., and graduated from Southside High School in 2007. My B.S. degree in animal science with a minor in equine science allowed me to fulfill the pre-veterinary program requirements. This fall I begin my studies at Louisiana State University Veterinary School. I have enjoyed my time at the University of Arkansas, becoming involved in clubs such as the Pre-Veterinary Club, Alpha Zeta, and Sigma Alpha. Through an exchange program set up by the Dale Bumpers College, I was able to complete an internship at the Royal School of Veterinary Studies in Scotland and gained valuable research experience at the same time.

This particular research experience could not have been achieved without the help and guidance of Dr. Charles Rosenkrans, Dr. Tes Sales, Dr. Elizabeth Kegley, and Dr. Jeremy Powell.

INTRODUCTION

Milk, cheese, and other dairy products are important sources of nutrition for people around the world. Despite record-breaking high milk prices, dairy profitability has been limited due to high feed prices; therefore, it is important to consider alternative feed options. One such option is tall fescue (*Lolium arundinaceum* (Schreb.), *Darbyshire*), a cool season grass commonly found in the Midwestern and Southeastern United States (Bouton, 2000). Success of tall fescue is due in no small part to its symbiotic relationship with an endophytic fungus (*Neotyphodium coenophialum*), which produces alkaloids to protect the plant from pests, stress, drought, and heat (Glenn et al., 1996). Ergot alkaloids increased animal respiration rates and rectal temperatures, decreased animal weight gain, and caused rough hair coats (Nihsen et al., 2004). One management practice to reduce the effects of grazing toxic tall fescue is to stockpile tall fescue in the fall and graze it during the winter months instead of feeding hay. By exposing the toxic fescue to the weather, the ergot alkaloids produced by the endophytic fungus are degraded naturally (Kallenbach et al., 2003).

Lactate dehydrogenase (LDH) activity is associated with cattle profitability (Looper et al., 2002), and polymorphisms in the LDH promoter are associated with serum LDH activity (Maekawa et al., 2002). Stress in general, and ergot alkaloids specifically, results in elevated LDH activity in serum. However, physiological and genetic relationships among LDH polymorphisms, LDH activity, and heifer growth and health have not been established. The objective of this research was to evaluate the polymorphic nature of the promoter/enhancer regions of the LDHB gene in dairy heifers and the relationship among LDHB genetic polymorphisms, immune function, and heifer growth while grazing stockpiled tall fescue.

MATERIALS AND METHODS

Animals. Cattle used in the experiment were Holstein-Jersey crossbred dairy heifers (n = 27) approximately nine months of age. Before the experiment, they were dewormed with ivermectin in the fall, and had grazed mixed grass paddocks or were fed similar hay at the Arkansas Agricultural Research and Extension Center located in Fayetteville. The trial began on December 6 and proceeded through February 28 for a total of 84 days.

Heifers were stratified by weight into eight tall fescue pastures. Tall fescue cultivars were Kentucky 31 (KY31; n = 4 pastures) and HiMag 4 (HiMag; n = 4 pastures). Cultivar KY31 is a wild-type endophyte-infected tall fescue and HiMag is a non-toxic endophyte-infected tall fescue (Nihsen et al., 2004). Based on visual evaluation (step point analysis), the KY31 stands were 81% tall fescue and the HiMag stands consisted of 80% tall fescue at the initiation of stockpiling. All heifers were fed a corn-based supple-

ment at approximately 0.8% body weight (BW) daily prorated for feeding five days each week. Automated watering devices and tanks were utilized to allow free access to water. Blood and animal weights were collected on days 0, 28, 56, and 84.

Digital scales were used to determine individual heifer body weight. Scales were calibrated before each weigh date and tare was established in between each heifer. On each weigh date, all heifers were gathered and weighed in random order. Heifers were sorted into their original pasture groups and returned to their original pasture after all heifers were weighed.

Pasture Design. Eight pastures (1.62 hectares each) were used in the trial. Pastures were separated by high-tensile electric fence, and each individual pasture was subdivided into thirds with poly-wire electric fence. During days 0-28 heifers were allowed to graze the first third of the pasture, on day 28 the poly-wire fence was moved to allow the grazing of an additional one-third of the pasture, and on day 56 the poly-wire fence was removed. Since no back fence was used, the cattle had access to all previous pasture sections as the trial progressed.

DNA Preparation, Amplification, and Sequencing. Blood was collected from the cattle through jugular venipuncture, into tubes containing ethylenediaminetetraacetic acid (EDTA; Vacutainer, Becton-Dickson, Rutherford, N.J.). The whole blood was centrifuged at 700 × *g* for 25 min and the plasma decanted and stored at -20 °C. Buffy coats were removed and stored at -80 °C until thawed for DNA extraction. Genomic DNA extraction was accomplished using the Purgene Genomic Purification Kit (Qiagen, Valencia, Calif.). The extracted DNA was stored at -20 °C until used for amplification.

Specific DNA primers for bovine LDHB were designed based on the GenBank accession number for human LDHB (NW 001495085) and cattle genome build 1.1 (Bovine Genome Database, Georgetown University, Washington D.C.). Primer design was accomplished using Primer 3 software (Center of Genome Research at the Whitehead Institute for Biomedical Research, Cambridge, Mass.). Cattle LDHB primers for the promoter region were: LDHB BovinePro forward: 5'-ACACACCAGCAGCATCTCAG-3' and LDHB Bovine 36 reverse: 5'-GATAAGGGCTGCACGAAGAC-3'. The amplicon size was 457 base pairs.

Extracted DNA was thawed and mixed with PCR water and the contents of the Biolase Red DNA Polymerase kit (Bioline, Randolph, Mass.) in concentrations of 74 µL of PCR water, 10 µL of 10 × NH_4 buffer, 3 µL of $MgCl_2$, 2 µL of LDH forward primer, 2 µL of LDH reverse primer, 2 µL of Red Taq, a protein dye, and 5 µL of DNA. The total amount of 100 µL was split into two tubes of 50 µL each to improve amplification when loaded into the PCR instrument.

Amplification of the promoter sequences by polymerase chain reaction (PCR) was performed using a PTC-100 Peltier Thermal Cycler (Bio-Rad, Hercules, Calif.). The amplification protocol began with 2 min heating at 98 °C followed by 35 cycles of 94 °C for 30 s, 1 min at 55 °C, and 1 min at 68 °C, and a final elongation step held for 10 min at 68 °C. Amplification products were visualized using gel electrophoresis (1.2% agarose gels in 1.0 × Tris/Borate/EDTA (TBE)) and stained with ethidium bromide.

Amplicons were verified using the gel electrophoresis and purified with QIAquick PCR Purification Kit (Qiagen, Valencia, Calif.). Concentration of the purified amplicons was determined with a DNYA Quant Fluorometer (Hoefer, San Francisco, Calif.) followed by sequencing at University of Arkansas DNA Resource Center. Submitted samples were subjected to both forward and reverse sequencing using the Applied Biosystems Big Dye Chemistry on an ABI 3130xl (Applied Biosystems, Foster City, Calif.). The resulting sequences were analyzed using ClustalW2 program (European Bioinformatics Institute, Cambridge, UK), which provided multiple alignments and identified nucleotide polymorphisms.

Blood Sample Analysis. Accutrend™ Lactate Analyzer (Roche Diagnostics, Alameda, Calif.) was used to determine lactate concentrations (mmol/L) in whole blood. Plasma protein concentrations, and lactate dehydrogenase activity (forward (LDHf) and reverse (LDHr)) were determined using modified colorimetric kinetic assays on a Spectra Max 250 (CV within 10%; Molecular Devices, Sunnyvale, Calif.). Forward reaction of LDH was determined using reagents β-nicotinamide adenine dinucleotide (0.0194 mmol/L; Sigma, Saint Louis, Mo.) and pyruvic acid (16.2 mmol/L; Sigma). Samples were analyzed at 340 nm four times one minute apart.

Reverse reaction of LDH was determined using reagents NAD^+, Free Acid (7 mmol/L; Calbiochem, San Diego, Calif.) and L-lactic acid lithium salt (50 mmol/L; Tokyo Kasei Kogyo Co., Tokyo, Japan). Samples were analyzed at 340 nm three times 30 s apart. When catalyzing the oxidation of lactate to pyruvate, LDH simultaneously reduces NAD^+ to NADH (Katz and Sahlin, 2002). The rate of reduction of NAD^+, measured by determining the rate of increase in absorbance at 340 nm, was directly proportional to LDH activity. Lactate dehydrogenase activity was expressed using the International Unit (IU), which is the amount of enzyme that catalyzes the transformation of one µmole of substrate per minute under defined conditions.

Total protein concentrations were determined using Pierce commercial BCA Protein Assay Kit (#23225; Thermo Fisher Scientific Inc., Rockford, Ill.). Both LDHf and LDHr were corrected for plasma protein concentration and were expressed as international units per milligram of plasma protein. Blood cell differential counts were determined within

four hours of collection using a Cell-Dyne 3500 hematology analyzer (Abbott Laboratories, Abbott Park, Ill.).

Statistical Analysis. Data were analyzed using analysis of variance with pasture as the whole plot experimental unit, and heifer weights, blood cell distributions, and enzyme activities as repeated measures. Pasture within cultivar was considered a random affect. Effects of cultivar, LDHB genotype (heterozygous (Del/In), and homozygous insertion (In/In)), days on trial, and their interactions were determined on dependent variables. When F-tests were significant ($P < 0.05$) means were separated using multiple *t*-tests, while *P*-values between 0.1 and 0.05 were defined as a tendency.

RESULTS AND DISCUSSION

Distribution of heifer LDHB genotype was the same ($P = 0.98$) across the two tall fescue cultivars (Table 1). Heifer weight increased ($P < 0.001$) with days on trial (207, 236, 260, 277 ± 5 kg, respectively, for d 0, 28, 56, and 84). In addition, weight was affected by an interaction ($P < 0.01$) between LDHB genotype and cultivar (Fig. 1). Heifers with heterozygous (Del/In) genotypes grazing HiMag were heavier than all other heifer groups.

Both LDHf activity (pyruvate to lactate) and LDHr activity (lactate to pyruvate) fluctuated ($P < 0.001$) over time, and plateaued at days 56 and 84 (Fig. 2). Heifer LDHB genotype tended ($P = 0.1$) to affect LDHf activity (12.2 vs. 11.7 IU/mg ± 0.29, respectively, for Del/In and In/In). In addition, LDHr activity was affected ($P < 0.05$) by LDHB genotype. Heifers that were In/In had lower ($P < 0.05$) plasma LDHr activity than heifers that were heterozygous (3.0 vs. 3.2 IU/mg, respectively). Heifer plasma lactate concentrations increased ($P < 0.01$) with days on trial (Table 2), and were affected ($P < 0.05$) by an interaction between LDHB genotype and cultivar (Fig. 2).

Concentration of red blood cells (RBC), whole blood hemoglobin content, hematocrit percent, and concentration of platelets were affected ($P < 0.05$) by days on trial (Table 2). Red blood cell concentrations were affected ($P < 0.01$) by an interaction between LDHB genotype and tall fescue cultivar; however, based on diagnostic lab values, all heifers had RBC concentrations within "normal" range (Fig. 3). Heifers grazing KY31 had higher ($P < 0.05$) whole blood hemoglobin concentrations than heifers grazing HiMag (12.4 vs. 11.3 g/dL ± 0.2), and In/In heifers had higher ($P < 0.05$) whole blood hemoglobin concentrations than Del/In heifers. Hematocrit percentages were larger ($P < 0.05$) in heifers grazing KY31 when compared with heifers grazing HiMag pastures (35.4% vs. 32.2%).

White blood cell (lymphocytes, neutrophils, basophils, eosinophils, and monocytes) concentrations were affected by the number of days on trial (Table 2). An interaction between tall fescue cultivar and LDHB genotype affected ($P < 0.01$) white blood cell concentrations (Fig. 4). Lymphocyte concentrations were affected ($P < 0.01$) by an interaction between tall fescue cultivar and LDHB genotype (Fig. 5). The concentration of monocytes was affected by LDHB genotype; specifically, In/In heifers had a larger ($P < 0.01$) concentration of monocytes than Del/In heifers (1.0 vs. 0.85 ± 0.06 × 10^4/μL). The In/In heifers had more ($P < 0.05$) basophils than Del/In heifers (0.15 vs. 0.12 ± 0.01 × 10^4/μL). Percentage of basophils also was affected ($P < 0.05$) by an interaction between tall fescue cultivar and days on trial (Fig. 6).

Heifers grazing HiMag that were heterozygous had the greatest weight gain. Lactate concentrations appeared to be inversely related to weight gain, with In/In heifers grazing HiMag having the highest lactate concentrations and heterozygous heifers grazing HiMag the lowest lactate concentration. This supports other findings related to high blood lactate concentrations, such as decreased reproductive performance and profitability in beef cattle (Looper et al., 2008). An increase in lactate concentrations in cattle has been associated with nutritionally induced muscular dystrophy (Kursa, 1975). These findings suggest that the more stress to which the cow is subjected requires the cow to use more energy.

Erythrocyte and hemoglobin concentrations were affected by LDHB genotype and tall fescue cultivar. All blood cell and component concentrations were within the accepted "normal" range. Higher RBC and hemoglobin concentrations for homozygous heifers grazing KY31 are consistent with results from other studies. A three-year study was conducted to determine the long-term effects of grazing toxic tall fescue on steers—one result of which was a higher RBC concentration in steers grazing toxic fescue (Oliver et al., 2000). An interaction between genotype and cultivar was noted on RBC concentration in this study, but the reason for the interaction is still unclear and warrants further study. A similar interaction was found between genotype and cultivar on white blood cell concentrations, but there were no overarching tendencies found.

Cow health and reproduction are acutely related to dairy herd profitability. Based on blood cell differential counts, genotyping for LDHB may be related to dairy cow health; although, that linkage will need additional testing for definitive relationships. Stockpiling tall fescue resulted in acceptable dairy heifer gain and immune function based on white blood cell distributions. However, many questions remain pertaining to the use of stockpiled toxic tall fescue as a management tool for developing dairy heifers. Specifically, whether or not ergot alkaloids would be stored in the fat of the heifer only to be released later, causing health issues for her, or deposited in her milk and result in a human health issue?

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Table 1. Distribution of heifers by LDHB genotype on tall fescue cultivars.

LDHB Genotype	HiMag (n)	KY31 (n)
Heterozygous (DI)	6	7
Homozygous insertion (II)	7	7

Table 2. Effects of trial days on heifer weights and blood characteristics.

	Day of Trial				Pooled
Trait[a]	0	28	56	84	SE
Body wt, kg	212 d[b]	241 c	265 b	283 a	4.9
LDHf, IU/mg	11.3 b	10.8 b	13.2 a	12.5 a	0.3
LDHr, IU/mg	2.8 b	2.8 b	3.5 a	3.4 a	0.1
Lactate, mM	2.6 b	3.3 a	3.4 a	3.4 a	0.2
Protein, mg/ml	83 a	87 a	69 b	70 b	1.8
RBC, # x $10^6/\mu$L	8.0 c	8.4 bc	8.7 ab	9.1 a	0.21
Hemoglobin, g/dL	10.5 d	11.8 c	12.3 b	12.7 a	0.2
Hematocrit, %	30.3 c	32.6 b	34.6 a	36.2 a	0.77
Platelets, k/uL	561 a	513 a	396 b	512 a	34
WBC, # x $10^4/\mu$L	8.9 b	11.0 a	11.3 a	8.3 b	0.49
Lymphocytes,# x $10^4/\mu$L	5.9 c	7.4 b	9.6 a	4.7 d	0.5
Neutrophils, # x $10^4/\mu$L	1.6 c	2.0 b	0.5 d	2.6 a	0.18
Eosninophils, # x $10^4/\mu$L	0.04 c	0.48 a	0.26 b	0.17 bc	0.06
Monocytes, # x $10^4/\mu$L	1.3 a	1.0 b	0.8 c	1.0 bc	0.07

[a]Items were determined on the stated trial days. Heifer (n = 27) body weight, white blood cell (WBC) and red blood cell (RBC) counts in whole blood. Serum concentrations of lactate, protein, and lactate dehydrogenase (LDH) activites are presented. The LDH activities forward (LDHf) and reverse (LDHr) were corrected for serum protein concentration.
[b]Means in a row followed by the same letter are not significantly different at $P < 0.05$.

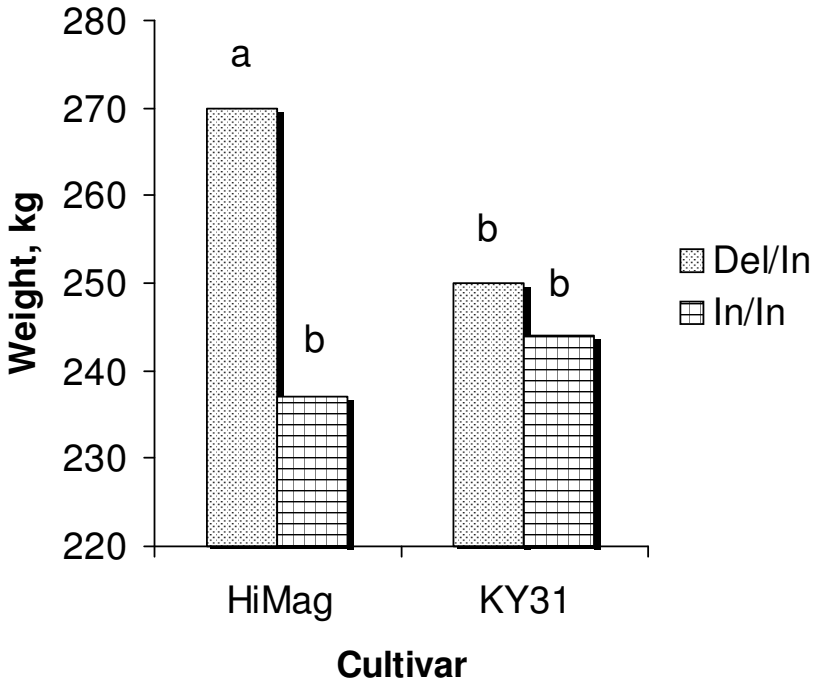

Fig. 1. Interactive effects of LDHB genotype (Del/In or In/In) and tall fescue cultivar (KY31 or HiMag) on heifer weight. Values in columns without a common superscript letter differ ($P < 0.01$).

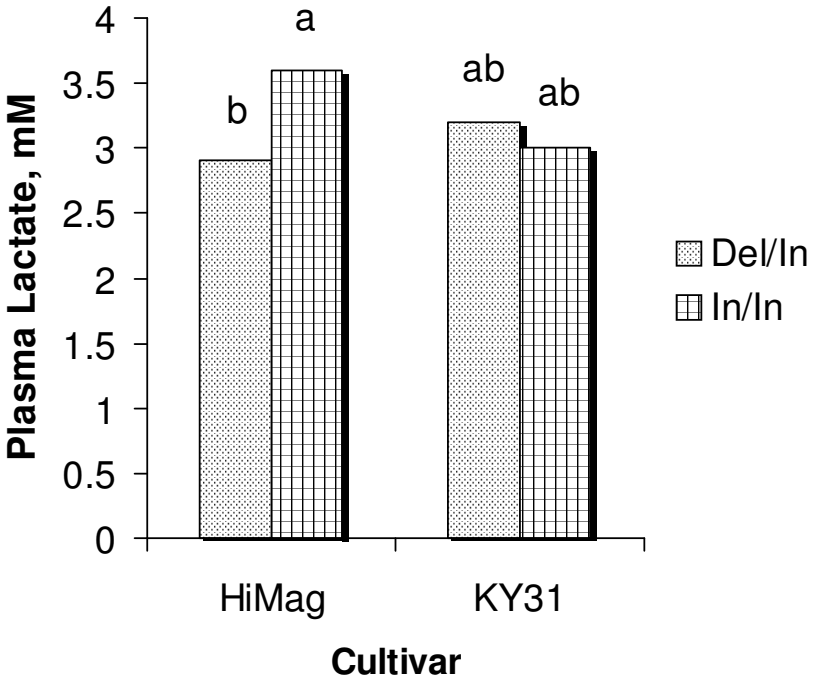

Fig. 2. Interactive effects of LDHB genotype (Del/In or In/In) and tall fescue cultivar (KY31 or HiMag) on plasma lactate concentrations. Values in columns without a common superscript letter differ ($P < 0.01$).

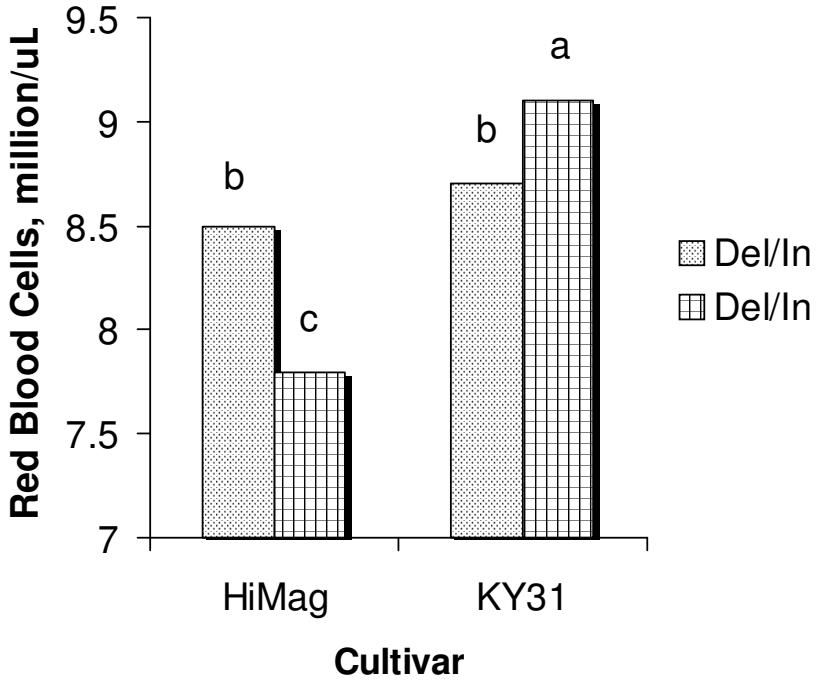

Fig. 3. Interactive effects of LDHB genotype (Del/In or In/In) and tall fescue cultivar (KY31 or HiMag) on circulating red blood cell concentrations. Values in columns without a common superscript letter differ ($P < 0.01$).

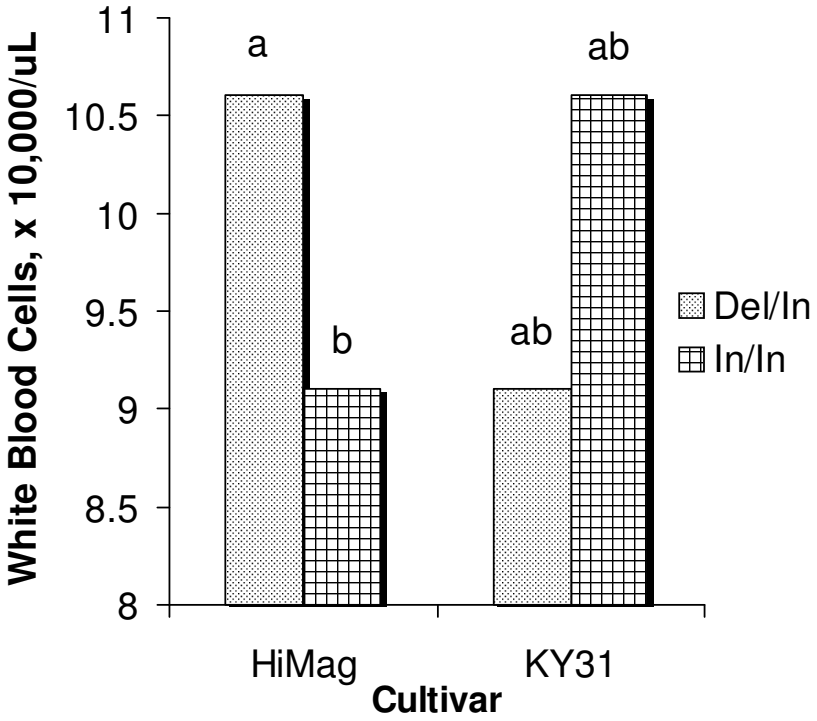

Fig. 4. Interactive effects of LDHB genotype (Del/In or In/In) and tall fescue cultivar (KY31 or HiMag) on white blood cell concentrations. Values in columns without a common superscript letter differ ($P < 0.01$).

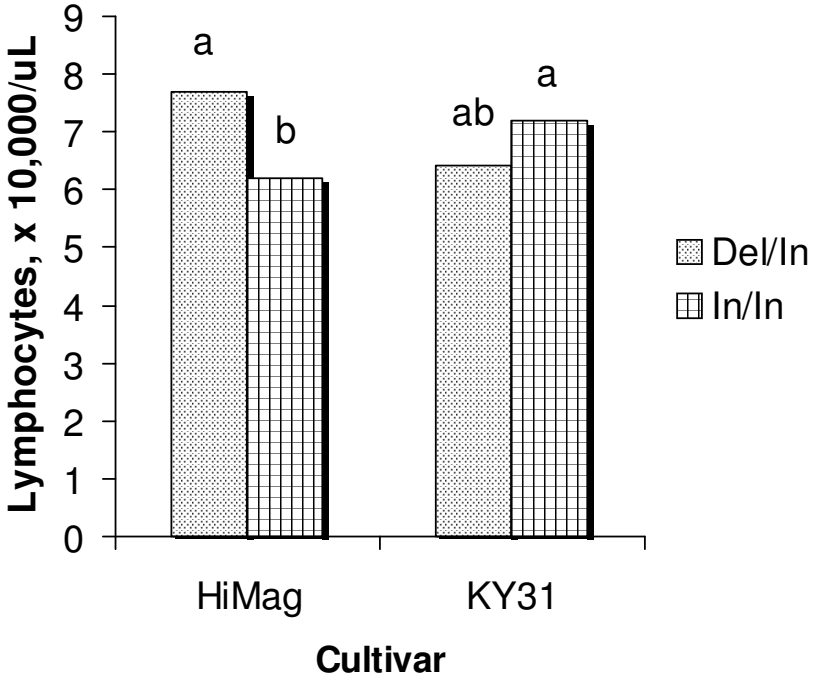

Fig. 5. Interactive effects of LDHB genotype (Del/In or In/In) and tall fescue cultivar (KY31 or HiMag) on lymphocyte percentages. Values in columns without a common superscript letter differ ($P < 0.01$).

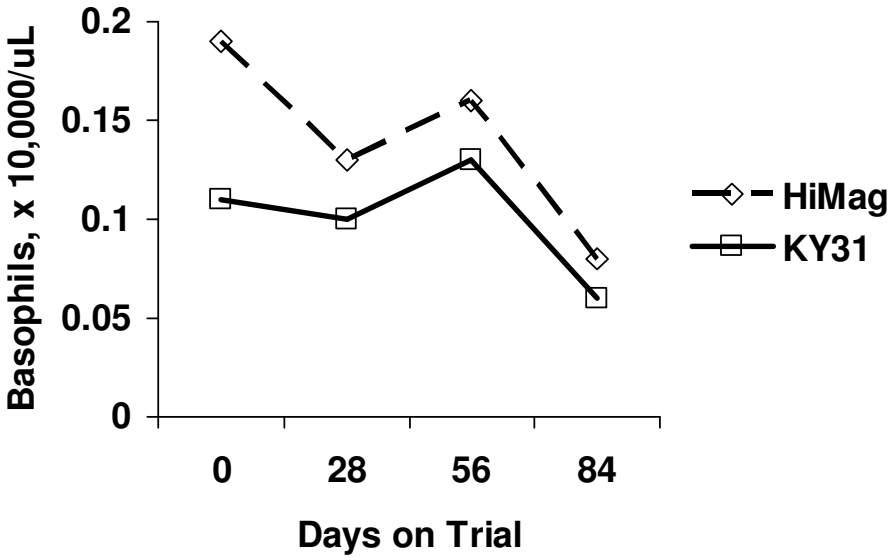

Fig. 6. Effects of time and tall fescue cultivar (KY31 or HiMag) on the percentage of basophils.